ZOONOTIC DISEASES OF PUBLIC HEALTH IMPORTANCE

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20th July, 2016
दिनांक/Dated......................

Foreword

Zoonotic diseases are major public health problems not only in India but also in different parts of the world. Over the last two decades, there has been considerable change in importance of certain zoonotic diseases in many parts of the world, resulting from ecological changes such as urbanization and industrialization.

This manual deals with various aspects of the zoonotic diseases to provide necessary background to the reader which shall help in better understanding of the diseases like Plague, Japanese Encephalitis, Leishmaniasis, Rabies, Leptospirosis and Dengue Fever etc. which have major public health concerns in India. Also, recent epidemics of Ebola, Zika Virus disease in various parts of the world have served as a reminder of the existence of infectious diseases and of the capacity of these diseases to occur unexpectedly across the boundaries. These diseases have contributed to the definition of new paradigms, especially relating to the diagnostic capacity and epidemiology. Hence, early diagnosis and treatment of zoonotic diseases is of paramount importance. Moreover the feasibility of integrating human, animal, and environmental health also needs to be emphasized so that one health approach becomes major element of disease control and prevention strategies.

I congratulate Director, NCDC and his team for bringing out a manual on **"Zoonotic Diseases of Public Health Importance" (Third Edition).** I am confident that this manual will provide an overview to the public health experts, laboratory professionals, State directorates, veterinary experts, airport health officials, medical and paramedical professionals about various aspects of emerging and re-emerging zoonotic diseases.

(Dr. Jagdish Prasad)

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Preface

Many emerging and re-emerging diseases of humans are zoonotic in nature. Their worldwide distribution, lack of effective surveillance, and problems in timely diagnosis pose significant issues to public health.

The arboviral and zoonotic diseases are a huge challenge in India leading to increased morbidity and mortality in humans. Also, worldwide occurrence of a significant number of outbreaks of new and emerging zoonotic diseases, like Ebola, Zika Virus Disease and Crimean Congo Haemorrhagic Fever, over the past few years is a matter of concern for India. As these diseases come from animals, prevention and control strategies need to be multi-sectoral and require the coordinated and combined efforts of veterinary, environment, forest, agriculture, climate change, and health sectors.

I commend the sincere and dedicated efforts of Dr S. Venkatesh, Director NCDC and his colleagues in bringing out this third and updated edition of the manual of **"Zoonotic Diseases of Public Health Importance"**. This manual will provide valuable insight to a variety of professionals in health, animal husbandry and environment & forest sectors: policy-makers and administrators, medical and veterinary officers, and public health specialists, on epidemiology, laboratory aspects and preventive and control measures of Zoonotic diseases of Public Health importance.

(N.S. Dharmshaktu)

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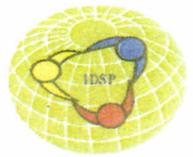22—शाम नाथ मार्ग, दिल्ली — 110054
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Acknowledgement

India has witnessed a number of outbreaks of arboviral and zoonotic diseases in the last two decades. Prevention and control of these diseases needs intersectoral coordination between health and veterinary sectors and involvement of epidemiologists , microbiologists, veterinary officers, entomologists, etc. Joint orientation of various sectors is needed to understand the knowledge pertaining to the source, transmission, pathogenicity, treatment and control of zoonotic diseases.

The first two editions of the manual on Zoonotic Diseases of Public Health Importance were published at the National Centre for Disease Control in 2000 and 2006 respectively. The third edition of this manual has been updated to provide a detailed insight into the epidemiology, diagnostic techniques, surveillance, prevention and control of various zoonotic diseases such as Dengue, Chikungunya, Japanese Encephalitis, Rabies, Leptospirosis, Crimean Congo Haemorrhagic Fever, etc.

In this edition, chapters on Ebola Virus Disease, Zika Virus Disease and Crimean Congo Haemorrhagic Fever have been added and chapters on Rabies, Leptospirosis and other Arboviral Diseases have been updated with detailed information about newer epidemiological aspects and recent diagnostic, therapeutic and prophylactic regimens. References for further reading have also been appended. We duly acknowledge the reference data/material obtained from IDSP, NVBDCP and CDC for this manual.

The efforts of Dr. Dipesh Bhattacharya, Former Additional Director & Head, Dr. Mala Chhabra, current Additional Director & Head of the Division of Zoonosis assisted by their colleagues in bringing out this manual in a short span of time is highly commendable.

I hope this manual will be useful in providing necessary technical information for medical and veterinary officers, public health specialists and laboratory personnel. Besides serving as a reference book, the manual will also form valuable resource material for training programmes and self learning.
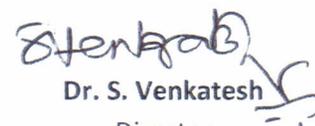
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ZOONOSES - GENERAL ASPECTS

1.1 DEFINITION

The word 'Zoonosis' (Pleural: Zoonoses) was introduced by Rudolf Virchow in 1880 to include collectively the diseases shared in nature by man and animals. Later WHO in 1959 defined that Zoonoses are "those diseases and infections which are naturally transmitted between vertebrate animals and man". Zoonoses include only those infections where there is either a proof or a strong circumstantial evidence for transmission between animals and man.

1.2 ZOONOSES - AN INTERNATIONAL PROBLEM

Historically, zoonotic diseases had a tremendous impact on the evolution of man, especially those cultures and societies that domesticated and bred animals for food and clothing. Zoonoses are among the most frequent and dreaded risk to which mankind is exposed. Zoonoses occur throughout the world transcending the natural boundaries. Their important effect on global economy and health is well known, extending from the international movement of animals and importation of diseases to bans on importation of all animal products and restrictions on other international trade practices. So, zoonoses no longer are solely a national problem. For effective control of zoonoses global surveillance is necessary.

With recognition of inter-relationships between countries, the internationalization of control efforts have become more relevant to technical, economic and social fields. The control of zoonoses retains its prominent place among the actions of international agencies according to the health and economic problems specific to each region.

1.3 ZOONOSES- AN EMERGING PROBLEM

Over the last two decades, there has been considerable change in the importance of certain zoonotic diseases in many parts of the world, resulting from ecological changes such as urbanization, industrialization and diminishing proportion of persons working in the so-called primary sector.

We do not know with what challenge nature will confront us in the world of constant interference with ecology. Most of the infections of man that have been discovered in the last twenty years are shared with lower animals and a number of other diseases previously thought to be limited to man have now been found to be zoonoses. Reference may be made to various types of encephalitis, eosinophilic meningitis, capillariasis, anisakiasis, lyme disease, monkeypox diseases in humans, lassa fever, Marburg disease and Ebola for all of which an animal link has been established.

Among those zoonoses recognized today as particularly important are anthrax, plague, brucellosis, Bovine tuberculosis, leptospirosis, salmonellosis, spotted fever caused by Rickettsiae, rabies, several common athropod borne viral infections (arboviral infection), certain parasitic diseases, especially cysticercosis, hydatid disease, trypanosomiasis and toxoplasmosis.

1.4   CLASSFICATION

With the advanced laboratory techniques and increased awareness among medical and veterinary scientists, ecologist and biologists, more than 300 zoonoses of diverse etiology are now recognised. Thus, a very large number of zoonoses calls for classification especially for easy understanding. These are classified as follows:

1.4.1   According to the etiological agents

Bacterial zoonoses	e.g. anthrax, brucellosis, plague, leptospirosis, salmonellosis, lyme disease
Viral zoonoses	e.g. rabies, arbovirus infections, KFD, yellow fever, influenza, CCHF
Rickettsial zoonoses	e.g. murine typhus, tick typhus, scrub typhus, Q-fever
Protozoal zoonoses	e.g. toxoplasmosis, trypanosomiasis, leishmaniasis
Helminthic zoonoses	e.g. echinococcosis (hydatid disease), taeniasis, schistosomiasis, dracunculiasis
Fungal zoonoses	e.g. deep mycosis - histoplasmosis, cryptococcosis, superficial dermatophytes.
Ectoparasites	e.g. scabies, myiasis

1.4.2   According to the mode of transmission

1.4.2.1 Direct zoonoses
These are transmitted from an infected vertebrate host to a susceptible host (man) by direct contact, by contact with a fomite or by a mechanical vector. The agent itself undergoes little or no propagative or developmental changes during transmission, e.g. rabies, anthrax, brucellosis, leptospirosis, toxoplasmosis.

1.4.2.2 Cyclozoonoses
These require more than one vertebrate host species, but no invertebrate host for the completion of the life cycle of the agent, e.g. echinococcosis, taeniasis.

1.4.2.3 Metazoonoses
These are transmitted biologically by invertebrate vectors, in which the agent multiplies and/or develops and there is always an extrinsic incubation (prepatent) period before transmission to another vertebrate host e.g., plague, arbovirus infections, schistosomiasis, leishmaniasis.

1.4.2.4 Saprozoonoses

These require a vertebrate host and a non-animal developmental site like soil, plant material, pigeon dropping etc. for the development of the infectious agent e.g. aspergillosis, coccidioidomycosis, cryptococosis, histoplasmosis, zygomycosis.

1.4.3   According to the reservoir host

1.4.3.1Anthropozoonoses

Infections transmitted to man from lower vertebrate animals e.g. rabies, leptospirosis, plague, arboviral infections, brucellosis and Q-fever.

1.4.3.2 Zooanthroponoses

Infections transmitted from man to lower vertebrate animals e.g. streptococci, staphylococci, diphtheria, enterobacteriaceae, human tuberculosis in cattle and parrots.

1.4.3.3 Amphixenoses

Infections maintained in both man and lower vertebrate animals and transmitted in either direction e.g. salmonellosis, staphylococcosis

1.5   FACTORS INFLUENCING PREVALENCE OF ZOONOSES

1.5.1   Ecological changes in man's environment

With the expansion of human population, man is forced to exploit the virgin territories and natural resources like harnessing the power of rivers, constructing roads and pipelines through virgin or thinly populated areas, clearing, irrigating and cultivating new land, deforestation. All this would lead to entering of humans in the unaccustomed ecosystem in which potential pathogens form part of the biotic community (natural focus).

Large scale expansion of agricultural and engineering resources, construction of dams, artificial lakes, irrigation schemes, clearing of forests -all these lead to changing of the biting habits of the blood sucking vectors and alteration in the population of reservoir animals which has led to the spread of leptospira, tuleraemia, helminthic infections etc.

1.5.2   Handling animal by-products and wastes (occupational hazards)

There is significantly higher attack rates in workers during the course of their occupation than the rest of the population, e.g. anthrax in carpet weavers, live stock raisers and workers with animal hair in the textile industry, leptospirosis in rice field workers, listeriosis in agricultural workers, erysipeloid in butchers and fish merchants, tularemia and trypanosomiasis in hunters, creeping eruptions in plumbers, trench diggers etc. Other examples of zoonoses as occupational hazards are Q-fever in abattoir and rendering plant workers, jungle yellow fever and tick borne diseases in wood cutters, salmonellosis in food processors, bovine tuberculosis in farmersetc.

1.5.3 Increased movements of man

Land development, engineering project work, pilgrimages, tourism, etc. expose the people to contaminated food and water leading to diseases like amoebiasis, colibacilliosis, giardiasis, salmonellosis, shigellosis, etc.

1.5.4 Increased trade in animal products

Countries which import hides
, wool, bone meal, meat, etc. from an area where some of the zoonoses are endemic, are likely to introduce the disease into their territories, e.g. salmonellosis, foot and mouth disease, anthrax, Newcastle disease etc.

1.5.5 Increased density of animal population

Animals may carry potential risk of increased frequency of zoonotic agents in man e.g. dermatophytosis, tuberculosis, brucellosis etc.

1.5.6 Transportation of virus infected mosquitoes

Aircraft, ship, train, motor and other vehicles bring the viruses in to a new area, e.g. yellow fever Chikungunya fever, dengue fever etc.

1.5.7 Cultural anthropological norms

In Kenya, people allow the dogs and hyenas to eat human dead bodies infected with hydatidosis. This helps to perpetuate the transmission cycle of the disease.

1.6 ZOONOSES AS A PUBLIC HEALTH PROBLEM

Although poorly documented, zoonotic diseases are a major public health problem in India. Plague has killed nearly 120 lakhs people since 1898. Rabies continues to be a serious health problem in the country. Approximately 20,000 deaths due to rabies are estimated to occur every year while more than 17 lakhs persons bitten by suspected rabid animals seek antirabies vaccination at rabies treatment centres. Typhus killed many people during World War-I. Brucellosis alone is estimated to cause annual loss of approximately 300 lakhs man days in addition to an annual economic loss of Rs.2400 lakhs through brucellosis in cattle and buffaloes. Japanese encephalitis is another emerging zoonotic disease in India causing several outbreaks and considerable morbidity and mortality. Studies on reservoir of this disease are yet in conclusive, Kala-azar although proved zoonotic all over the world, continues to be non zoonotic in India in spite of the epidemiological evidence suggesting it to be zoonotic. Cutaneous leishmanias is which was hither to consider an anthroponosis in India has been proved to be a zoonosis recently with the Indian desert gerbil *Merriones hurriane* as the animal reservoir. It is not surprising, that in India, where approximately 80%of population lives-in rural areas in close contact with large domestic animal population (5120 lakhs approximately, 7290 lakhs poultry and equally large populations of wild and semi-wild animals) abundance of vectors because of suitable climate, low socio-economic conditions and lack of proper medical care, zoonotic diseases assume great public health significance. However, because of inadequate diagnostic

facilities, unfamiliarity of physicians with these diseases and lack of co-ordination between physicians, veterinarians, and epidemiologist, the extent of their existence is obscured.

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CHAPTER –II

SAFETY PRECAUTIONS IN THE LABORATORY

Biosafety in a microbiological laboratory' is very essential and basically depends on three components:

- Basic standard of laboratory design, operation and equipments.
- Selection and use of essential biosafety equipment.
- Safe laboratory procedures.

An exhaustive review of each component is beyond the scope of this manual but practical and Easily achievable safe laboratory guidelines are listed below:

- Avoid mouth pipetting

- Avoid eating, drinking, smoking and storing eatables in the laboratory.

- Decontaminate the working area at least once a day and more frequently after the spillage of potentially infective material.

- Wash your hands with soap and water after handling the infectious material.

- Wear laboratory coats/gowns in the laboratory and these should not be taken outside.

- Use gloves for all those procedures that may involve accidental or direct contact with blood or infectious materials.

- Decontaminate all liquid or solid waste before disposal.

- Perform all technical procedures in a way that minimizes the aerosol formation.

- Provide adequate training to the staff in laboratory safety procedures

- As far as possible actively immunize the workers against the diseases the materials of which are handled by them.

- Employ only medically fit staff to work in clinical laboratories.

- Report accident and illness promptly to the concerned officials.

- Provide ample space preferably with an exhaust fan and adequate illumination for safe conduction of laboratory procedures.

- Design smooth and easily cleanable walls, ceilings and floors and tabletops, which should be impermeable to liquids and resistant to chemicals and disinfectants.

- Ensure a dependable and good quality water supply.

- Make suitably equipped 'first aid' facilities readily accessible.

- Provide the staff safe laboratory equipments e.g. pipetting aids, safety cabinets, screw cap tubes and bottles, loop, incinerator if possible, and autoclaves, etc.
- Carry out periodic health and medical surveillance of the workers to exclude the highly susceptible individuals.
- Provide safety systems for covering fire and electrical emergencies.
- Control rodents and insects in the laboratory.
- Do not permit the entry of the experimental animals, which are not to be used in the laboratory.

In case of spillage of culture material, blood or blood products, pour 1% sodium hypochloriteover the spillage and leave it for 15minutes and then wipe it.

Further Reading

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RABIES

3.1   INTRODUCTION

Rabies is an acute viral disease, which causes encephalomyelitis in virtually all the warm blooded animals including man. The causative agent is found in domestic and wild animals, and is transmitted to other animals and to humans through close contacts with their saliva (i.e. bites, scratches, licks on broken skin and mucous membranes).

Rabies is an important zoonotic infection in which man is dead end of the infection and hence does not play any role in its spread to new hosts. In most of the developing countries, dogs are the principal reservoirs of rabies (**canine rabies**) whereas sylvatic rabies involving animals such as foxes, racoons and coyotes are principal wild animals reservoirs of this disease in developed countries.

Rabies has terrified man since antiquity. The fear is by no means unfounded since the disease is invariably fatal and perhaps the most painful and horrible of all communicable diseases in which the sick person is tormented at the same time with thirst and fear of water (hydrophobia). Till date no treatment has succeeded in curing hydrophobia and inspite of great strides in the prevention of rabies, with few exceptions, the disease is no less a global problem now than it was almost a century ago.

3.2   CAUSATIVE AGENT

Rabies virus belongs to the family Rhabdoviridae and genus Lyssavirus (Lyssa: Greek: rabies). This RNA virus is bullet shaped, round at one end and flat at the other. the virus is covered with a lipid envelope having spike like projections.

3.2.1 Susceptibility to physical and chemical agents

The rabies virus is highly resistant against cold, dryness and decay. In cadavers, it remains infectious for weeks. This virus is highly thermolabile with a half-life of approximately 4 hours at 40^0C and 35 seconds at 60^0C. Serum proteins and other chelating agents diminish thermal inactivation. In brain tissue at room temperature it can survive upto 1-2 weeks.

The rabies virus remains stable for several days at $0\text{-}4^0$C, indefinitely at -70^0C and when freeze dried. The virus cannot withstand pH less than 4 or more than 10. It is also susceptible to the action of oxidizing agents, most organic solvents, surface acting agents, and quaternary ammonium compounds. Proteolytic enzymes, ultraviolet rays and X-rays rapidly inactivate rabies virus. Soaps and detergents are effective against rabies virus because of their lipid eliminating property, which destroys the outer covering of the virus.

3.2.2 Excretion of rabies virus

Virus is excreted by the rabid animal mainly in saliva. It is present in the saliva of the dogs for 2-3 days before the appearance of clinical features. It remains in the saliva till the animal dies. Death usually occurs within one week of onset of clinical manifestations.

3.3   EPIDEMIOLOGY

3.3.1 Global status

Worldwide the number of human rabies deaths is estimated to be between 35,000 and 50,000 annually. Rabies occurs in all continents with the exception of Australia and Antarctica. Several countries are currently free of rabies. Even in infected countries the disease is not uniformly distributed. Areas free of disease, of low and high endemicity and areas with epizootic outbreaks can be found in many countries. In Africa and Asia (with few important exceptions such as Japan and Singapore) rabies is prevalent in almost whole of the territory with a stable pattern. Most of the countries of Americas and Europe report occurrence of disease in limited or border areas.

3.3.2 Rabies in India

Rabies is responsible for extensive morbidity and mortality in India. The CBHI provisionally reported around 379 deaths due to hydrophobia during 2003. The estimated number of deaths per year is, however, around 20,000. Almost 18 lakhs people annually receive post exposure prophylaxis against rabies following bite or exposure to rabid or suspected rabid animal.

With the exception of Andaman & Nicobar islands and Lakshadweep islands, human cases of rabies are reported from all over the country. The cases occur throughout the year. 96% of the mortality and morbidity is associated with dog bites. Cats, wolf, jackal, mongoose and monkeys are other important reservoirs of rabies in India. Bat rabies has not been conclusively reported from India.

3.3.3 Mode of transmission

Rabies virus is predominantly neurotropic and kills the host in short period after it has entered the neurones. Before death, from the brain virus reaches salivary glands and is excreted in saliva. The saliva gains entry into another host through a preexisting breach in skin when mere licking or contamination is adequate or the bite of the rabid animal creates a mechanical breach of skin through which the rabies virus gains entry. Virus may be present in the saliva for many days before clinical signs appear and it may be steadily or intermittently secreted until just before death. Report of pre clinical periods of virus secretion in saliva range from 3 days in cats, 12 days in Mexican free tail bats, 14 days in dogs infected with an Ethiopian virus isolate to 29 days in foxes. Infection has been documented in personnel receiving corneal grafts and organs from rabies cases.

3.4   PATHOGENESIS

On entering into human body, rabies virus multiplies at local site of inoculation prior to its spread towards brain via the nerves. Within the brain, virus spreads from infected to contagious cells. There may be regional differences in the intensity with which areas of brain become

infected. The main areas affected are usually the cerebellum, hypothalamus, hippocampus and scattered neurons in the reticular formation. It may be that aggression in rabies is related to the presence of virus in mid brain raphe nuclei and medical hypothalamus, since these are two inhibitory centres of aggressive behaviour. It may also be that the distribution of virus in the brain has a bearing as whether the disease becomes the manifest in dumb or classical furious rabies. It does not follow the haematogenous spread. The movement of the virus is extremely slow which results into a long incubation period. This fact helps in initiating immunoprophylaxis even after the causative agent has invaded the body.

3.4.1 Incubation period

The average incubation period is between 30-90 days. Factors which may influence the length of the incubation period include the site of bite, the amount of virus in saliva of the bitting animal, the virus strain, and the age and immune status of the victim. It is shorter in case the bite is closer to brain and massive dose of virus has been inoculated. Incubation period as short as 10 days and as long as 2 years have been reported.

3.5 CLINICAL FEATURES IN MAN

The first symptom to appear may be pain and tingling in the affected limb, especially around the site of bite. This is seen in 35-65% cases. Hydrophobia is the best known symptom of this disease and is pathognomonic for rabies.

3.5.1 Hydrophobia

Hydrophobia is usually the only neurologic abnormality found in patient presenting with furious rabies. It is due to a violent jerky contraction of the diaphragm and accessory muscles of inspiration that is triggered by the patient's attempts to swallow liquid and by a variety of other stimuli such as strong current of air, loud noise and bright light. Hydrophobia is usually not associated with pain in neck or throat. It is also not a conditioned reflex caused by aspiration of liquid into trachea.

3.5.2 Differential diagnosis

Before the appearance of hydrophobia and in those cases where it does not manifest, rabies needs to be differentiated from other clinical conditions which are as follow:

Initial phase	Lockjaw
	Encephalitis
	Hysteria
Paralytic phase	Acute polyneuritis
	Poliomyelitis
	Belladona poisoning
	Delerium tremors

Rabies post vaccinal encephalomyelitis

3.6   RABIES IN ANIMALS

3.6.1   Clinical features in dogs

After an incubation period of around 3 months (range 10 days to 6 months), dog may manifest one or more of the following clinical features. There may be change in behavior of dog, change in bark tone, change in feeding habits, the animals may go off feed and eat abnormal objects. They may develop fever, vomiting, excessive salivation, paralysis of lower jaw, anxiety, restlessness, convulsions, paralysis leading to death with in 5-7 days on onset of disease. There is however no hydrophobia in animals.

Rabies in dogs is also classified as dumb (predominantly paralytic manifestation with docile behavior of animal) or furious (mainly convulsions and aggressive behavior with greatly exaggerated biting tendencies).

3.6.2   Clinical features in cats and cattle

Rabid cats show extreme aggressiveness, great sensitivity to touch/voice, profuse salivation and may attempt to attack dog or man.

In cattle, rabies is manifested as abnormal movements of posterior extremity, foamy yellow froth from mouth and decrease in yield of milk.

Milk of rabid cattle has been shown to have viable rabies virus and its ingestion in raw form may require post exposure treatment in those individuals who have ulcers or abrasions in mouth or pharynx. Otherwise the gastric juice destroys the rabies virus. Pasteurization and cooking also kill the virus.

3.7   POST-EXPOSURE TREATMENT IN HUMANS

Because of long incubation period, which is typical of most cases of human rabies, it is possible to institute prophylactic post exposure treatment. This must be started at the earliest to ensure that the individual will be immunized before the rabies virus reaches the Central Nervous System.

3.7.1   Decision to treat

In a rabies endemic country like India, where every animal bite is potentially suspected as a rabid animal bite the treatment should be started immediately. To bring out uniformity globally, the WHO recommended classification of animal bite for post-exposure treatment should be followed (Table-3.1).

Table 3.1:   WHO Guide for post-exposure treatment against rabies

Category	Type of contact with a suspect or confirmed rabid diagnostic or wild[a] animal, or animal unavailable for observation	Recommended treatment
I	Touching or feeding of animals Licks on intact skin	None, if reliable case history is available
II	Nibbling of uncovered skin Minor scratches of abrasions without bleeding Licks on broken skin	Administer vaccine immediately[b] Stop treatment if animal remains healthy throughout an observation period[c] of 10 days or if animal is killed humanely and found to be negative for rabies by appropriate laboratory techniques
III	Single or multiple transdermal bites or scratches Contamination of mucous membrane with saliva (i.e. licks)	Administer rabies immunoglobulin and vaccine immediately[b] Stop treatment if animal remains healthy throughout an observation period[c] of 10 days or if animal is killed humanely and found to be negative for rabies by appropriate laboratory techniques

[a]   Exposure to rodents, rabbits and hares seldom, if ever, requires specific anti-rabies treatment
[b]   If an apparently healthy dog or cat in or from a low-risk area is placed under observation, the situation may warrant delaying initiation of treatment
[c]   This observation period applies only to dogs and cats. Except in the case of threatened or endangered species, other domestic and wild animals suspected as rabid should be killed humanely and their tissues examined using appropriate laboratory techniques

Source: Guidelines for post-exposure treatment in 8th Report of the WHO Expert Committee on Rabies, WHO Technical report Series 824, 1992

Although unvaccinated animals are more likely to transmit rabies, vaccinated animals can also do so if the vaccination of the biting animal was ineffective for any reason. The risk of dog being infected with rabies is greatly reduced when it appears healthy and there is confirmed history of vaccination with minimum of two immunizations with potent rabies vaccine in last two years. The treatment should be started immediately after the bite. The treatment may be discontinued if animal involved (dog or cat) remains healthy throughout an observation period of 10 days. The observation period is valid for dogs and cats only. Bite by all wild animals should be treated as category III exposure. It should be noted that bites by rats, mice, squirrel, hare and

rabbits seldom require treatment. Bat rabies has not been conclusively proved in India and hence exposure does not warrant treatment.

It is re-emphasized that the treatment should be started as early as possible after exposure, but it should not be denied to person reporting late for treatment.

The post-exposure treatment is a three pronged approach. All three carry equal importance and should be done simultaneously.

- Management of wound
- Passive immunization
- Active immunization

3.7.2 Management of animal bite wound

Wound toilet

Since the rabies virus enters the human body through a bite or scratch, it is imperative to remove as much saliva, and thereby the virus, from the wound as is possible by an efficient wound toilet that should not involve additional trauma. Since the rabies virus can persist and even multiply at the site of bite for a long time, wound toilet must be performed even if the patient reports late.

This can be done by prompt and gentle thorough washing with soap or detergent and flushing the wound with running water for 10 minutes. If soap and detergent are not immediately available wash with running water for at least 10 minutes. Avoid direct touching of wounds with bare hands. Considering the importance of this step the anti rabies clinics should have wound washing facilities.

The application of soil, chillies, oil etc. is unnecessary and damaging. In case soil, chilies, oil etc. have been applied on the wound, enough gentle washing with soap or detergent to remove the extraneous material especially oil should be done followed by flushing with copious amount of water for 10 minutes immediately .

It should be noted that the immediate washing of the wound is a priority. However, the victim should not be deprived of the benefit of wound toilet as long as there is an unhealed wound, which can be washed even if the patient reports late. The maximum benefit of the wound washing is obtained when fresh wound is cleaned immediately. **Suturing** of wound should be avoided as far as possible. If unavoidable, minimum loose sutures should be applied after adequate local treatment along with proper infiltration of anti rabies serum.

Cauterization of wound is no longer recommended as it leaves very bad scar, and does not confer any additional advantage over washing the wound with water and soap. Inj. tetanus toxoid should be given to the unimmunized individual. To prevent sepsis in the wound, a suitable course of an antibiotic may be recommended.

Application of antiseptic
After thorough washing and drying the wound, any one of the available chemical agents should be applied: Savlon (in appropriate recommended dilution), Dettol (in appropriate recommended dilution), povidone iodine, alcohol etc.

Local infiltration of rabies immunoglobulins

In category III bites rabies immunoglobulins should be infiltrated in the depth and around the wound to inactivate the locally present virus.

3.7.3 Passive Immunization by rabies immunoglobulin

Antirabies serum/ERIG: The antirabies serum provides passive immunity in the form of ready-made antirabies antibody to tide over the initial phase of the infection. Antirabies serum (ARS) has the property of binding with the rabies virus, thereby resulting in the loss of infectivity of the virus. A purified version of this antirabies serum called as equine rabies immunoglobulins (ERIG) is also now available.

Human Rabies immunoglobulins (HRIG): HRIG are free from the side effects encountered in a serum of heterologous origin, and because of their longer half life, are given in half the dose of equine antirabies serum. The antirabies sera should always be brought to room temperature (20 – 25^{0}C) before use.

Dose of rabies immunoglobulins : The dose of equine anti rabies serum is 40 i.u. per kg body weight of patient and is given after testing of sensitivity, upto a maximum of 3000 i.u.   The ARS produced in India contains 300 i.u. per ml.   The dose of the human rabies immunoglobulins (HRIG) is 20 i.u. per kg body weight (maximum 1500 i.u.). HRIG does not require any prior sensitivity testing. HRIG preparation is available in concentration of 150 i.u. per ml.

In Category III of animal bites, the antirabies serum after sensitivity test is infiltrated in and around the wound even if the lesion has begun to heal followed by administration of antirabies vaccine.

Tolerance and side effects. With HRIG, there may be transient tenderness at the injection site and a brief rise in body temperature which do not require any treatment. Skin reactions are extremely rare. HRIG must never be given intravenously since this could produce symptoms of shock, especially in patients with antibody deficiency syndromes. With antisera of equine origin, in addition, anaphylactic shock may occur and thus sensitivity testing is mandatory before giving ERIG. Skin test may be performed as per the manufacturer's instructions given in the product insert. Otherwise as a general guidelines the heterologous immunoglobulin may be diluted 1:10 in sterile physiological saline and 0.1-0.2 ml may be given intradermally in the dorsal aspect of the forearm. An equivalent intradermal injection of physiological saline solution may be used as a control. The reading made 15 minutes later may be considered to be positive if erythema (>6 mm), local odema or systemic reaction is observed and the control is negative.

 A negative skin test must never reassure the physician that no anaphylactic reaction will occur. Those administering ERIG should always be ready to treat early anaphylactic reactions with adrenalin. The dose is 0.5 ml of 0.1 percent solution (1 in 1000, 1mg/ml) for adults and 0.01 ml/kg body weight for children, injected subcutaneously or IM. If patient is sensitive to ERIG, HRIG should be used.

Serum sickness occurs in 1% to 6% of patients usually 7 to 10 days after injection of ERIG, but it has not been reported after treatment with HRIG.

The total recommended dose of immunoglobulin must not be exceeded as it may reduce the efficacy of the vaccine. If the calculated dose of immunoglobulin is insufficient to cover infiltration in all wounds, sterile saline can be used to dilute 2 or 3 fold to permit thorough infiltration.

If immunoglobulin was not administered when vaccination was begun, it can be administered upto the seventh day after the administration of the first dose of vaccine. Beyond the seventh day, Rabies Immunoglobulin (RIG) is not indicated since an antibody response to anti rabies vaccine is presumed to have occurred.

Immunoglobulin should never be administered in the same syringe or at the same anatomical site as vaccine.

3.7.4 Active immunization - Antirabies vaccines

The currently available antirabies vaccines in India can be grouped on the basis of the substrate used to grow the vaccine virus strain.

Name of the vaccine	Fixed virus strain	Substrate	Available
1. <u>Neural tissue vaccine</u> BPL inactivated sheep brain vaccine (Semple type)	PV – 11	Sheep brain	Production stopped since December 2004
• <u>Cell Culture vaccines</u> i) Human Diploid Cell Vaccine (HDCV)	Pitman Moore (PM)	MRC-5	Imported
ii) Purified Chick Embryo Cell Vaccine (PCEC)	LEP-Flury	Primary SPF chick embryo cells	Produced locally in pvt. Sector
iii) Purified Vero Cell Rabies Vaccine (PVRV)	Pitman Moore (PM)	Vero Cells	Imported + produced locally in public sector
3. Purified Duck Embryo Vaccine	Pitman Moore (PM)	Duck Embryo	Imported

3.7.4.1 Neural Tissue Vaccine (NTV)

The Neural Tissue Vaccines (NTV) have been effective but as it contains less antigen, daily injections for 10-14 days followed by boosters are required to produce a protective level of antibody. The production of NTV has been stopped in India in view of serious Neuroparalytic reaction since December 2004.

3.7.4.2   Tissue Culture Vaccines (TCV)

There has been a growing use of Tissue Culture Vaccines (TCV) in India. Three type of vaccines that are currently available are:

- Human diploid cell strain vaccine (HDCV)
- Purifed chick embryo cell vaccine (PCEC)
- Purified Vero cell vaccine (PVRV)

As recommended by the WHO Expert Committee on Rabies (1992), the course for postexposure prophylaxis should consist of five injections (Day O, 3, 7, 14 and 28) and the sixth injection (D90) should be considered as optional. (should be given to those individuals who are immunologically deficient, and are at the extremes of age and on steroid thereapy). Day 0 indicates date of first injection

The dose of the vaccine per injection is 1 ml for HDCV and PCEC vaccines and 0.5 ml for PVRV irrespective of age and weight of vaccinee. The dose of PVRV produced by PII Coonoor is 1 ml per injection.

Indications.   All cases of animal bites, irrespective of severity of exposure, require the same number of injections and dose per injection.   The Category III require administration of rabies immunoglobulins as discussed earlier. The general indications remain same as discussed under neural tissue vaccines.

Site of inoculation.   The deltoid region is ideal for the inoculation of these vaccines. Gluteal region is not recommended because the fat present in this region retards the absorption of antigen and hence impairs the generation of optimal immune response.

Storage and transportation.   Though tissue culture vaccines are marketed in freeze dried (lyophilized) form which is more tolerant of vagaries of temperature, yet it is recommended that these vaccines should be kept and transported at a temperature range of 2-8°C. Freezing does not damage the vaccine but there are chances of breakage of ampoule containing the diluent.

Reconstitution and storage.   The lyophilised vaccine should be reconstituted with the diluent provided with the vaccine immediately prior to use. However, in case of unforeseen delay it should not be used after 6-8 hours of reconstitution.

Protective level of antirabies antibody. Humoral antibodies are believed to play important role in protection against rabies and a titre of 0.5 i.u./ml or more in serum is considered as protective.

Adverse effects with tissue culture vaccines.   The tissue culture vaccines are widely accepted as the least reactogenic rabies vaccines available today. Various studies have now shown that adverse effects can be either general in nature or allergic in origin. The general adverse reactions include sore arm, headache, malaise, nausea, fever and localised oedema at the site of injection. Symptomatic treatment may be needed.

3.7.4.3 Purified Duck Embryo Vaccine (PDEV)

A purified version of duck embryo vaccine (PDEV) has been prepared because of the excellent yield of rabies virus obtained from embryonated eggs and the economy in production. the PDEV has been claimed to be as immunogenic and safe as other anti-rabies Tissue Culture Vaccine. Its dosage and schedule is similar to that of tissue culture vaccine.

3.7.5 Intradermal regimen

Recently the administration of tissue culture vaccine by intradermal route has been evaluated in humans in an effort to reduce the number of vaccination and amount of vaccinee thereby considerably reducing the cost. Two intradermal regimens have been demonstrated to be immunogenic 2-2-2-0-1-1 and 8-0-4-0-1. However, it requires especially trained staff to administer intradermal injections and hence feasibility of this regimen in small rural hospital is questionable. The reconstituted vaccine should be used as soon as possible and no later than 6 to 8 hours if kept at $4\text{-}8^0C$ as there could be a risk of contamination. It has also been seen that sero conversion with intra dermal route is low when simultaneous anti malarial treatment with chloroquin is being used. WHO strongly advocates the use of TCV for post exposure prophylaxis by intradermal route. Sri Lanka, Phillipines and Thailand have already taken up intra dermal route for PET. This route, however has not yet been cleared by Drug Controller General of India. It would need to be validated by conducting separate trials for these regimens.
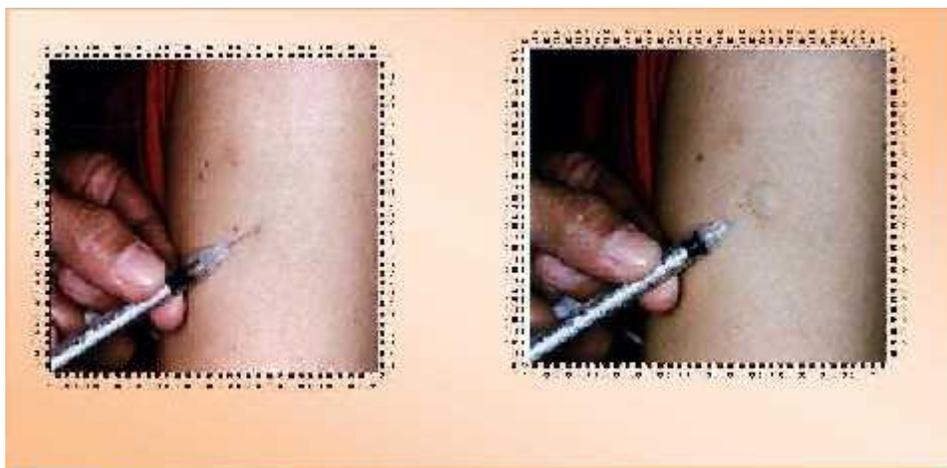
Fig 3.1. ID inoculation

3.7.6 Post exposure therapy for previously vaccinated persons

Managing re-exposure following post-exposure treatment with TCV

If re-exposed, persons who have previously received full post-exposure treatment with a potent cell-culture vaccine should be given only two booster doses, intramuscularly on days 0 and 3, but no rabies immunoglobulin.

Managing exposure following pre-exposure prophylaxis with TCV

If after recommended pre-exposure prophylaxis, a vaccinated person is exposed to rabies, a proper wound toileting should be done and two IM doses of Tissue Culture Vaccine be given on days 0 and 3. Treatment with RIG is not necessary

3.7.7 Approach to a patient requiring rabies immunoglobulins when none is available

In circumstances where no immunoglobulins are available greater emphasis should be given to proper wound toileting followed by Essen schedule of Tissue culture vaccine with double dose on day 0 at 2 different sites intramuscularly (0 day – 2 doses on left and right deltoid, 3, 7, 14 and 28 days).

3.7.8 Management of animal bite exposure to pregnant women and lactating mothers

Pregnancy and lactation are no contraindications for rabies vaccination. Post-exposure prophylaxis against rabies takes preference over any other consideration since it is a life saving procedure. Moreover, rabies vaccine does not have any adverse effect on fetus, mother-to-be and the course of pregnancy. Hence complete post-exposure treatment should be given depending on the category of the exposure.

3.8 PRE-EXPOSURE PROPHYLAXIS

Pre-exposure prophylaxis may be offered to high risk group like laboratory staff handling the virus and infected material, clinicians and para-medicals attending to hydrophobia cases, veterinarians, animal handlers and catchers, wildlife wardens, quarantine officers and travellers from rabies free areas to rabies endemic areas. Pre-exposure immunization should be three full IM dose of TCV given on day 0, 7 and 28 or 0, 28 and 56 followed by booster at one year and then a booster every three years.

Laboratory staff and others at high continuing risk of exposure should have their neutralizing antibody titres checked every 6 months. If it is less than 0.5 i.u./ml a booster dose of vaccine should be given. Such individuals on getting exposed to rabies virus after successful pre-exposure immunization require only two booster injections of vaccine given on days 0 and 3 without any anti rabies serum.

3.9 CONTROL OF RABIES

Rabies is primarily a disease of animals and control measures have to be directed towards the natural reservoir of the disease. Wild animals act as important and frequent reservoirs of disease in developed countries where as developing countries till have canine rabies as their major problem. The issue of control of rabies in wild animals is quite complicated and tremendous research is being conducted to develop effective tools to understand its dynamics. Development of suitable oral vaccines and appropriate delivery systems are the areas in which notable progress has been made.

Any strategy for control of rabies in developing countries shall have following four components:

- Epidemiolgocial surveillance
- Mass vaccination
- Dog population management and

- Community participation

3.10 LABORATORY DIAGNOSIS

3.10.1 Collection, Preservation, Packing and Transportation of Specimen

Acute infectious nature of rabies does not require further elaboration. Therefore, in the collection of specimen from suspected cases of rabies-human or animal, it must be borne in mind that highly dangerous material is being handled. It is imperative to follow all precautionary measures during collection, packing, transportation and handling of specimen to avoid any serious mishap.

3.10.1.1 Specimen from Human beings
From a clinical case (hydrophobia) the antemortem specimen that may be collected include saliva, corneal smear, skin biopsy, hair follicles, blood and cerebrospinal fluid. Postmortem specimen shall be usually of brain or spinal cord and sometimes other organs too. The specimens are collected as follows:

Saliva/Sputum
Saliva is collected from under the tongue:
1. Wet a sterile cotton swab with tissue culture medium or physiological saline and remove excess medium by squeezing on the sides of the vial.

2. Swab under the tongue, rinse in the tissue culture medium or physiological saline containing 2% normal horse serum. (NHS)

3. Take another swab similarly and make two smears each on clean labelled glass slides.

4. Air dry the glass slides for 10minutes.

5. Discard the swabs in suitable disinfectant.

6. Treat the slides immediately with chilled acetone and process or wrap in paper and despatch to the laboratory.

Often due to dehydration, there is very little saliva in the mouth. The patient, if responsive, may be asked to cough and spit in petri dish or beaker. Mix the sputum with a few ml of tissue culture medium or 2% NHS in physiological saline and transfer to screw capped vial.

Corneal Smears
a) Retract the eyelids with thumb and one finger and press a clean marked slide against the cornea.

b) Prepare two smears on each slide taking care to apply sufficient pressure to get the smear.

c) Avoid exerting too much pressure as it may damage the eye.

d) Air-dry the smears for 10-15minutes at room temperature.

e) Treat with chilled acetone and process further

Skin Biopsy

With very fine sharp scissors collect small pieces of skin from the site of bite and the face near the mandible. Preserve in a vial containing 50% glycerol saline (prepared by mixing equal volumes of glycerol and physiological saline and sterilized by autoclaving).

Hair Follicle

Pluck a few hairs with the help of forceps from the face and/or behind the ear. Put in a vial containing 50% glycerol saline.

Cerebrospinal Fluid (CSF)

The CSF in acute phase of the disease is processed for isolation of the virus and in the later phase for antibodies. It is collected by lumbar puncture. Usually no preservative is used but, if required, 50% glycerol saline may be used.

Blood

Acute phase venous blood specimen is collected as soon as possible with the usual aseptic precautions. If the patient survives for several days, a second sample is taken. In case the patient recovers another blood sample is taken before discharging the patient.

Urine

a) Collect in wide mouth containers.

b) Mix with equal quantity of tissue culture medium

c) Centrifuge immediately & discard the supernate.

d) Mix sediment with 2-3 ml of tissue culture medium and transfer to screw capped vials.

Brain

The brain is collected at autopsy. Many times the relatives do not agree for a full postmortem. In such cases Vim-Silverman needle maybe used to collect a small piece of brain sample, which is then put in 50% glycerol saline.

3.10.1.2 Collection of specimen/rom suspected rabid animals

The specimen useful for proper diagnosis of rabies in animals are mainly brain and salivary glands. Though it is risky to collect antemortem specimen, if required, saliva and corneal smears may becollected as already described. The better course, however, is to permit the animal to die natural death unless otherwise required (see microscopic examination). Facilities for removal of the animal brain and salivary glands are are not available in the laboratory and hence the whole brain or salivary glands should be sent to the laboratory after post-mortem.

If it is not possible to send the whole brain, pieces from Ammons horn of hippocampus, cerebrum, cerebellum, pons and medulla may be included.

3.10.1.3 Preservation

If possible the samples of brain and salivary glands may be sent in widemouth leakproof containers

Preserved on ice. However, if the samples are to be sent long distance these may be preserved by Use of following:

a) 10% formol saline/Zenker's fluid for half of the brain

b) 50% glycerol saline for other half of the brain and salivary glands.

c) Tissueculturemedium2%NHS saline for saliva, CSF, urine etc.

3.10.1.4 Labelling

All the specimen e.g. slides, vials must be labeled with number of specimen, name of the patient, or species of the animal, type of preservative used etc. Permanent markers should be used. The parcels should also be labeled properly.

3.10.1.5 Information To Be Enclosed
a) Hydrophobia:- name, age, sex, treatment taken, exposure to animal etc. may be enclosed.
b) Animal:- The species and breed of animal, contact with other animals, symptoms, mode and date of death, vaccination status etc.

3.10.1.6 Packing
a. It should preferably be wide mouth leakproof plastic containers.

b. Seal the mouth of the container with tape or sealing paraffin.

c. Pack in plastic bags and put in thermocol box with sufficient ice.

d. If sending by post pack in sturdy wooden boxes with sufficient packing material (preferably absorbent cotton/saw dust/paddy husk).

3.10.1.7 Transportation
a) By courier
b) By air/by post.

Utmost urgency should be exhibited in trasportation of these specimens because any undue delay, especially in tropical climates, shall witer away the cooling effect of ice and result into putrefaction of the sample making it unsuitable for the diagnosis.

3.10.2 Laboratory tests

Many tests have been reported from time to time for the detection of rabies antigen/virus, assessment of rabies antibodies, study for the morphology and growth characteristics of rabies virus. The use of these tests in a diagnostic laboratory depends on the availability of appropriate facilities. The tests may be for detection of rabies antigen in ante-mortem specimens like corneal smear, CSF of saliva etc. or postmortem specimens which include brain and salivary glands. The rapidity of test and early reporting help in a big way in the treatment of humans and animals bitten by rabid animals.

Following laboratory tests are usually employed for studies on rabies :-

3.10.2.1 Negri Bodies Examination
This test is simple and quick in diagnosis of rabies and can be performed easily and quick report can be given. The intracytoplasmic inclusion bodies called "Negri bodies" can be detected by using various stains of which Seller's stain is the simplest and widely used. Both impression smears and sections of tissue can be stained. The presence of these bodies depends on the time of death and the course of the disease. The chances of detection of Negri bodies increase if the animal is permitted to die its natural death. Nearly 70% of the specimen from rabid animals can be diagnosed by this test. Other encephalopathic changes can also be studies from the stained preparations.

3.10.2.2 Fluorescent Antibody Test (FAT)
The test is more specific being an immunological reaction between specific antigen and antibody. It requires slightly more time than Negri bodies examination but is quick enough of being importance in early diagnosis. Direct FAT, which is commonly used, requires an antibody conjugated to a dye fluorescein isothiocyanate (FITC). The test is performed with the help of an ultraviolet lamp attached microscope. Indirect FAT can also be used for detection of antibodies in the serum.

3.10.2.3 Mouse Inoculation
The white laboratory mice are susceptible to rabies virus infection and develop illness after inoculation of any material having viable rabies virus. The test requires approximately 21-30 days for completion and this is the biggest disadvantage with this ideal test.

3.10.2.4 Serum Virus neutralization Test
The test is very specific and is an antigen antibody reaction. This test may he used for identification of the isolated virus or for assessment of antibodies present in the serum or other body fluids. The test is mainly performed *in vivo.*

3.10.2.6 Counter Immunoelectrophoresis (CIE)
The test is quick, economical and simple to perform and can be sued for assessment of antibodies in the serum. Being a specific antigen antibody reaction it is used as a serum neutralization test also.

3.10.2.7 Enzyme Linked Immunosobent Assay (ELISA)
The test uses the property of certain enzymes to act on their substrates to give out coloured visual end products. The test being very sensitive requires standardization for specificity. It can be used for assessment of antibodies as well as detection of rabies virus antigen.

3.10.2.12 Electron Microscopy
This requires processing of tissue and virus suspension in a specific procedure and is helpful in study of structure and morphology of rabies virus. A sophisticated electron microscope and skilled persons with training are pre requisites. Its main application is undertaking fundamental research.

3.10.2.13 Tissue culture Techniques

The use of tissue culture cells is made both for diagnosis by detection of rabies virus in inoculated cells by FAT and for assessment of antibodies add commonly known as Rapid Fluorescent Focus Inhibition Test (RFFIT).

3.10.2.14 Polymerase Chain Reaction

The test is quick, sensitive and specific but it technically demanding. Viral RNA is extracted from the tissue and is converted to c-DNA, which is then amplified and detected (RT-PCR). Besides diagnosis of infected samples, PCR is useful for characterization of infecting rabies virus strain and molecular epidemiology. However, it is feasible only in the reference laboratories.

3.10.3 Principles and procedures of commonly used techniques

3.10.3.1   Seller's Staining for Detection of Negri Body

Principle

Many virus infections are associated with presence of inclusion bodies in the infected cells. These inclusion bodes may be intranuclear or inteacytoplasmic – acidophilic or basophilic in nature dpending on their reaction with the stains used. The presence of rabies infection can be demonstrated by the presence of intracytoplasmic inclusion bodies commonly known as Negri bodies. The intracellular nature of Negri bodies may be altered in impression smears dur to the rupture of cells when these may be found both intracellular and extracellular. The Negri bodies are aciodphilic in staining reaction and are seen as pink to purplish-pink in colour in differential stains that use basic fuchsin or eosin with methylene blue as their base.

Reagents and Equipment

a) Seller's stain

b) Light microscope

c) Glass slide (5 x 7.5 cm and 2.5 x 7.5 cm)

d) Scissors and forceps

e) Filter paper

Preparation and Staining of Slides

The brain sample received in the laboratory may be unpreserved or preserved in 50% glycerol Saline or 10%formolsaline. For demonstration of Negri bodies the unpreserved specimen can be processed as such. The formalin preserved specimen can be used for histopathology. The glycerinated specimen can be used for making impression smears and biologic test like unpreserved specimen but the presence of glycerine interferes with the adherence of tissue to the glass slide. Therefore, the glycerine must be washed off by immersing the tissue pieces in physiological saline for 30minutes or more during which at least three changes of physiological saline are required to wash off the glycerine.

The procedure for preparation of smear and its staining is as follows:

a) With a pair of scissors make a longitudinal incision about 3-5 cms from the occipital pole into the dorsal surface of cerebral hemisphere approximately 2 cm lateral to midline of the brain. .

b) Cut through to lateral ventricle.

c) Widen the opening to expose the hippocampus on ventricle floor.

d) The hippocampus can be seen as white, glistening, semicylinderical and curved body.

e) Cutout a small piece of hippocampus (0.5-1cm) and place it on a spatula/filter paper with cut surface facing upwards.

f) Place the filter paper on a glass slide (5 x 7.5cm)

g) Lightly sponge the cut surface with the edge of a filter paper to remove blood.

h) Press a clean microscope slide on the tissue piece on spatula/filter paper to get an impression smear.

i) Make at least 3 smears on each slide.

j) While the smears are wet, flood the smear with working stain.

k) Stain for 2-3 seconds.

l) Quickly wash with tap water by gently flushing the slide.

m) Air dry the smear.

n) Examine under oil immersion.

Observations
Following may be observed:

Nerve cells	:	Blue cytoplasm and dark blue nucleus
Stroma	:	Pink
Erythrocytes	:	Copper coloured
Negri bodies	:	Magenta to dark red with dark blue or black inner granules

Similarly smears can be prepared from cortex and cerebellum and examined for Negri bodies.

Other Inclusion Bodies
Some of the inclusion bodies in normal brains of cats, foxes, mice etc. may appear similar to the Negri bodies to an inexperienced worker. The inclusion body due to some other diseases in dogs e.g. distemper may also stain similar to Negri bodies. However, these can be differentiated from Negri bodies on the basis of following features:

Negri bodies	Other Inclusion bodies
a) Oval,round or elongated	Oval or round
b) Size 3-20J..lm	Small to large

c) Presence of basophilic inner granules and heterogenous matrix	Homogenous matrix without granules
d) Less refractile	More refractile
e) Mostly abundant in hippocampus	Rare in hippocampus

3.10.3.2 Fluorescent Antibody Test

Principle

Rabies specific antibodies and antigen when mixed and kept under optimum conditions combine to form an antigen-antibody complex. This complex is not visible to naked eye. To detect this complex use is made of dyes like fluorescein isothiocynate (FITC) which emit fluorescence when exposed to ultraviolet rays. The FITC is tagged with anti rabies antibody to form the conjugate. If the detection of antigen is a one step procedure, it is called a direct fluorescent antibody test (FAT).If more than two steps of combination of antigen and antibodies are required for detection, it is known as indirect immunofluorescent test (IFAT)and is employed for detection of rabies antibodies.

Reagents and Equipment
  a)   Fluorescent microscope

  b)   Incubator

  c)   Antirabies FITC conjugate (pretitrated)

  d)   10%eggyolk in phosphate buffer

  e)   Normal mouse brain emulsion (NMB)

  t)   Infected or rabid mouse brain suspension (1MBor RMB)

  g)   Phosphate buffer saline pH 7.5

Procedure
  a) Prepare two impression smears of approximately 1cm diameter about 1.5cm from each end of the labelled slides. Prepare at least 4 slides each from the cerebrum, hippocampus, midbrain, cerebellum and medulla of brain.

  b) Air-dry the smears for 25-35 minutes at room temperature.

  c) Immerse the slides in coplin jars containing chilled acetone in a deep freeze at
  d) -20°C to -25°C.

  e) Keep for 4 hrs to overnight.

  f) Drain off the acetone and store slides at-20°C till stained.

  g) Make the requisite dilution of the conjugate separately in two tubes containing NMB and 1MB respectively.

  h) Keep in an ice bath for 30 minutes.

i) Take out the impression smear slides from deep freeze and dry at room temperature for 20 minutes. Include known positive and negative smears also.

j) Mark the outline of the smears with grease marking pencil or wax pen.

k) To the smear away from labelled side add properly mixed conjugate-1MB mixture with the help of a pasteur pipette.

l) To the smear near to labelled side add conjugate-NMB mixture.

m) Place the slides in a chamber with moist filter paper at the bottom.

n) Cover the chamber and keep at 37°Cfor 30 minutes.

o) Wash the slides in 0.01 M phosphate buffered saline (pH 7.5) for 5 minutes.

p) Repeat washing.

q) Remove from buffer and dip in ajar of distilled water for 5 minutes with gentle shaking (Step n, 0 and p can also be performed by gentle agitation using a magnetic stirrer).

r) Remove from distilled water and dry the slides at room temperature.

s) Mount in 90% buffered glycerol (pH 8.5).

t) Examine under a microscope with ultraviolet source of light (fluorescent microscope), the known positive, known negative and test slides. A dark ground condenser is useful.

Observations

Rabies antigen is seen as fine dusty particles emitting bright to dull yellowish green fluorescence.

A rabies positive smear will show fluorescence on the NMB-conjugate treated side. There is almost no fluorescence on the RMB-conjugate treated smear.

3.10.3.3 Polymerase Chain Reaction Test

Principle

Rabies virus is an RNA virus and hence before amplification the target RNA segment is converted
to c-DNA with the help of enzyme reverse transcriptase (RT). c-DNA is then amplified using Taq polymerase. This is called RT-PCR.

Primers

For diagnosis of rabies and rabies related isolates, regions on N gene are selected as primers since

N gene is highly conserved region on the viral genome and it is transcribed in the highest amounts

during multiplication.

N((+) sense   :   (587) 5'-TTT GAG ACT GCT CCT TTT G-3' (605)

N 2 (-) sense :   (1029) 5' - CC CATATAGCA TCC TAC- 3' (1013)

For typing and molecular studies the highly variable area, rabies pseudogene is selected. It is a non protein-coding region and is highly susceptible to mutation and therefore is most suitable for detecting strain variation and closely related isolates.

G (+) sense   :   (4665) 5'-GAC TTG GGT CTC CCG AAC TGG GG-3' (4687)

L (-) sense   :   (5543) 5' -CAAAGGAGA GTTGAG ATTGTAGTC-3' (5520)

Procedure
- RNA extraction
- c-DNA synthesis
- c-DNA amplification
- Detection of PCR products

RNA extraction
Homogenize the infected brain tissue with lysine buffer to lyse the cells, cell membranes and nuclear membrane. This can be done by manual method, I-phenol, 2 phenol/chloroform (1:1) and l-chloroform as described by Tord oe tal or by RNA extraction kits (TRlzol, Life technologies)

c-DNA synthesis
Anneal the RNA (lmg) with the N_1 primer (100mg) at 65°C for 3 minutes. After chilling on ice, add murine leukemia virus reverse transcriptase and incubate for 90minutesat 42°C.

c-DNA amplification by PCR
To the c-DNA reaction, add PCR buffer containing 100 mg of N_2 primer and 2 units Taq polymerase and subject it to five initial cycles of denaturation (D) 60 seconds at 94°C, annealing (A) 90 seconds at 45° C and elongation (E) 90 seconds at 72° C followed by 30 additional cycles of D for30 seconds at 94°C, A for 20 seconds at 50°C and E for 60 seconds at 72°C.Carry out the final elongation at 72°C for 10 minutes.

Detection of PCR product by polyacralmide gel electrophoresis
To 1.2% agrose gel add 0.5mg/ml.of ethidium bromide and pour in trough with combs to make wells. Add approximately 250 ml of 1X electrophoresis buffer to there servoir tank. Mix 20ml of PCR product with 2m! of loading dye (containing bromophenol blue, xylene cynol and glycerol) and load in the well. Add 100 bp DNA ladder to the first lane. Switch on the power supply, adjust to 100V and leave it for electrophoresis. Switch off the power supply when the dye front is about *0.5* cm from the positive end. View the gel in UV transillumination. DNA segment can be seen at 430 bp.

Precautions

- Always wear gloves while handling the samples and reagents
- Frequently change gloves while handling RNA
- Carry out high-speed mixing and centrifugation procedure in tightly closed container and in a biological safety cabinet.
- Avoid mouth pipetting
- Always include a positive control (infected mouse brain) a negative control (uninfected mouse brain) and a negative PCR control (Add water instead of the template) in each run of PCR.

Uses

PCR is probably not going to be very practical diagnostic tool. Its main use will be for strain differentiation while using it as an epidemiological marker.

3.10.3.4 Biological Test
Introduction

The growth of rabies virus, just lie any other virus takes place only in living tissues. These may be animals, developing chick embryo or tissue culture. Rabies virus has been found to be pathogenic to all mammals when given by intracerebral route. However, the animals commonly used for experimental studies of rabies are mouse, rat, guinea pig, hamster, rabbit and dog. The white laboratory mice are susceptible to infection and are easy to handle in routine laboratory examination of specimen. Suckling mice are more susceptible than the adult but a regular supply of these may not be possible in all the laboratories. The presence of rabies virus in any specimen can be detected by intracerebral inoculation of specimen into mice and observing the animals for sufficient period for the development of sickness and death due to rabies.

Reagents and Equipment

    a)   2% Normal horse serum (NHS) in distilled water.
    b)   3-4 weeks old mice and mice cages.
    c)   Centrifuge.
    d)   Centrifuge tubes.
    e)   Pestle mortar, tissue grinder or omni mixer.
    f)   Scissors and forceps.

Procedure
Brain

    a)   Aseptically collect 3-4 gm or pieces of brain tissue of approximately 1 cm diameter cut out from different areas of the brain, i.e. hippocampus, cerebrum, cerebellum, medulla, pons etc.

    b)   By weighing, calculate the exact weight of the tissue pieces.

    c)   Homegenise these pieces to make a fine paste with the help of a pestle mortar/tissue grinder/electric grinder/omnimixer.

    d)   Add enough chilled distilled water containing 2% inactivated normal horse serwn (serwn should be collected from horses not vaccinated against rabies) to make a 10% suspension. Mix thoroughly while adding the diluent.

e) Transfer to a sterile 15 ml centrifuge tube.

f) Centrifuge at 1000-1500 rpm for 5 minutes.

g) Collect the supernatant with the help of pipette in a Bijou bottle/halftube kept in ice bath.

h) Add 100 units/ml penicillin and 50~g/ml of streptomycin and keep for 30 minutes

i) Inoculate into 3-4 weeks old white mice by intracerebral route with 1f4 inch long 26/27 gauze needle in 0.03 ml dose/mice. Use at least eight mice for each inoculum.

Observe for 21 days for signs like roughening and loss of lustre of the fur, tremor, hyperexicitability, arching of the back, convulsions, paralysis of the hind legs and death.

k) Check any mouse dying after 72 hours for presence of Negri bodies in brain by Seller's staining or antigen by FAT.

Salivary Gland

Grind the salivary glands with the help of sterile sand or sterile coarse glass powder in a pestle and mortar and repeat the processes from step 5 onwards as described for brain.

Further Reading

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LEISHMANIASIS

4.1 INTRODUCTION

Leishmaniasis is an infectious disease caused by the protozoan parasite leishmania. -Across the tropics three different diseases are caused by various species of genus Leishmania. These are:

Visceral leishmaniasis: *L. donovani*
Cutaneous leishmaniasis: *L. tropica*
Mucocutaneous leishmaniasis: *L.braziliensis*

Though these species are morphologically very similar, yet produce three strikingly different diseases. The infection may be predominantly visceral as in kala-azar or visceral leishmaniasis; it may be restricted to the skin, producing the chronic ulcer of the oriental sore, or it is concentrated in the mucuous membranes which are gradually eroded to produce the grossly disfiguring and often fatal condition known as espundia. Also, subsequent to the curing of a visceral leishmaniasis infection, parasites may occur in large numbers in the skin, to which they are restricted, causing the condition post kala-azar dermal leishmaniasis (PKDL).

4.2 EPIDEMIOLOGY

Disease Burden:

Global scenario:-

The leishmaniasis have an immense geographical distribution in the tropics and sub tropics of the world extending through most of the Central and South America, part of North America, Central South East Asia, India, China, the Mediterranean region and Africa. Australia, the Pacific islands and Japan are free of leishmaniasis.

The annual incidence of reported global KA cases is 58,200 of which 42,619 (above 70%) is contributed from the Indian Subcontinent (ISC). The revised global and ISC annual incidence estimate of KA is 201,500–378,500 and 160,000–320,000, respectively, of which more than 90% cases occur in six countries, Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan. There are currently no accurate data on the burden of Post Kala-azar Dermal Leishmaniasis (PKDL). VL–HIV co-infection has also emerged as a serious concern and is reported from 36 countries. There is a strong need to establish surveillance for both the conditions. An estimated 1470 lakhs people in 119 districts in 4 countries, namely Bangladesh, Bhutan, India and Nepal, are at risk with an estimated 20,000 new cases each year. India alone accounts for about 50% of the global burden. The proportion of unreported cases is yet to be established but estimates range from 0.2 to 4 times of the reported cases.

Indian scenario:-

In India, visceral leishmanaisis is a major public health problem. The resugence of kala-azar in India, beginning in the mid 1970's, assumed epidemic proportions in 1977 and involved over 1,10,000 cases in man.

Kala-azar is at present endemic in 54 districts in the country of which 33 districts of Bihar, 4 districts of Jharkhand, 11 districts of West Bengal besides occurrence of sporadic cases in 6 districts of eastern Uttar Pradesh. The state of Bihar alone contributes more than 70% of total KA reported from the four states. In 2013, the cases have declined by 38% and 33% respectively in comparison with the year 2011. Mortality has also reduced from 90 deaths in 2011 to 20 in 2013. The same trend is observed during 2014 so far.

4.3 CAUSATIVE AGENT

The causal agent of VL and PKDL in India is the parasite Leishmania donovani. There are two morphologically distinct stages in the life cycle of the parasite, namely:

• Amastigote (aflagellate) or Leishmania stage: occurs in man
• Promastigote (flagellate) or Leptomonad stage: occurs in gut of (a) sandfly and (b) artificial culture

Amastigote stage:

The amastigote is the intracellular, non-motile form in the vertebrate host, and it divides by longitudinal binary fission at 37°C.This intracellular stage is found particularly in the reticuloendothelial system. The amastigote is also called the Leishman-Donovan (LD) body. This stage is represented by round or oval body measuring about 2 to 6 micrometers in length and 1 to 3 micrometers in width. Within cytoplasm, a relative large nucleus, a prominent kinetoplast, and a short axoneme, the last of which consists of a rod like body and a dot like basal body are present. Both nucleus and kinetoplast contain deoxyribonucleic acid. The parasite prefers to stay in macrophages like monocytes, lymphocytes and the cell of bone-marrow, spleen and liver.

Promastigote stage or Leptomonad Stage:

These forms are found in culture medium as well as digestive tract of sand fly. The promastigote has a single free flagellum arising close to kinetoplast at the anterior end and possesses marked motility. The average length is 15-20 μm and 1.5-3.5 μm in width. The flagellum measures 15-28 μm. In old culture, these promastigote tend to form rosettes with their flagella centrally directed. Short, board and rounded forms may be seen in these old cultures.

Man is the only known host of Leishmania donovani in India. People of all ages are susceptible to the disease but it occurs mainly in older children (between 5-14 years of age) and young adults. Sub-clinical cases of Kala-azar have been reported from Bihar. The disease is widely prevalent among individuals with low socio-economic status, poor standard of living;

malnutrition etc. Susceptibility of the host appears to be a critical factor in determining infection and the disease. The way of acquiring the infection is however, not well understood.

4.3.1 Vector

Visceral Leishmaniasis or Kala-azar is transmitted by the bite of infected sandflies. Phlebotomus argentipes is the only known vector of Kala-azar in India. The seasonal prevalence of this species varies from area to area depending upon the ecological conditions. Disease transmission is highest in the rainy season. The vector breeds in humid soil rich in organic matter and near cattles sheds and mud- houses. It rests most commonly in cracks & crevices of thatched mud-houses. The peak biting time of the vector is around midnight. The extrinsic incubation period in the vector sand flies vary from 4-25 days which is the time required for the vector to become infective after an infective blood meal.
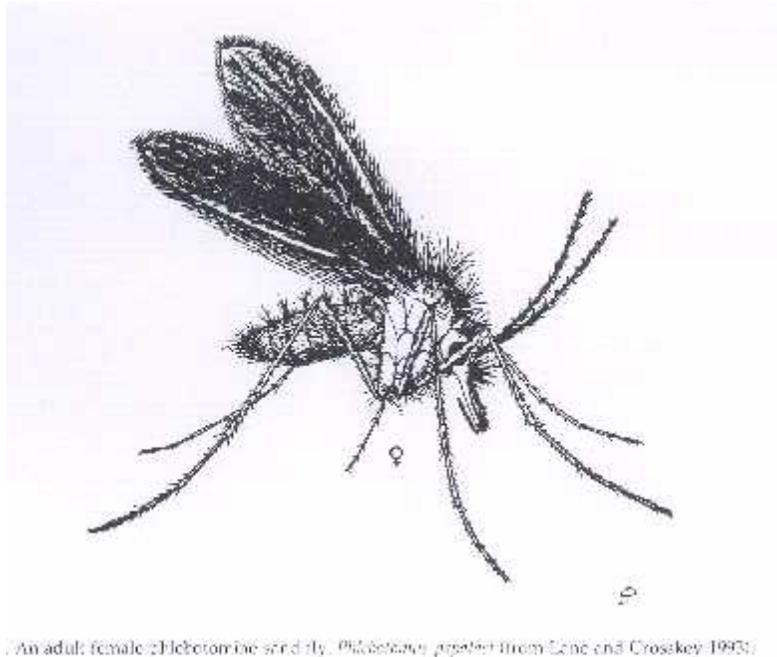
An adult female phlebotomine sand fly, *Phlebotomus argentipes* (from Lane and Crosskey 1993)

4.3.2 Environmental and Ecological factors

The prevalence of vector is dependent upon the environmental factors which include humidity, temperature and rainfall. The ecological factors like alluvial soil, kuccha mud-houses and large scale vegetation also influence build up of vector density.

The parasite primarily infects reticulo-endothelial system and may be found in abundance in bone marrow, spleen and liver. Post Kala-azar Dermal Leishmaniasis (PKDL) is a condition in which the Leishmania donovani invades cells of skin and develops lesions. This results in skin manifestations of PKDL. Some of the Kala-azar cases manifest PKDL after a few years of treatment. Recently it has been claimed that PKDL may appear without passing through visceral stage
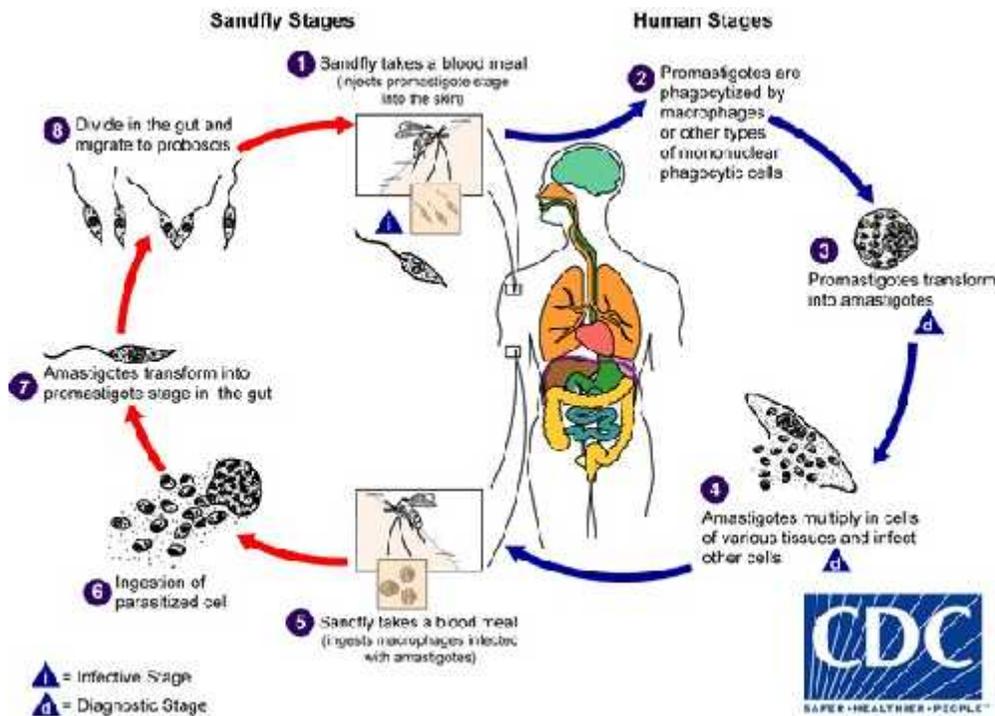
Sandfly Stages

1. Sandfly takes a blood meal (injects promastigote stage into the skin)
8. Divide in the gut and migrate to proboscis
7. Amastigotes transform into promastigote stage in the gut
6. Ingestion of parasitized cell
5. Sandfly takes a blood meal (ingests macrophages infected with amastigotes)

Human Stages

2. Promastigotes are phagocytized by macrophages or other types of mononuclear phagocytic cells
3. Promastigotes transform into amastigotes
4. Amastigotes multiply in cells of various tissues and infect other cells

▲ = Infective Stage
▲ = Diagnostic Stage

Fig 4.1 : Life cycle of *leishmania* parasite (available at http://www.cdc.gov/dpdx/leishmaniasis/index.html).

Kala-azar in India has a unique epidemiological feature of being anthroponotic, i.e. human beings are the only known reservoirs of infection. The female sandflies pick up the amastigote stage (LD bodies) of the parasite while feeding on an infected human host. The parasites undergo development and multiplication in the gut of sand flies to become numerous flagellates (Promastigote or Leptomonad stage) which migrate to their mouthparts. The cycle in the sand flies is completed in about 8 days. Infection is transmitted to healthy human beings when such infective sand flies bite them.

4.4 CLINICAL FEATURES

Kala-azar being a chronic disease, incubation period significantly varies. Generally it varies from 1-4 months but in reality the range is from 10 days to 2 years, however in India the range varies from 4 months to 1 year. Kala-azar is reported amongst all age groups, however in earlier surveys carried out for the purpose of research children in the age group of 5-9 were most afflicted with male to female ratio of 2:1.

The endemic visceral leishmaniasis has a usual incubation period of 3-6 months, which may range from 10 days to 2 years. The patient presents with symptoms of malaise, weight loss, anorexia, cough, diarrhoea and fever. Liver and spleen are enlarged and generalised lymphadenopathy may be present. Darkening of skin of the face, hands, *feet* and abdomen is common in India and these changes are accompanied by signs of malnutrition.

Generalised immunosuppression is also associated with active visceral leishmaniasis and many patients present with secondary infections. Massive fatal gastro-intestinal haemorrhage may occur. In untreated patients, death ensues in as many as 90% cases, which is usually attributed to the concomitant infections.

In many cases, however, the parasites are not completely eliminated and may recrudescence in the skin giving rise to post kala-azar dermal leishmaniasis (PKDL).

This feature of the disease in Indian sub-continent appears within 2 years of recovery of Kala-azar, initially as a faint mottling of the skin. The chronic lesions consist of multiple nodular infilterations of the skin, usually without ulceration. Hypopigmented or erythematous macules on any part of the body may later become nodular, especially on the face. Parasites are numerous in the lesion as well as in normal skin but bone marrow and organ biopsy are negative.

The incubation period of the zoonotic cutaneous leishmaniasis is usually less than 4 months. In this disease painless multiple lesions appear. Some lesion may be severely inflammed and ulcerated and heal in 2 - 8 months.

4.5 LABORATORY DIAGNOSIS

Leishmaniasis, especially kala-azar may present with various clinical manifestations which run across a wide spectrum and at the same time may simulate many other tropical diseases. The prevalence of these diseases in areas endemic for leishmaniasis further impedes in establishing a correct diagnosis. Many a times a patient may have to undergo therapy for diseases like malaria, enteric fever, amoebiasis, pulmonary tuberculosis, brucellosis etc. before he is administered anti kala-azar treatment. This "hit and trial" therapy methods have a wide range of financial, ethical and above all physical implications for the patient, all of which can easily be avoided if the help of laboratory is sought in reaching at the final diagnosis.

In non-endemic areas, laboratory assumes greater importance because otherwise one may not even sufficiently suspect the diagnosis of kala-azar and the patient may not get the treatment for an inordinate long time.

4.5.1 Specimen

The diagnosis of leishmaniasis consists of demonstration or isolation of the parasite from blood or biopsy material and demonstration of Leishmania specific antibodies in the serum. In addition there are a few non-specific tests based upon the deviations in the normal blood picture and the serum proteins, which may also aid in diagnosis.

For the above tests, the following clinical specimens have to be collected:
i)   Blood (serum)
ii)   Biopsy/Aspirate from - Bone marrow
iii)   Spleen.
 Rarely biopsy of lymphnode may also be taken.

i) Blood
It can be collected either through a venipuncture or by finger prick method. By venipuncture 4-5 ml blood should be collected in a plain vial. Allow it to clot and separate the serum.

By finger prick method blood is collected on filter paper. It carries the advantage of easy transportation and requires less storage space. In the laboratory the serum is eluted from filter

paper strips by dipping the circle in 1 m! phosphate buffered saline (pH 7.2) and keeping in refrigerator at +4°C overnight. Squeeze the circle within the test tube and discard the paper.

Since one circle soaks 0.1 ml of blood and final elution of serum is 20µl, the final dilution of serum after elution shall be I :50.

ii) Bone Marrow Aspirate
It can be collected from

a)   Mid - sternal region, a little away from the mid line at the level of second or third intercostal space.

b)   Posterior iliac crest puncture, 1 cm below the superior iliac spine.

iii) Spleen Puncture
When spleen is considerably enlarged it is one of the most valuable method for establishing the parasitological diagnosis of kala-azar.

4.5.2 Demonstration of the parasite

The conclusive evidence in the diagnosis of kala-azar is the demonstration of the parasite. It can be achieved in either of the following ways:

4.5.2.1 Direct
a)   Microscopic examination of the stained film
b)   Culture of the parasite

For the aforesaid, the following clinical specimens are tested:

a)   Blood - Blood film and blood culture
b)   Marrow - Biopsy/Aspirated material obtained by sternal or iliac crest puncture
c)   Splenic tissue - Biopsy material obtained by splenic puncture.

Direct examination
Make smears and stain with Leishman or Giemsa stain. Examine for amstigote forms of the parasite

Giemsa Staining
a)   Fix the film, with pure methyl alcohol or ethyl alcohol for 3 to 5 min and allow it to dry.
b)   Dilute Giemsa stain by adding 1 drop to each 1 ml of neutral or faintly alkaline (pH 7-7.2) distilled water.
c)   Pour diluted stain over the film (about 5 ml/film is required) and keep for 30 to 45 minutes.
d)   Then flush the slide in a gentle flow of tap water.
e)   Examine the stained film under oil immersion lens.

Amastigote forms of the parasite (L.D . Bodies) can be easily demonstrated.

Leishman Staining

a) Pour Leishman's stain over the dried film and allow it to remain for 30 seconds.
b) Dilute the stain with twice its volume of distilled water which should be neutral or slightly alkaline (pH 7-7.2). Prevent drying
c) Allow the diluted stain to remain on the slide for 10 to 15 minutes.
d) Hold the slide under an open tap and flush the stain in a gentle flow of water.
e) Keep the slide in an upright position to drain and dry.
f) Examine the dried stained film under oil immersion and look for L.D. bodies.

JSB Staining

This method of staining method was developed at National Centre for Diseases Control. As compared to the conventional Gimesa and Leismania staining, this method is quicker .

J.S.B. I

A. Measure 500 c.c. of water in a flask with measuring cylinder.
B. Add 0.5 gm. Methylene blue in 500 c.c. of water, shake well to dissolve.
C. 3 c.c. sulphuric acid 1% should then be added in three parts (1 c.c. at a time)and solution stirred thoroughly.
D. 0.5 gm potassium dichromate is added and mixed by shaking till blue colour turns violet and precipitate forms.
E. Now 3.5 gm. Disodium hydrogen phosphate dehydrate is added and solution is shaken till precipitate dissolves.
F. Put this solution on low flame. Note time when boiling starts and boil slowly for 1 hour.

A GOOD SOULTION IS PERCIPITIATE FREE AND VIOLET IN COLOUR

Note: Boil the solution on low flame using condenser, otherwise more water will evaporate changing its concentration.

J.S.B. II

Dissolve 1 gm eosin in 500 c.c. of water. Allow to mature for 48 hours before use.

Buffer water:- Take distilled water. If not available, well or tap water filtered through filter paper. Add specified quantity of buffer salt in 1000 or 2000 c.c. of water as required and indicated above. pH of the solution should be approximately 6.2 to 6.8.

A mature solution after it cools down should be violet in colour and deep blur with violet iridescence- If it is blue or green, it should be thrown away and a fresh stain may be made.

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PREPARATION OF J.S.B. STAINS

J.S.B.I.   **INGREDIENTS**

1. METHYLENE BLUE (MEICINAL)   0.5 gm
2. SULPHURIC ACID (H_2SO_4) 1%   3 c.c.
3. POTASSIUM DICHROMATE ($K_2CR_2O_7$)   0.5 gm
4. DISODIUM HYDROGEN PHOSPHATE
    DIHYDRATE ($Na_2HPO_4.2H_2O$)   3.5 gm

5. DISTILLED WATER   500 c.c.

J.S.B.II.

1. EOSIN YELLOW (WATER SOLUBLE)   1.0 gm
2. DISTILLED WATER   500 c.c.

BUFFER WATER

1. WATER   1000c.c. 2000c.c.
2. DISODIUM HYDROGEN PHOSPHATE
    DIHYDRATE($Na_2H PO_4.2H_2O$)   0.22 gm   0.47 gm

3. POTASSIUM ACID PHOSPHATE (KH_2PO_4) 0.74 gm   0.752 gm

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4.5.2.2 Culture techniques
Primary Isolation from Aspirate:
a) Under all aseptic precautions inoculate 1-2 drops of bone marrow or splenic aspirate in culture media like Tobies medium
b) Incubate at 22-25°C for 3-4 days.
c) Examine a drop from culture for promastigotes by making a wet smear and its examination under microscope.
d) If promastigotes are present, fresh subcultures are made.
e) Strains can be maintained by doing sub-cultures every 10 - 15 days.

Primary isolation from blood:
The parasite can be isolated from blood in large number of cases. The only disadvantage of this method is that it is the slow process and takes a long time i.e. about a month.

a) Under all aseptic precautions collect 1-2 ml of blood and dilute with 10 ml of citrated saline solution (0.85% normal saline containing 2% sodium citrate).
b) Allow the cells to settle either by keeping it overnight at 22°C in an incubator or by centrifugation.
c) Inoculate the cellular deposit in culture media.

d) Incubate at 22-25°C for 1 to 4 weeks.
e) At the end of each week examine a drop from culture medium for promastigotes by making a wet smear.

4.5.2.3 Serological techniques

i) Indirect Fluorescent Antibody Test (IFAT)
Principle
Antibody in patient serum can be detected using fluorescein conjugated anti immunoglobulin. Patient serum is applied directly to the slides coated with leishmania antigen and than visualised by treatment with a fluorescein conjugated anti immunoglobulin.

Interpretation of Results:
Serum sample showing fluorescence is taken as positive for leishmania antibodies and the highest dilution showing fluorescence is the antibody titre. The test is positive very early in disease, even before the appearance of symptoms and become negative within 6 months of cure.

ii) Enzyme Linked Immunosorbent Assay (ELISA):
Principle:
Patient serum is added to leishmania antigen bound to microtitre plate. After washing, antibody bound to antigen is revealed by adding an enzyme lablelled anti human immunoglobulin and enzyme substrate.

Interpretation
Visually positive and negative sample can be differentiated by development of brown colour. Use of ELISA reader or spectrophotometer increase the sensitivity of the test and antibody titre can also be assessed. The test becomes positive very early in disease and is negative soon after the cure.

iii) rK-39 Rapid Diagnostic Test:
Principle: The kala-azar dipstick rapid test is a immunochromatographic assay for qualitative detection of antibodies to L. donovani in human serum. The assay is for aid in the presumptive diagnosis of VL. During testing the serum sample reacts with the dye conjugate (Protein AColloidal Conjugate, which has been pre-coated in the test device). The mixture then migrates upwards on the membrane chromatographically by capillary action to react with rK39 antigen on the membrane and generate a red line. Presence of this red line indicates a positive result while its absence indicates a negative result. Regardless of the presence of antibody to VL antigen, as the mixture continues to migrate across the membrane to the immobilized chicken antiprotein a region, a red line at the control line will always appear. The presence of this red line serves as a verification for sufficient sample volume and proper flow and is a control for the reagents.

It is commercially available as Kalaazar Detect Rapid Test. It is a qualitative immunochromatographic assay for detection of antibodies to leishmana. K39 is an epitope apparently conserved on amastigotes of Leishmania species that cause visceral infection; by use of laboratory ELISA testing, circulating anti-K39, IgG is detectable in 95%- 100% of patients who have kala-azar, irrespective of geographic region. Using K39 antigen-impregnated nitrocellulose strips developed for field conditions, fingerstick-obtained blood and serum samples tested from Indian subjects demonstrated a positive anti-K39 immunochromatographic reaction in 362 patients with aspirate-proven kala-azar; with an estimated sensitivity of 100% and a specificity of 97%. The strip testing proved simple to perform and yielded results within five minutes. The rapid diagnostic test when evaluated in comparison to the diagnostic performances of DAT, based both on freeze-dried and liquid antigens, on parasitologically

confirmed Kala-azar and Post-Kala-azar Dermal Leishmaniasis (PKDL), the sensitivity of the tests was almost 100%, rK39 was found, to be more sensitive. It was concluded that these tests are comparable to parasitology in terms of their sensitivity and can replace parasitology as the basis for a decision to treat visceral leishmaniasis at peripheral health centers in endemic areas. Exclusion Criteria: The rK39 is not to be used in the following cases:

- Kala-azar relapses
- In cases of kala-azar re-infection
- kala-azar and HIV co-infection

4.5.2.4 Other Indirect Evidences
i) Changes in blood picture

Leucocyte count reveals leucopenia (neutropenia) with marked diminution of neutrophil granulocytes accompanied by a relative increase of lymphocytes and monocytes. Eosinophil granulocytes are absent. During the course of the disease there is a progressive diminution of leucocyte count falling to I OOO/cmm of blood or even below that.

Erythrocytes also decrease in number. The proportion ofleucocytes to erythrocytes is greatly altered and may be about 1 :2000 to l: lOOO (Normal 1:750).

ii) Non - specific Serological tests

These tests depend on rise in gamma-globulin levels and become positive when disease is of three months duration. Different tests employed are:

iii) Aldehyde test
a) Take 1 to 2 ml of serum in a small glass test tube.
b) Add a drop or two of 40% formalin.

Jellification of milky white opacity like the white of hard-boiled egg within 2 to 20 minutes indicates a positive test.

iv) Antimony Test
a)   Take 1-2 ml patient's serum in a small glass test tube.
b)   Add few drops of 4% urea stibamine solution.

Formation of profuse flocculant precipitate indicates positive test. This test is less reliable than aldehyde test.

However, a major disadvantage with these tests is their positivity in many other diseases where albumin: globulin ration is reversed and are positive only when the disease 00 months duration.

4.5.2.5 Other Diagnostic tests

The laboratory tests, which are described, previously are well established and commonly used. There are certain other tests, which can be used for the diagnosis of leishmaniases.

i) Direct Agglutination Test.

This test has been introduced recently. Since it detects IgM antibody, the titres are high in acute cases as compared to patients with chronic disease. Various dilutions of the patient's serum are mixed with suspension of L.donovani grown in Tobie's diphasic medium. The serum parasite

mixture is allowed to settle for 4 hours at room temperature after which it is kept at 4°C overnight. The readings are taken against a dark background and the settled parasites with irregular edge indicate agglutination.

ii) Indirect Haemagglutination Test

It employs soluble antigen from promastigotes after absorbing it with sheep erythrocytes. No cross reactions with tuberculosis or leprosy are seen. The test gives excellent results in Indian kala-azar, PKDL as well as cutaneous leishmaniasis.

iii) Recent Molecular Techniques

In recent years diagnostic procedures based on detection of leishmanial nucleic acid has been developed.The introduction of DNA probes and the advent of the polymerase chain reaction (PCR) have made it possible to detect and identify small numbers of parasites in tissues from infected humans. DNA detection procedures could also be used to detect parasites in reservoir hosts, in other animals, and in infected sandflies. DNA hybridization technique has also been used for detection of parasite DNA in infected tissues.

The above mentioned methods are available only at few centres mainly as research tools.

4.5.2.6 Laboratory Diagnosis Of Cutaneous Leishmaniasis

Laboratory diagnosis of cutaneous leishmaniasis consists of demonstration of parasite in the lesion:

a)   Collect material from the indurated edge of the sore by needly biopsy.
b)   Inoculate some material in NNN or Tobie's medium for culture.
c)   Spread rest of the material on microscope slide.
d)   Stain the smear with Leishman or Giemsa stain.
e)   Examine under the microscope for amstigote forms of the parasite.

In some lesions there are innumerable parasites, while in others prolonged examination of the slide may be necessary before amastigotes are seen.

4.6 TREATMENT

Treatment for cutaneous, mucocutaneous and visceral leishmaniasis-

The drug of choice in the treatment of cutaneous, mucocutaneous and visceral leishmaniasis is pentavalent antimonials. The initial treatment should be based on daily injection of 20 mg of antimony per kg body weight for a period of 30 days. A resistence of upto 1 mg per kg body weight for 5-15 weeks depending on response. 60% has been reported to pentavalent antimonials in a dose of drug for treatment of kala azar is pentavalent sethionate. Amphotericini B has also been found to be very effective in treatment of Kala azar but its use is limited due its toxicity and side effects.

Miltefosin a new drug which can be administered orally is an effective treatment for Kalaazar. The dose is 100-200 mg for 28 days.

Treatment of cutaneous leishmaniases-

For treatment of cutaneous leishmaniases mepacrine (5%), meglumine antimoniate (85 mg Sb/ml) and sodium stibogluconate (100 mg Sb/ml) are the drugs of choice. All these are injected intralesionally. Mepacrine is injected three times a day at 3 - 5 days interval and antimony compounds are repeated once or twice, if necessary, at intervals of 1 - 2 days.

4.7 SURVEILLANCE AND CONTROL

Continuous surveillance is the most important factor in epidemiological study and control measures against leishmaniasis. At the same time this is perhaps the most neglected aspect in most of the countries. It should comprise activities such as prompt field investigation of all suspected as well as definite cases together with laboratory confirmation of presumptive diagnosis and prompt dissemination of relevant information to all responsible for control of disease. With the progress in control activities, a corresponding intensification of the surveillance is also called for. Surveillance also helps in investigating the factors that contribute to the epidemics or endemicity of leishmaniasis. A minimal surveillance system must be set up to warn of further outbreaks and to monitor the effects of continued control measures. The control of leishmaniasis can be directed on the following lines:
a)   Case detection and prompt treatment
b)   Vector control
c)   Reservoir control

Department of National Vector Borne Disease Control Programme undertakes surveillance and control of Kala-azar. The goal is to eliminate Kala azar from the country by 2010.

Kala-azar Case Definition for enrolling a subject for dipstick testing is –"A case presenting to a clinician with a fever of more than two weeks duration, with splenomegaly and not responding to the full course of anti-malarials" should be subjected to rK39 dipstick test.

The expert group on the introduction of rapid diagnosis and miltefosine into the programme had recommended at least five percent of the suspected kala-azar cases, conforming to the case definition of kala-azar and reporting to the PHC for rapid diagnosis should be subjected to splenic aspirate examination.

The cases, to be referred for splenic aspiration, will be only those reporting to the Additional PHC.s within very short distances of the higher centers of diagnosis and treatment. The Chief Medical Officer, of the district will designate such centers, and referrals will only be made to such centers, after ensuring that splenic aspiration is to be conducted by experienced and skilled personnel, in
properly equipped laboratories. In case a suspected patient of kala-azar referred for splenic aspiration is found to be negative for parasites, he/she may be referred to a higher center, according to the discretion of the medical officer.

4.8 KALA-AZAR ELIMINATION PROGRAMME IN INDIA

Initial target of Elimination set by NHP (2002) was 2010 which later got extended to 2015 in 12th Financial Plan Document.

- Target for elimination to reduce incidence of the disease to less than one case per 10000 population at block PHC level.
- The elimination strategies include surveillance, case detection and treatment, integrated vector management and cross cutting interventions like capacity building, IEC/BCC, monitoring & evaluation and operational research.
- Altogether 54 districts (33 in Bihar, 11 in West Bengal, 4 in Jharkhand and 6 in Uttar Pradesh are endemic for KA.
- Sporadic case reported from Assam, Himachal Pradsh, Jammu & Kashmir, Kerala and Uttarakhand.

The National Vector Borne Disease Control Programme (NVBDCP) is an umbrella programme for prevention & control of vector borne diseases and an integral part of the India's National Health Mission (NHM). The National Health Policy (NHP) of 2002 has set goal for achieving elimination by the year 2010 which was later extended to 2015 in 12th Financial Year Plan document.

National Kala-azar Elimination programme

The Government of India (GOI) launched a centrally sponsored Kala-azar Control Programme in the endemic states in 1990-91. The GoI provided drugs, insecticides and technical support and state governments provided costs involved in implementation. The program was implemented through
State/District Malaria Control Offices and the primary health care system. The programme brought a significant decline in Kala-azar morbidity, but could not sustain the pace of decline for long. The National Health Policy-2002 set the goal of Kala-azar elimination in India by the year 2010 which was revised to 2015. Continuing focused activities with high political commitment, India signed a Tripartite Memorandum of Understanding (MoU) with Bangladesh and Nepal to achieve Kala-azar elimination from the South-East Asia Region (SEAR). Elimination is defined as reducing the annual incidence of Kala-azar to less than 1 case per 10,000 population at the sub-district (block PHCs) level in Bangladesh and India and at the district level in Nepal. Presently all programmatic activities are being implemented through the National Vector Borne Disease Control Programme (NVBDCP) which is an umbrella programme for prevention & control of vector borne diseases and is subsumed under National Health Mission (NHM).

Goal

To improve the health status of vulnerable groups and at-risk population living in Kala-azar endemic areas by the elimination of Kala-azar so that it no longer remains a public health problem.

Target

To reduce the annual incidence of Kala-azar to less than one per 10,000 populations at block PHC level.

Objective

To reduce the annual incidence of Kala-azar to less than one per 10 000 population at block PHC level by the end of 2015 by:

- reducing Kala-azar in the vulnerable, poor and unreached populations in endemic areas;
- reducing case-fatality rates from Kala-azar to negligible level
- reducing cases of PKDL to interrupt transmission of Kala-azar and
- preventing the emergence of Kala-azar and HIV/TB co-infections in endemic areas.

In India, cutaneous leishmaniasis has been restricted to dry western half of the Indo-gangetic plain including dry areas bordering Pakistan extending from Amritsar to Kutch and Gujarat Plains. To the east the cases have been reported from as far as Delhi and Varnasi in U.P. hI 1971, an outbreak of cutaneous leishmaniasis was reported from rural areas of Rajasthan and in Bikaner town. Sporadic cases have been reported from Fazilka (Punjab), Rajasthan Canal zone in Hanumangarh, Jodhpur city and rural & urban areas of Bikaner district in Rajasthan.

Further Reading

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PLAGUE

5.1 INTRODUCTION

Plague remains a major life threatening infection of human beings and animals. The disease took a heavy toll of life in the early part of the century with an estimated 125 lakhs human deaths in the last pandemic, which started in 1889 and involved widely scattered areas in the country. There was a decline in the incidence of disease in early fifties and last human case of plague was reported in 1966 from Mulbagal, Kolar district in Karnataka. However, outbreaks of suspected Plague occurred in some parts of the country viz. Attibele, Dharampuri at South India trijunction of Tamil Nadu in May 1984 and at Tangnu village of Himachal Pradesh during September 1983, but *Y. pestis* could not be isolated to establish the diagnosis of plague.

Human plague again struck the country after a lapse of 28 years when in August 1994 cases of bubonic plague were reported from Mamla village of Beed district in Maharashtra and of pneumonic plague from Surat in Gujarat in September 1994. Subsequently, suspected cases of pneumonic plague were reported from Delhi, Varanasi, Karnataka and other states. A total of 876 cases and 54 deaths wre reported. After a gap of 8 years an outbreak of pneumonic plague occurred in Himachal Pradesh with 16 cases & 4 deaths. A localized outbreak of bubonic plague was reported from Uttarakhand in 2004 with 8 cases & 3 deaths. NCDC is monitoring the surveillance of plague in south India trijunction, Maharashtra, Gujarat, H.P. and Uttarakhand.

5.2 EPIDEMIOLOGY

Plague is an acute, onset disease caused by *Yersinia pestis* which is transmitted to man by the bite of infected rat flea. It is primarily a zoonoses being a disease of rodents. Man is affected incidentally.

5.2.1 Vector

Fleas are the natural vectors of plague in the world. Nearly 132 species of fleas all over the world have been incriminated as vectors of plague. In India, the fleas considered important for plague transmission are *Xenopsylla cheopis, X astia* and *X brasiliensis.* Out of these *X cheopis* is the principal vector.

5.2.2 Reservoirs

A large number of mammals may be infected with Y.pestis. More than 200 animals have been incriminated so far. Dogs may become infected without showing any sign of illness and act as sentinel animals. Serotesting of their blood can be an indicator to assess the prevalence of plague in that area. Cats, pigs, cattle, sheep, goat have been infected experimentally. Rodents are the true natural hosts of Y.pestis. At least 220 species of rodents which inhabit mountains, plains, deserts, cultivated fields and forests in both temperate and tropical climate are known to be infected with plague bacillus. Tatera indica among the wild rodents and R.norvegicus and R.rattus among the commensal rodents are found to play an important role as reservoir of Y. *pestis.*
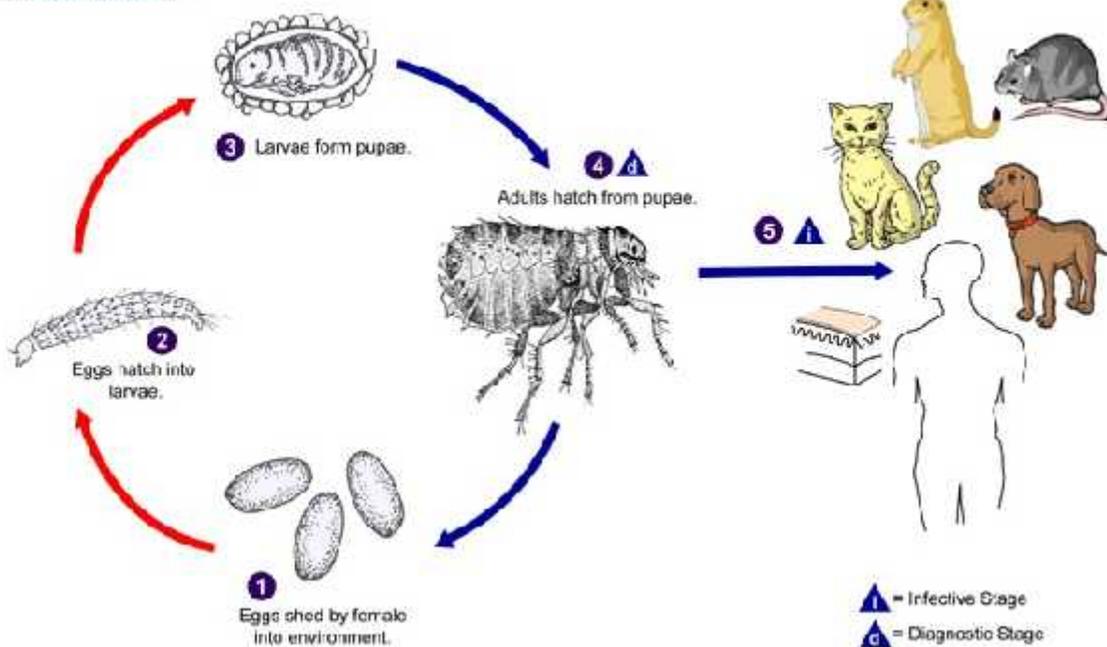
Fig 5.1: General Flea Life Cycle (available at: http://www.cdc.gov/dpdx/fleas/)

The epidemiology of plague is extremely complex. Infection depends upon its maintenance on a great variety of rodents and insects which differ from country to country and from time to time. Ecological studies point to a multiplicity of factors concerned in the fluctuating balance that exists between rodents of greater and lesser degree of susceptibility to the plague bacillus and in the degree of risk to which man is exposed. Plague exists in two forms.

5.2.3 Sylvatic Plague

The sylvatic plague is maintained in relatively resistant hosts called permanent reservoir hosts. These transmit the infection to less resistant animal hosts resulting in epizootics. When infection is endemic in sylvatic rodents, it may remain smoldering for a long time before circumstances favour its epidemic spread. During this time human beings in villages may occasionally contract the disease through handling of an infected animal. The disease does not become epidemic in man unless infection spreads to what is sometimes termed as 'liasion rodent, namely a species of rat that comes in contact with man.

5.2.4 Domestic plague

Domestic plague is intimately associated with man and rodents living with man. It occurs when infection is picked up from permanent reservoir hosts by semidomestic rodents which in turn transmit it to the commensal rodents and then to man. The ratfall is the harbinger of domestic

rodent plague and a sign of imminent outbreak of epidemic bubonic plague in man. Rodents are also known to transmit the infection to remote areas causing pandemics.

5.3 CAUSATIVE AGENT

Plague is caused by *Yersinia pestis.* The organism was discovered in 1894 in Hong Kong by Yersin and Kitasato. It is a short, thick cocco- bacillus measuring 1.5-2 x 0.5-0.7 microns in size. It has rounded ends, which on staining give a characteristic bipolar appearance to the organism. It is Gram negative, nonmotile and non-sporing. True capsules are seen in living tissues but are less readily seen in culture. The optimum temperature for growth is 28^0C but at this temperature, capsular material (envelope substance) is not well developed. For that, the organism should be grown at 37°C. The capsular material is important for antigenicity and protectivity and is used for preparation of Fraction I antigen for serological test and preparation of plague vaccine.

The organism is pathogenic to common laboratory animals like rats, mice, guinea pigs etc. The inoculated animal dies in 3-4 days time. Necropsy findings reveal marked local inflammation at the site of inoculation with enlarged and congested lymphnodes and may show greyish white patches in the tissue. The characteristic coccobacilli are seen in large numbers in films made from local lesions, lymph nodes, spleen pulp and heart blood.

5.4 CLINICAL FEATURES IN MAN

The incubation period of bubonic plague is 2-6 days while that of pneumonic plague is 2-4 days or may be shorter.

Bubonic plague

This is the commonest form characterised by high fever, toxemia and painful enlargement of lymph glands, which are called buboes. Buboes may appear commonly in the groin or in the axillary or cervical region depending upon the site of entry of the organism. The glands gradually become soft and suppurate, and there is marked haemorrhagic tendency with variable septicemia. Mortality is 25-30% in untreated cases.

Pneumonic plague

It is due to droplet infection. It is a severe form of disease with high fever and cough with blood tinged tenacious sputum. The disease is highly contagious resulting in explosive outbreaks. Recovery is rare, and death occurs within 2-4 days.

Septicemic plague

It is a variant of bubonic plague where there is overwhelming septicemia overrunning the lymph nodes. Consequently, lymph node enlargement is not prominent but the patient is highly toxic. This form also results in a very high mortality.

Pestis minor

It is an ambulant plague, a mild form of the disease with fever and buboes which may suppurate. Symptoms are very mild so that the patient does not go to bed.

5 .5 SIGNS IN RODENTS

In rodents dying of plague, necropsy reveals enlargement of lymph nodes with periglandular inflammation and edema that is more frequent in the cervical lymph nodes due to tendency of the rat flea to bite at the neck region. There is pleural effusion, enlargement of spleen with small white areas in the pulp, liver is congested and mottled, congestion and hemorrhages under the skin and in internal organs. *Y. Pestis* can be demonstrated in material obtained from these organs.

Plague in other animals

Besides rodents, plague can affect other animals too. Domestic animals, rabbits, camels etc., are all susceptible to infection

5.6 LABORATORY DIAGNOSIS

The laboratory diagnosis of plague must be rapid because of the risk for contact and conclusive because of the fear this disease evokes in people and authorities. When plague is suspected, clinical specimen should be collected urgently and specific antimicrobial treatment begun without waiting for laboratory report.

Specimen for Diagnosis

Material for laboratory diagnosis of plague can be obtained from the following sources:
- Human beings suffering from infection
- Post-mortem specimen
- Specimen from rodents
- Flea specimen
- Soil specimen

Human Specimen

Appropriate specimen should be examined for evidence of plague if a person resides in, or has a recent travel history to a plague-infected area; and presents with symptoms suggestive of plague (fever, sepsis syndrome, lymphadenopathy and/or acute pneumonitis). Specimen should be obtained from appropriate sites for isolating the bacteria. As far as possible these should be collected before initiation of antimicrobial therapy.

Laboratory specimen required for Y.pestis are shown below:

Clinical presentation	Specimen
Bubonic	• Bubo fluid/aspirate
	• Serum
Pnemonic	• Sputum
	• Bronchial/tracheal washing
	• Serum
Septicaemic	• Blood
Post-mortem	• Autopsy from lymph node
	• Lungs
	• Bone Marrow

5.6.1 Collection of material

The preferred specimen for microscopic examination and isolation from a bubonic case is aspirated fluid from the affected bubo, which should contain numerous organisms.

Blood cultures should be taken whenever possible. Organisms may be seen in blood smears if the patient is septicemic. Bacteria may be intermittently released from affected lymph nodes into the blood streams. Therefore, a series of blood specimen taken 10-20/30 minutes apart may be productive in the isolation of Y.pestis.

Sputum/throat swabs taken from pneumonic plague patients may contain too many other organisms so wayson stain is the test of diagnostic value; these smears should be examined with more specific direct fluorescent antibody (DFA) test also. Preferably, a bronchial/tracheal washing should be taken from pneumonic plague patient for culture. In biopsies or in specimen taken after post-mortem where recovery of live organisms is difficult, lymphoid tissue, lung and bone marrow samples may yield evidence of plague infection by DFA test or by detection of Y.pestis by PCT test.

Rodent and Flea Specimen

Appropriate tissues should be taken from animals for detection of Y.pestis. Lymphoid tissues-liver, spleen, lymph nodes-should be removed to test for the presence of F-1 antigen by DFA test. Attempts to recovery Y.pestis should be made by culture of the tissue and by inoculation into susceptible laboratory mice. Bone marrow from dissected animal carcasses may yield positive results when other tissue are not available. Serum and blood specimen may be taken for detection of antibody.

Flea should be indentified and placed in pools by species. A flea pool is then triturated and inoculated into the laboratory mice for isolation of plague bacteria. In case where no cultutes or serum specimen are available for testing both animal and flea material may be tested by polymerase chain reaction (PCR) to detect plague DNA in the specimen.

5.6.2 Packaging and transportation of Specimen

Packaging

The etiological agent of plague Yersinia pestis is classified as biosafety Class II agent.
The requirement for this class of infectious material, in brief, are:
- A water-tight primary receptacle
- A water-tight secondary receptacle
- An absorbent material, which must be placed between the primary receptacle and the secondary packaging. The absorbing material must be adequate to absorb the entire contents of all primary receptacles.
- Outer packaging must be at least 100 mm in the smallest overall external dimension
- Itemized list of contents must be enclosed
- Outside package must be marked with identification of the infectious substance, volume of contents name and telephone number of sender.

All materials should be double packed in crush-proof and leak proof containers. Infectious agents, serum or culture vessels should be labeled with alcohol-resistant, permanent ink marker as to the contents and dated. The materials should be sealed with para film or tape to prevent inadvertent leakage and then wrapped materials are placed in a leak proof bag, soaked with disinfectant and sealed. The sealed bag is then placed in the first of two crush-proof containers, sealed and placed into the second crush proof container. Depending on the specimen and the destination, the double container is sent in a sealed box with or without coolant.

Do not use wet ice for packaging, because it melts and leaks. Cool packs are acceptable but must be placed in a box so that, upon thawing they do not permit movement of materials within the box. Containers must be especially well sealed if they are being shipped under dry ice conditions. Dry ice evaporates, thus when packing, this must be taken into consideration.

Local Surface Transport

Examples include transport of specimen from a doctor's office/clinic to a laboratory, from a hospital to a diagnostic laboratory and from one laboratory to another. A hospital, a laboratory, a health service or other approved agency or organization may operate such courier services.
The principle of safe transport by this means is the same as for air or international transport – the material should not have any possibility of escaping from the package under normal conditions of transport.
The following practices should be observed:
- Specimen containers should be watertight and leakproof;
- If the specimen container is a tube, it must be tightly capped and placed in a rack to maintain it in an upright position;
- Specimen containers and racks should be placed in robust, leak-proof plastic or metal transport boxes with secure, tight fitting covers;
- The transport box should be secured in a transport vehicle.
- Each transport box should be labelled appropriately consistent with its contents
- Specimen data forms and identification data should be accompany each transport box.
- A spill kit containing absorbent material, a chlorine disinfectant, a leak proof waste disposal container and heavy duty reusable gloves should be kept in transport vehicle.

5.6.3 laboratory criteria for diagnosis

Suspected Plague

1. Clinical symptoms that are compatible with plague,e.g. fever, sepsis syndrome, lymphadenopathy and/or acute pneumonitis in a person who resides in or recently travelled to a plague endemic area.
2. If gram negative and/or bipolar-staining coccobacilli are seen on a smear taken from affected tissues, e.g.

  - Bubo(bubonic plague)
  - Blood(septicemic plague)
  - Tracheal/ lung aspirate (Pneumonic plague).

Presumptive Plague

- Y.pestis F1 antigen detected in clinical materials by direct fluorescent antibody testing, or by some other standardized antigen detection method; or
- Isolate from clinical specimen demonstrates biochemical reactions consistent with Y.pestis or PCR positivity; or
- A single serum specimen is found positive for diagnostic levels of antibodies to Y.pestis F1 Antigen, not explainable on the basis of prior infection or immunization.

Confirmed Plague

- Isolate indentified as Y.pestis by phage lysis of cultures; or
- A significant (equal or more than 4-fold) change in antibody titre to the F1 antigen in paired serum specimen.

Specimen submitted to the laboratory are processed, in general, for isolation, identification and confirmation as per the flow chart. A smear is prepared and stained. Specimen are directly cultured and/or inoculated in laboratory mice, isolates recovered from mice and culture are further processed for identification.

5.6.4 Test Procedures

5.6.4.1 Smear Examination
The diagnostic value of smears is considerable. During epidemics or epizootics, it is often possible to arrive at a preliminary diagnosis by the examination of smears that show the presence
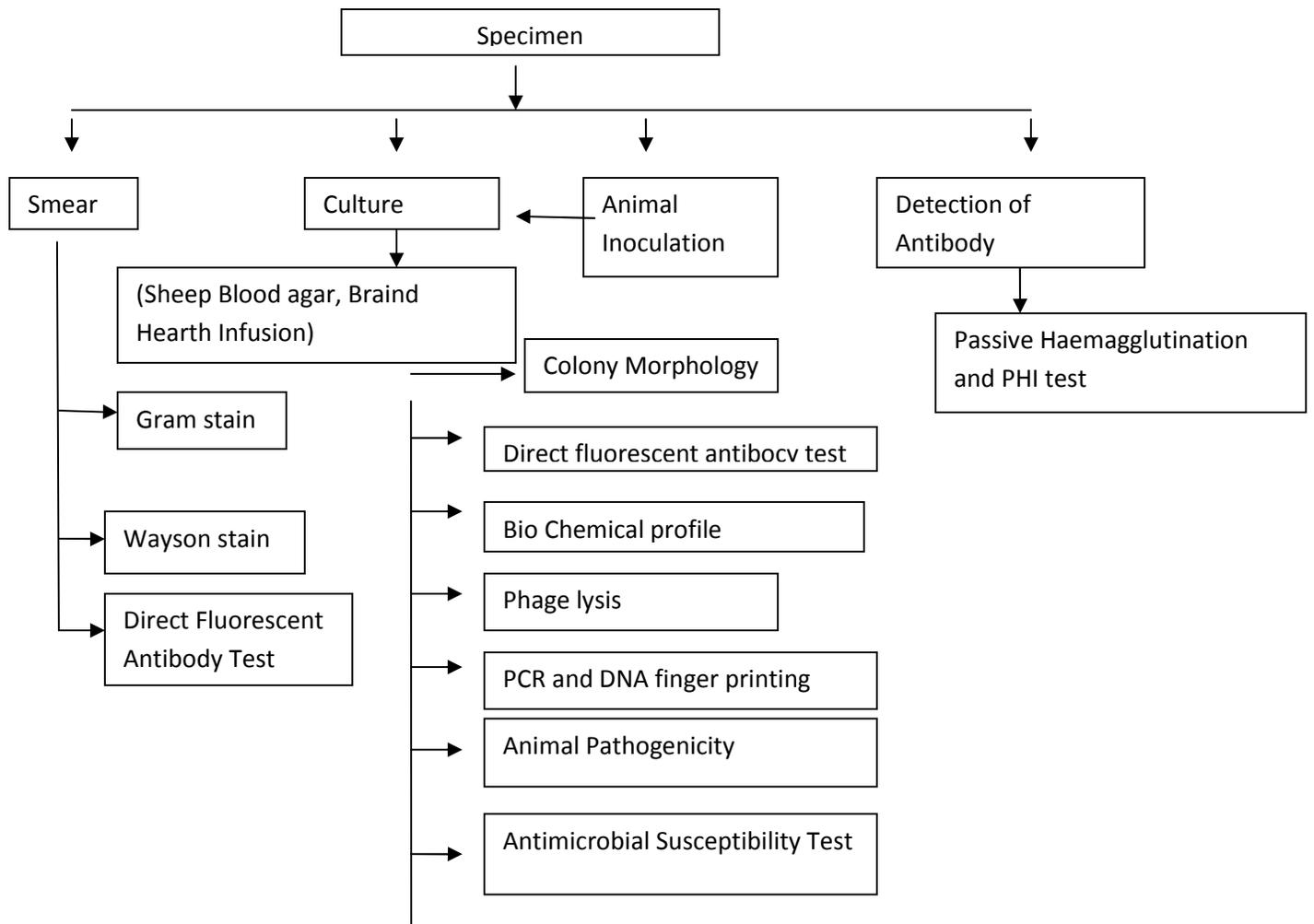
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                           ┌──────────────────┐
                           │     Specimen     │
                           └──────────────────┘
                                    │
        ┌──────────────┬────────────┼──────────────────────────────┐
        ▼              ▼             ▼                              ▼
  ┌─────────┐    ┌──────────┐  ┌──────────────┐         ┌──────────────────┐
  │  Smear  │    │ Culture  │◄─┤   Animal     │         │  Detection of    │
  └─────────┘    └──────────┘  │ Inoculation  │         │  Antibody        │
       │              │        └──────────────┘         └──────────────────┘
       │     ┌─────────────────────────────┐                     │
       │     │ (Sheep Blood agar, Braind   │                     ▼
       │     │  Hearth Infusion)           │          ┌──────────────────────┐
       │     └─────────────────────────────┘          │ Passive Haemagglutination │
       │                    │    ┌──────────────────┐  │ and PHI test         │
       │                    └───►│ Colony Morphology │ └──────────────────────┘
       ▼                         └──────────────────┘
  ┌────────────┐          │
  │ Gram stain │          ├──►┌────────────────────────────────┐
  └────────────┘          │   │ Direct fluorescent antibocv test │
       │                  │   └────────────────────────────────┘
       ▼                  ├──►┌──────────────────────┐
  ┌──────────────┐        │   │ Bio Chemical profile │
  │ Wayson stain │        │   └──────────────────────┘
  └──────────────┘        ├──►┌──────────────┐
       │                  │   │ Phage lysis  │
       ▼                  │   └──────────────┘
  ┌────────────────┐      ├──►┌────────────────────────────┐
  │ Direct Fluorescent │  │   │ PCR and DNA finger printing │
  │ Antibody Test  │      │   └────────────────────────────┘
  └────────────────┘      ├──►┌──────────────────────┐
                          │   │ Animal Pathogenicity │
                          │   └──────────────────────┘
                          └──►┌───────────────────────────────┐
                              │ Antimicrobial Susceptibility Test │
                              └───────────────────────────────┘
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Fig. 5.2 Chart for laboratory investigation

of characteristic bipolar stained plague bacilli. The smears of clinical material should be prepared as early as possible after collection. Delay in preparation of smears from specimen in transport medium etc. may be result in change in the classical morphology of the bacilli. Smears may be prepared from bubo aspirates, spleen, liver blood, sputum and culture isolates.

For the fixation of the smear, alcohol should be used preferably because it promotes good bipolar staining of the organisms and kills the bacteria.

The staining of the smears can be done by Gram stain, Wayson stain or Direct Flourescent Antibody((DFA) stain.

Gram Stain

- Prepare a thin smear from the specimen on the side.
- Heat fix the smear by passing the slide quickly through a flame
- Cover the smear with crystal violet for 1 minute, followed by iodine for 2 minutes. Rinse off with tap water.
- Decolorise the smear with acetone for 30 seconds, rinse with tap water.

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- Counterstain the smear with saffranin for 30 seconds, rinse with tap water. Air dry and examine the smear using oil immersion.

Interpretation

The presence of 1.0-2.0µm X 0.5µm fat, gram negative(pink) and perhaps bipolar staining bacilli seen mostly as single cells or short chains along with clinical symptoms compatible with plague suggest that specimen may contain plague bacilli.

Quality Control

Gram staining to test specimen should be carried out with parallel staining of known gram positive (Staphylococcus aureus, Bacillus subtilis) and gram negative (Esch.coli, Klebsiella pneumoniae) organisms to ensure proper staining results.

Wasyson stain

- Prepare a thin smear or impression touch preparation on a slide. Let it dry.
- Fix the smear in absolute methanol for a minimum of 3 minutes. Let the methanol evaporate completely.
- Flood the smear with Wayson stain for only 5-10 seconds.
- Wash the slide with tap water. Let the stained smear air dry. Examine under oil immersion.

Interpretation

Bipolar stained safety pin fat bacillus is characteristics of Yersinina pestis. All Yersinia pestis may stain as bipolar cells, but all bipolar staining cells are not Y.pestis. When stained smear reveals the cells with the characteristics safety pin morphology, further evaluation by more specific staining by DFA, culture isolation and identification must follow.

Quality Control

Yersinia spp. (bipolar staining) and Esch. Coli or B. subtilis(non bipolar staining) cells should be stained to assist interpretation of bipolar characteristics.

5.6.4.2 Direct Fluorescent antibody Staining(DFA)
- Prepare thin tissue (spleen and liver) impression smears, lymph node aspirate, blood or culture on the slide. Let the smear air dry.
- Heat fix the smear by passing the slide through flame, cool, then mark the smear area with wax pencil.
- Add 20µl of conjugate to the heat fixed smear and incubate for 30 minutes at ambient room temperature. Cover the slide to prevent evaporation of the conjugate.
- Soak the slide in Phosphate Buffered Saline(PBS) (pH 7.2-7.4) for 10 minutes. Discard the PBS from the container and refill with fresh PBS. Soak the slide for 10 more minutes.
- Remove the slide from PBS and air dry. The slide may be examined directly under a fluorescent microscope. Alternatively, in case of UV microscope light source, add a drop of mounting fluid(PBS+glycerol), place a cover lip on top and examine the slide.

Interpretation

The positive slide will be seen as bright, intense green staining around the bacterial cell. If clinical specimens are from patients who have received antibiotic therapy, or if the specimen have not been preserved properly, fluorescence may be seen as small green spots of fragmented F-1 antigen. Because DFA positively is only a presumptive diagnosis for the presence of Y.pestis, attempts should be made to isolate the organism.

5.6.4.3 Isolation and Identification of Y.pestis

For isolation all clinical specimen, rodent organs, tissues recovered from inoculated mice are streaked onto two 6 percent sheep blood agar (SBA), BIN agar plates for incubation at 28^0C and 37^0C for at least 24-48 hours.

Examine the plates for characteristic colonies. After completion of work decontaminate the plates by autoclaving before discarding the contents.

Interpretation

Y. pestis grows as grey white, translucent colonies, usually too smart to be seen as individual colonies at 24 hours. After incubation for 48 hours, colonies are 1-2mm in diameter, grayish white to slightly yellow colour and more opaque. Under 4x enlargement, after 48-72 hours the colonies have a raised, irregular, "fried egg" morphology. Colonies also can be described as having "hammered copper" shiny surface. There is no haemolysis of the sheep RBCs. The growth on SBA should be examined by DFA test, biochemical reactions and bateriophage lysis test for confirmation as Y.pestis.

Quality Control

Each batch of plates must be checked for sterility by incubating plates at 37^0C for 24 hours.

Each batch of plates must be checked to ensure that it supports the growth of the bacteria like Staphylococcus aureus, Esch.coli and K.pneumoniae.

5.6.4.4 Mouse Inoculation

Laboratory mice inoculation has proven to be useful method in the recovery of plague bacilli from other contaminating agents especially if the submitted material are thought to be contaminated with other organisms or are compromised because of deterioration.

Procedure

- A fluid suspension of the inoculum is prepared in saline. Tissue samples are prepared by grinding small pieces ($0.5cm^2$) into a mortar with a few drops of saline. Cultures only need to be suspected in saline and mixed prior to inoculation.
- Inject 0.1-0.5ml of suspension subcutaneously in the lower abdomen. Two mice are injected with 0.1ml of prepared inoculums from culture of purified material. Whereas for tissue upto 0.5ml of suspension should be injected. For extremely contaminated specimen percutaneous inoculation is recommended.
- Mice are labelled and placed in cages with sufficient food and water. Mice are checked twice for signs of illness or death. Surviving mice are kept for 3 weeks.
- If mice are found dead or moribund during the test period, a postmortem examination is conducted. The microscopic appearance of the liver and spleen is observed and a section

of the organ tissue excised. Impression smears of the tissues are taken for DFA testing and the remaining tissues are processed for culture and isolation.

- Discard the mouse caracasses by incineration.

Interpretation

Y.pestis is easily isolated when passaged through susceptible, laboratory raised, specific pathogen free mice. Inoculated mice typically become moribund within first 3 to 4 days post inoculation and ultimately die from disseminated infection. Spleen and liver are enlarged, hemolytic and sometimes contain multiple focal abcesses. Tissue samples taken from the mice are a good source of nearly pure Y. pestis culture. If mice do not yield and infective isolate, indirect evidence of Y.pestis infection may be obtained by testing the serum of surviving mice for antibodies to Y.pestis.

Mouse morbidity/mortality plus DFA positive liver or spleen tissue are presumptive evidence for plague.

Quality Control

Mouse handling conditions must comply with local animal ethical committee guidelines. Since the mouse inoculation test is dependent on visible morbidity and mortality as the outcome, mouse colonies should be inoculated with known virulent. Y.pestis to test their sensitivity to infection prior to use.

5.6.4.5 Specific Bacteriophage Lysis Test

Bacteriophage typing of bacteria is a common method by which bacteria are identified, grouped or sub-typed. Y.pestis cells are sensitive to lysis by temperature specific bacteriophage; thus, this feature can be used to confirm its identification. Plague bacteria are susceptible to bacteriophage lysis at 20-25^{0}C and 37^{0}C. Y.psuedotuberculosis may be differentiated from Y.pestis by the loss of lysis at incubation temperatures of less than 28^{0}C.

The bacteriophage lysis test should be performed on a pure or nearly pure culture to confirm the presence of the plague bacilli. Enriched medium like 6% SBA, is preferred for use in the test since Y.pestis grows slower on standard media(nutrient agar (NA), trypticase soy agar or BHI agar).

The test described here uses a bacteriophage impregnated filter paper strip as the test reagents. The paper strip method is preferred to the drop method, in which a spot of liquid bacteriophage culture is dropped onto a lawn of cells and lysis is visualized by a clear zone where the bacteriophage was placed. Depending on the seeding concentration of the lawn of cells, spot-lysis results may not be sharply distinguishable.

Procedure

- Duplicate SBA or NA plates should be inoculated with thin (<5-mm width) parallel lines of test cultures. Up to a total of 8 lines may be placed on the agar surface with the last two lines to contain the control cultures, Y.pestis A1122 and Y.pseudotuberculosis.
- Place a bacteriophage-impregnated paper strip perpendicular to the lines of culture. Gently tap the paper strip onto the agar surface so that full contact is made. Tape to seal the plates. Incubate one plate at 20-25^{0}C (this is usually the same ambient room temperature), and the other plate at 37^{0}C for 18-24 hours.

- Observe for lysis at the area where the bacteriophage paper strip intersects the culture line.
- Decontaminate plates by autocalving before discarding.

Interpretation

Results of lysis may be less obvious at 24 hours when standard media are used; and if the culture was not pure, contaminations may overgrow and mask the lysis. Only Y.pestis cells are lysed by the bacteriophage at both 20-25^{0}C and at 37^{0}C. Lysis at both temperature, after 24 hours incubation, is a confirmatory test for plague; therefore, controls results must also be satisfactory before reporting the results. Prolonged incubation may lead to false interpretation because of the movement of bacteria cells on the filter paper strips, or if using the drop method, contaminants may overgrow the cleared area and mask lysis activity. Always ensure that the incubator temperatures are set correctly; unsatisfactory results usually are due to improper incubation temperatures.

Quality Control

The control of Y.pestis and Y.pseudotuberculosis strains must always be used, and the test must be set up for incubation at both temperatures for lysis. If lysis is not working, check for the efficacy of the bacteriophage paper strips, blood plates and incubator temperatures.

Biochemical Test

Standard Tube and Rapid Miniaturized Kits

Y.pestis is relatively inactive in routine biochemical tests; therefore, biochemical testing is not critical for the identification of plague bacilli. However, some of the tests are useful in differentiating Y.pestis from the other pathogenic Yersinia species (Y.pseudotuberculosis and Y.enterocolitica) and for determining the Y.pestis biotype based on the ability to utilize nitrate and glycerol.

When a pure culture is obtained, the biochemical profile of the suspect Y.pestis culture is generated to complete the culture identification process. Some minor differentiation in sugar fermentations and some enzymatic differences are used to create profile for an isolate.

Procedure

- Pure culture is grown for 24 hours at 37^{0}C in BHI broth or on SBA. A suspension of cells equal to 10^8 cells/ml is made in either diluent or in saline.
- Each biochemical tube in inoculated with 0.1ml of suspended culture, or inoculated as per manufacturer's instructions. Tubes/kits are incubated at 37^{0}C for 24 hours.
- Reaction to each tube is observed and recorded.
- At the completion of test, the tubes are decontaminated by autocalving before discarding.

The change in the pH indicator and substrates should be recorded and compared to known profiles of Y.pestis strains. The biochemical reactivity of plague strains is not very distinctive(Table 5.1). The unease test may be used to distinguish Y.pestis from the closely related Y.pseudotuberclosis; however, there are rare unease-positive isolates of Y.pestis. Glycerol fermentation and nitrate reduction test results distinguish the biotypes (Table 5.2).

Table 5.1: Biochemical reactions of Y.pestis and Y.pseudotuberculosis		
Test or property	**Y.pestis**	**Y.pseudotuberculosis**
Catalase	Positive	Positive
Coagulase	Positive	Positive
Indole	Negative	Negative
Oxidase	Negative	Negative
Urease	**Negative***	Positive
Adonitol	Negative	Positive
L-Rhamnose	Negative	Positive
Glucose	Negative	Positive
Mannitol	Negative	Positive
Melibiose	**Variable***	Positive
Sucrose	Negative	Negative
Arginine	Negative	Negative
Ornithine	Negative	Negative
Nitrate	**Variable***	Positive
*Indicate the more prominent features of Y.pestis		

Table 5.2: Differentiation of Y.Pestis biotypes		
Biotype	**Nitrate Reduction**	**Glycerol Fermentation**
Orientalise	+	-
Antiqua	+	+
Mediaevalis	-	+

Quality Control

For tube tests, each batch of medium should be tested with bacterial strains that are known to be positive or negative for the reaction to ensure appropriate reactivity. Check the reactivity of the medium to support growth and acid production by inoculating a tube with Yersinia pestis strain A1122, K.pneumoniae and Esch.coli. Incubate test tubes at 37^0C for 24-48 hours. Commercial kit ought to be tested with control strains to verify kit performance. If profiles of control strains cannot be properly generated, then the reagents, tube medium or identification kit, and technical problems must be examined and corrected.

Antimicrobial Susceptibility Test

Prompt diagnosis and appropriate antimicrobial treatment are important for successful resolution of plague infection. The drugs of choice of treatment of plague are streptomycin or gentamicin. Tetracyclines, sulfonamides and chloramphenicol are also very effective alternative drugs for treatment. Penicillins and cephalosproins are not recommended. Penicillins appear effective in inhibiting Y.pestis in vitro; however, this antibiotic is considered ineffective against human disease. Therefore, in vitro antimicrobial test results should be interpreted carefully. Although a multidrug – resistant isolate from Madagascar has been recovered from bubonic plague case, drug resistance has not been a problem in treating plague. Naturally occurring strains of resistant Y.pestis isolates are rare. The potential for the spread and public health implication of multidrug-resistance Y.pestis needs to be monitored by research and heightened surveillance.

The disk diffusion method for determining antimicrobial susceptibility is most commonly used method because of its simplicity. Disk diffusion results are reported as antimicrobial susceptible, intermediate or resistant, based on the size of the zone of inhabitation around the disk. The zone size is inversely proportional to the MIC levels, though MIC (µg/ml antibiotic) cannot be determined by this test. The antibiotic-containing disks are impregnated with a fixed concentration of antimicrobial agent, and these disks are purchased from commercial sources. The limiting factor of the disk diffusion test is the dependence on the availability of the variety of drug-impregnated disks.

Procedure

- Prepare inoculums by passing test and control isolates in Brain Heat Infusion (BHI) broth or on Sheep Blood Agar(SBA) and incubate for 24-48 hours at 37^0C. Standardize the inoculum by adjusting the turbidity to equal McFarland turbidity standard 0.5 in 2-5ml of saline in a 13x100mm sterile test tube.
- Pre warm Muller Hinton Agar(MHA) plates to ambient room temperature. Moisture on the surface of the plate will affect the test; therefore, ensure that the surface is dry before inoculation. Plates should be inoculated within 15 minutes of inoculum preparation.
- Vortex to mix the inoculums well. Dip a sterile cotton swab into the suspension; roll the swab several times firmly against the wall of the tube(above the liquid level) to remove excess liquid from the swab. Streak the swab over the entire surface of the agar plate 3 times, rotating the plate at 60^0C each time so that the inoculum is evenly streaked across the entire plate. Run the swab around the agar edge before discarding the swab in an appropriate container for decontamination and disposal. Let the sugar surface adsorb the inoculums for 5 minutes (with lids slightly ajar) before applying antimicrobial disks.
- Equilibrate the antimicrobial disks to ambient room temperature prior to use. Place the disks evenly on the inoculated agar surface. Keep about 24mm distance between two disks. Disks may be placed with sterile forceps or by using dispensing apparatus. With sterile forceps, gently tap down each disk onto agar surface to prevent slipping. No more than 12 disks should be placed on a 150mm plate and not more than 5 disks on a 100mm plate. Disks should not be moved once they have touched the agar surface since diffusion begins almost immediately upon contact with the agar.
- Invert and tape plate lid to bottom in two places for safety and incubate at 37^0C for at least 24 hours.
- Measure zones of inhibition and record results.
- Decontaminate plates before discarding.

Interpretation

The quality of the MHA plate and standardized inoculum are curical for the accuracy of antimicrobial susceptibility test results. Before recording results, ensure that the zones of inhibition are uniform and circular and that the lawn of growth is even and confluent. Results must be taken at 24 hours; tetracycline and chloramphenicol are bacteriostic; their effect is diminished beyond 24 hours at the levels used and may result in erroneous sensitivity level if measurements are taken too late. Measure the diameters of the zones(including the diameter of the disk) to the nearly whole millimeter with a ruler, sliding caliper or caliberate template. The zone edge measured should be an area where there is no obvious growth to the unaided eye. Small colonies detected by transmitted light or under magnification should not be taken as the edge of confluent growth. Any growth within the zone of inhibition would suggest resistance.

Colonies appearing in the zones should be subcultured and their identity should be verified and retested. The diameters measured should be interpreted based on the guidelines published by the NCCLS. The results are reported as susceptible, intermediate or resistant to the antimicrobial agents tested.

Quality Control

Each batch of MHA plates should be tested to ensure accurate performance by assessing diameter zones for standard organisms. These zones must be acceptable. A panel of cultures, Y.pestis strain A1122 (avirulent) and organisms that are susceptible and resistant to the antibiotics selected should be tested on the agar. Care must be given to ensure that the agar is at 4-mm depth; agar too deep may give false resistance results and thinner agar may given false susceptibility results. The pH of the agar must also fall between 7.2-7.4 since pH ranges that are too acidic/basic can adversely affect potency of drugs (gentamicin, which is an aminoglycoside, will lose potency at acidic pH). If the growth on the plate is not confluent, the results should not be taken and the test should be repeated.

The commercially purchased antimicrobial disks should be stored according to the manufacturer's recommendations and should not be used beyond the expiration dates. Each batch of new disks should be tested against the panel of standard organisms to ensure performance.

5.6.4.8 Serological Tests

Plague serology tests have been based on the detection of antibody to whole cells and to the Y.pestis – specific F1 antigen. The agglutination test is simple to perform, does not depend on a secondary labeled reagent and is not species dependent. Thus , this test has broader applicability in plague surveillance, where antibody detection of many mammalian species can be examined by the same format.

In the passive haemagglutination assay(PHA) test for plague, F1 antigen is coated onto gluteraldehyde-fixed Sheep Red Blood Cells (SRBCs) and used as the sensitized antigen. Serum, plasma or serum proteins extracted from whole blood dried on filter paper may be used as a source of antibody in the PHA. Nonspecific cross-reactivity may occur in agglutination tests giving rise to indeterminate results. Consequently, two control measures need to be included with the test: 1) Pre-adsorption of the serum with fresh SRBCs to remove non-specific cross-reactivity; and, 2) if the serum sample reacts positively, a F1 inhibition(PHI) test must also be run to verify the specificity of the agglutination test.

Procedure

Preparation of serum/plasma test samples
- Heat-inactive serum/plasma samples at 56^0C for 30 minutes.
- Add one drop (50µl) of packed, washed SRBCs to every 0.5ml of test sample. Adsorb for 30-60 minutes at ambient room temperature or overnight at 4^0C.
- Centrifuge at 100Xg for 10 minutes to sediment SRBCs; remove clarified test sample to a new vial. Sample is ready for testing. Hold serum/plasma at 4^0C temporarily until testing is completed.

Setting up the PHA/PHI Plate

- **Screening test** (4-wells PHA test). If there are many samples and if there is enough specimen volume for more than one test (at least 200µl), it may be useful to first screen the samples. Divide the 96-Well plate horizontally into two sections: top 4 rows and bottom 4 rows(this permits screening 10 samples plus one positive and one negative in each section).
- Add 25µl of Haemagglutination Diluent (HA) to all wells.
- To each first well, add 25 µl of serum.
- Set pipettor to 25 µl and serially dilute the samples through well 4, discard the last 25 µl.
- Add 25 µl of diluted Sensitized Sheep Red Blood Cells(SSRBCs) to all the wells, mix gently and incubate at ambient room temperature for 4 hours or overnight at 4^0C.

Standard test (8-wells PHA and 4-wells PHI). Divide the 96-well plate vertically into two compartments: one section with 8 columns and the other with 4 columns (can test 6 rows of test samples plus one positive and one negative control serum on each plate).

End Point of High Titer Specimen

On high titred specimen that exceed 8 wells of agglutination. It is recommended that the specimen be retested with 12 or more PHA wells and 12 PHI wells, along with the positive and negative controls.

Interpretation

Examine the wells for flocculent, lattice-type agglutination. Negative wells will have a sharp edged button or a small, well defined circle. Screening titer results of 1:10 are considered positive for plague since antibody to F1 antigen is specific. Because of likely nonspecific cross reactivity , all screening test – positive samples must be further tested by full end point dilution and PHI before reporting the final titer. Agglutination of true positives in the PHI buffer will be inhibited because free F1 antigen in the PHI buffer will react with anti-F1 antibodies and thus remove antibody that can react with the F1 bound to the SRBCs.

End point titer are then determined by taking the last PHA well showing full agglutination and subtracting from that the PHI wells showing agglutination. The resulting well number represents the end point titer. For example, if the PHA results for a specimen shows full agglutination at 6 wells and the PHI agglutination is at 2 wells then 6-2=4, the end point titer would be 1:32.

Quality Control

Each lot of SSRBCs & diluent must be tested with a control serum panel to ascertain that performance does not deviate from pervious lots. For each positive sample, care must be taken to achieve an end-point and ensure the specificity of the reaction. Specificity of the reaction must be tested by the PHI test to rule out non-specific reactivity.

5.6.4.9 Molecular Techniques

Molecular techniques are powerful tools that can provide information about the etiological agent that cannot be obtained by traditional diagnostic methods. When standard microbiologic methods

fail to yield a viable isolate, molecular-based tests may be the only means available to confirm the presence of Y.pestis

Molecular techniques used by the diagnostic laboratory have primarily been for the purpose of grouping isolates to gain more discriminatory power over the traditional biologic typing methods. Reproducibility is influenced by biological and technical variability; therefore, these techniques in the diagnostic laboratory should be selectively applied.

Polymerase Chain Reaction for the detection of selected Yesinia pestis genes

PCR methodology has provided exquisite discriminatory power in detecting low quantities of an infectious agent nucleic acid in a specimen. Because of PCR's theoretical ability to detect a single DNA copy, the use of PCR methodology in a diagnostic laboratory has to be rigorously controlled to prevent false – positive results.

Application
This method should be used for reference and experimental purposes. PCR should be used only if cultures cannot be recovered.

Procedure
For DNA template preparations, DNA extraction kits following manufacturer's guidelines should be used.

Recovery from Fresh Culture

BHI Broth –Harvest fresh (24-48 hours) Y.pestis cells from BHI broth. The amount of cells collected should have a turbidity equivalent to OD_{600} 0.2. Pellet cells by centrifugation at 800Xg for 5 minutes on the microfuge. Discard the supernatant broth.

SBA plate- Harvest cells using a calibrated 1 µl loop. Transfer a loopful of cells to 100 µl of lysis buffer. Cell pellet should equal to 10 µl volume of packed cells. Resuspend the pellets in 100 µl of lysis buffer. Vortex to get an even suspension. Add 1 µl of proteinase K. Vortex again. Incubate the tube at 37^0C for 1 hour. Heat at 95^0C for 2 minutes (to inactive proteinase K, and to separate the double standard DNA). Keep tube on ice if ready to use for PCR or store at -20^0C. DNA templates sometimes may have to be further purified and stored under specific conditions free of inhibitory agents or other impurities. Extract and purify DNA by adding 100 µl(Equal volume) of saturated phenol (pH 8). Vortex briefly to mix well, then spin in microfuge at 4^0C(in cold room) at top speed for 3-5minutes. The contents of the tube should separate to a clear top layer (DNA-containing aqueous phase), a middle layer if dense white pellicle(cell debris), and bottom layer(phenol).

Recovery of DNA Template after Phenol Extraction

Transfer the top aqueous layer on a new clean tube; add ether in the chemical hood. Vortex briefly to mix well, discard top layer (ether) and let the tube sit open in the air for 5 minutes to let ether completely evaporate. Add 2.5 volumes of salty ethanol(EtOH) (to 100 µl of extract, add 250 µl of salty EtOH), vortex to mix well, then place the tube under one of the following conditions to precipitate and store the DNA.

For Shortest Time: insert tube into dry ice to quick freeze for 5 minutes, This method gives the lowest recovery of DNA.
For Moderate Time: Place tube at -70^{0}C for 30 minutes
For longest Time: Place tube at -20^{0}C for 2 hours to overnight. This is the preferred method to collect and store DNA.

Collect DNA by centrifugation in the microfuge at the top speed for 15 minutes at 4^{0}C. Remove all traces if the EtOH. Then add 50µl of Tris- EDTA (TE) buffer (pH 8.0) and mix gently. Template is ready for PCR.

To make stocks of reference DNA, aliquot the template(above 50 µl) into 10 µl aliquots in microfuge tubes, then add 2.5 volumes of salty EtOH to tube. Invert to mix tube contents well; then plate at – 20^{0}C. To use stock, repeat this step and resuspended DNA in 10 µl of TE.

Preparation of PCR reaction or use ready-to-go beads following manufacturer's guidelines
1. **Prepare Master PCR mixed as follows:**

In µl	1 RXN	2RXN	4RXN	8RXN
10X PCR buffer	10	20	40	80
dNTP stock (10mM)	8	16	32	64
DTT(1M)	0.5	1	2	4
Taq polymerase(5U/ µl)	0.5	1	2	4
Distilled water	90	180	360	720
Keep on ice until ready to use				

Prepare reaction mix as follows in a 500- µl tube	µl
DNA template (10ng-100ng DNA)	10
+primer (positive or sense; 50-100 µM)	1
-primer (negative or anti-sense; 50-100 µM	1
Mix by vortex, quick spin down, and then keep on ice	

Always prepare one control tube with water in place of DNA
- Add 88 µl of Master PCR mix to each of the DNA/primer tubes, vortex to mix well and quick spin to collect sample to the bottom of the tube. Place the tube on ice.
- Overlay the tops of reactions with 50 µl of mineral oil or with paraffin (to prevent samples evaporation during thermocycler run).
- Using ready to go beads: Use the same reagents as above and add enough water to make a 20 µl total volume reaction following manufacturer's guidelines.

PCR Cycle

Segment	Time	Temperture	Explanation
1.	1m 10s	94^0C	Separate dsDNA
2.	2m	52^0C	Primer annealing*
3.	3m	72^0C	ds DNA synthesis
Extension	15s	72^0C	Fill in DNA reaction
Hold	Hold	4^0C	Holding until removal
* Annealing temperature depends on the primer pairs, Usually 52^0C is adequate			

- Remove tubes and pipet off the oil. Or, if using paraffin, leave it in the tube. Examine results by mixing loading dye with 10 µl of product and resolved in 0.85% agarose gel with ethidium bromide (EtBr), 100V for 30 minutes to 2 hours, depending on the concentration of the agarose gel.
- Store PCR reaction tubes at -20^0C. PCR reactionsd may be purified and processed for futher uses.
- Dispose EtBr-stained gel into appropriate toxic waste container.

Interpretation

Amplicon of the expected size would suggest a positive reaction. If the water blank control has a product, this would mean that the PCR run cannot be validated and must be repeated after reagent check and equipment cleansing have been done. Negative PCR results do not mean a true negative. PCR reactions are sensitive to the balance of template-to-primer ratios and the amount of magnesium or inhibitors present. If one or another component is off-balance, the results will be PCR negative. Each DNA template may need to be titrated to obtain the ideal reaction conditions.

Quality Control

Aerolisation and carry over of DNA template are the most common PCR problems leading to false positive results. PCR is so sensitive in detecting low amount of target DNA template therefore special attention must be given to work flow so that it is unidirectional starting from extraction, assembly, amplification to analysis. Separate physical areas must be dedicated for each work area as well separate pipettors set aside for each step. Pipettors used with DNA or primers must use barrier-tips to reduce unwanted DNA transfer. All equipments should be cleaned with 10 percent bleach solution followed by 70% alcohol wash.

5.6.4.10 Animal Pathogenicity test

Two animals can be used:
Guinea pigs
White mice

The diagnosis of isolated strain can be confirmed by animal inoculation. The animals are injected subcutaneously with exudate of bubo or with 24 hours growth in broth. Infected animals die within 2 - 5 days and post mortem shows:

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Local necrosis with oedema
Enlargement of regional lymph nodes
Hepatosplenomegaly

Impression smears prepared from heart blood, bone marrow or affected organs shall show presence of bipolar staining organisms.

In case of polycontaminated material, injection to guineapigs must be preferred to inoculation of mice due to extreme susceptibility of the mouse to associated pathogens such as pneumococci, anaerobes etc.

5.6.4.11 New Rapid Diagnostic Tests

ELISA based Rapid Seological Tests
ELISAs for detecting IgM and IgG antibodies and for antigen capture, are especially useful in laboratory diagnosis in early period of illness.

Dot ELISA kit for F1 antibody detection
The newly developed Dot ELISA kit for detection of antibodies to F1 antigen for serosurveillance and possibly for disease diagnosis employs a relatively less amount of F1 antigen and is workable with both the conventionally purified F1 antigen or the recombinant F1 antigen. The kit is fully field based and does not require any laboratory equipment for any specialized training. Test can be performed with a drop of blood without the need of serum separation and the results are obtained within 90 minutes.

Sandwich Dot ELISA for detection of F1 antigen
A sandwich Dot ELISA has been standardized for detection of F1 antigen from the blood following Y. pestis infection. Testing carried out in experimental animals revealed that the antigen detection by the test was comparable to isolation and immunofluorescent test and this could be promising rapid and simple test for routine adaption by the plague laboratories.

Methods for Rapid Identification of Y.pestis and enzyme substrate biochemical kit
For rapid presumptive identification of Y.pestis isolates from broth or agar media, a simple and convenient to use biochemical system has been developed based on 9 newly identified bacterial enzyme substrate reactions. Results are obtained within 30 minutes to 2 hrs. Moderately contaminated cultures can also been tested with this biochemical kit without need of purifying the miocroorganisms.

Monoclonal antibody sandwich Dot ELISAs
Recombinant outer membrane proteins of Y.pestis, namely Pia, Vops, VopM, VopV and hmsT that elicited immunodominant response were produced. Specific monoclonal and the polychonal antibodies generated against these proteins could be successfully utilized in developing sandwich Dot ELISA kits that could provide confirmatory identification of Y.pestis isolates within 2 hrs.

All the above mentioned tests need to be evaluated by WHO Collaborative Centres, before these are used for testing of samples and release of reports.

5.6.4.12 Safety in the Laboratory

Handling of Y.pestis or material suspected to be having this organism requires biosafety level II (BSL-II) environment and precautions. However, when large volumes of culture are handled, BSL-III facilities are required. Only trained personnel, in restricted area, should undertake plague diagnostic work. The infectious material must be handled only in biosafety cabinets (vertical laminar airflow under negative pressure and vented to the outside).

In accordance with national or institutional policy, chemoprophylaxis or immunoprophylaxis, with regular monitoring, should be provided to laboratory staff. They must wear gowns, gloves (with sleeves of gowns tucked into gloves) and masks (if aerosol generation is expected). Hand washing before leaving the laboratory should be mandatory.

A solution of 0.5% sodium hypochlorite should be used to decontaminate the laboratory surfaces as well as for managing the spills. In later case sodium hypochlorite should be sprayed into spill, left as such for 10 minutes and followed by a 70% alcohol wash. Other germicidal solutions containing phenol or quaternary ammounium compounds should be used in instances where hypochlorite is considered corrosive.

All contaminated material must be placed in highly visible biohazard bags. These should be filled only to two-third capacity, sealed with sterility indicator autoclave tape and autoclaved at 115^0C for 15 minutes before disposal. Sharps should be disposal of in rigid puncture-proof containers, labeled with biohazard sign and decontaminated with autoclaving.

5.6 TREATMENT

The response of plague to antibiotic treatment in dramatic and rapid. Chemotherapy must be started at the earliest suspicion of plague without waiting for laboratory confirmation as it will not only curtail morbidity and prevent mortality but also contribute towards containment of transmission of infection by rendering the patient noninfectious. Specific drugs recommended for treatment of plague are tetracycline, chloramphenicol, co-trimoxazole, sulphonamide and streptomycin. Penicillin is ineffective.

5.7 PREVENTION AND CONTROL METHODS

Long-term prevention and control of plague is primarily based on following activities:
- Surveillance
- Rodent control
- Vector control
- Chemoprophylaxis
- Immunoprophylaxis

Surveillance

Establishing and sustaining an effective surveillance system is a prerequisite for prevention and control of plague. A senior and experience officer should be identified at the district and state level to co-ordinate and supervise surveillance activities. As a first step, it is necessary to identify the natural foci of plague in the country and find out which local rodent and flea species should be targeted for extensive surveillance and control. It is also necessary to know the information on local landscape, human activity and host/vector ecology to design prevention and control strategies appropriate for a particular plague focus. Expertise available at the national level

(NCDC) may be sought for these activities and for training of core people involved in surveillance activities.

The surveillance activities should cover surveillance of rodents and vectors as well as laboratory and clinico-epidemioloical surveillance.It is emphazied that surveillance is not a one-time activity and has to be carried out on a continuos basis to be effective in prevention and control of plague.

Rodent Control

Rodents are the most adaptable among all mammals and form the most important reservoirs of infections transmissible to man. As with all wild animals, there is a delicate balance between their number and natural regulatory forces. However, during any disruption within the environment, rodents are the first to respond and adapt to new circumstances.

Rodent species which are synanthorpic (living together with man) viz. Rattus rattus, R.nervegicus, Mus.musculus, Bandicota bengalensis, B. indica etc. may act as reservoir of disease and play role in the spread and transmission of plague.

Rodent control activities should be undertaken during interepidemic period only. Killing of rodent during epidemic situations may result in large number of fleas leaving their dead rodents and biting human beings and transmitting the infection. Rodent control measures should, therefore, always be undertaken as a long-terms measure.

Vector Control

Flea control measures should be undertaken during following situations:

- As and when any locality/area rat fall.
- Increase in the population of fleas/any area reporting flea nuisance(increase of flea index).
- Flea (Xenopsylla cheopis) index found to be more than 1.0 through active surveillance in areas of known foci of natural plague.
- On receipt of specific information from local health authorities about flea nuisance and apprehending plague.

Chemoprophylaxis

Preventive therapy may be taken in the event of exposure to the bites of rodent fleas during an outbreak or to the tissues or fluids of a plague-infected animal, in the event of close exposure to another person or to a pet animal with suspected plague pneumonia. For preventive drug therapy, the preferred antibiotics are the tetracylines, chloramphenicol, or one of the effective sulfonamides. Chemoprophylaxis is given until one week after the exposure ceases.

Immunoprophylaxis

A formalin killed plague vaccine is produced in limited quantity by Haffkine Institute, Mumbai. It contains 2000 million organisms per ml.

Dosage: The adult dose is 0.5ml given subcutaneously followed by second dose of 1ml given after 7-14 days. Boosters are given every six months. Immunity appears after 5-7 days of

complete vaccination and lasts for about six months. Repeated boosters induce higher incidence of adverse reactions.

Adverse reactions: Vaccination may result in adverse effects of fever, headache, malaise, lymphadenopathy and erythema and indurations at the injection site. These reactions are more common after repeated injections. The vaccine should not be given to persons with unknown hypersensitivity to beef protein, soya, casein or phenol. Persons with history of severe local or systemic reactions to plague vaccine should not be revaccinated. Vaccine is contraindicated for pregnant women because of its potential risk to produce abortions or fatal abnormalities.

- Vaccination is of little use during human plague outbreaks, since a month or more is required to develop a protective immune response.
- Vaccination is recommended only for high risk group of individuals.
- Medical and public health workers who are dealing with patients of plague or who investigate plague outbreaks.
- Laboratory workers who handle plague cultures of plague infected materials.

It is unlikely that plague vaccine will protect completely a vaccine exposed to infection from patients with pneumonic plague. Therefore, all persons exposed to patients with suspected pneumonic plague should receive antibiotics regardless of their immunization status.

Universal precautions

Health workers handling plague cases or material infected with plague bacilli should take following precautions:

- Take chemoprophylaxis with tetracycline or co-trimoxazole mentioned above. Depending upon feasibility, such workers should be immunized with plague vaccine.
- Handle material suspected to be contaminated with plague bacilli very carefully. Such material should be incinerated or properly sterilized.
- A patient with pneumonic plague should be given respiratory isolation or cohorted. All workers coming in contact with patient of pneumonic plague or processing clinical material must wear mask, goggles and gloves and must take all biosafety measures.

Referal Centres For Plague

National Centre for Disease Control, Delhi
Plague Surveillance Unit, NCDC, Bangalore

Further Reading

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LEPTOSPIROSIS

6.1   INTRODUCTION

"Mud fever," "Pea Pickers Disease", "Canicola Fever", "Weil's Disease", "Haemorrhagic Jaundice", "Infectious Jaundice", "Swineherd's Disease", "Swamp Fever", and "European Swamp Fever" are all terms that were formerly used to describe Leptospirosis.

In 1882 Adolf Weil described a disease that he had observed two times in four patients in 1870. The symptoms were similar in all four patients and were very unique. The disease came with sudden fever, high temperature, tumor of spleen and icterus. In 1891 F. Muller described Schlamfieberepidemie in Schleisen, a disease with similar symptoms. Rimpau et al. described Feldfieber, which was a name that was meant for unicteric leptospirosis. Thus the disease, Leptospirosis, had been called with different names at different places over different periods of time. Weil had called it "Typhus biliosus nostras", others called it "Maladie de WeB", Infectious icterus" etc.

Leptospirosis is primarily a contagious disease of animals, occasionally communicable to humans, caused by a pathogenic *spirochete* of the genus *Leptospira* and is considered as the most widespread zoonotic diseases in the world. Leptospirosis is caused by *Leptospira interrogans,* a corkscrew-shaped bacterium (spirochete). Leptospirosis causing bacteria are common worldwide, especially in tropical countries with heavy rainfall.

Leptospirosis was recognized and identified as a separate disease entity roughly one and a half century ago and by around 1930s the dynamics of the infection including the portal entry of bacteria, reservoirs of infection, vehicles of transmission, mode of transmission etc. were known.

6.2   CAUSATIVE AGENT

Leptospirosis is a zoonotic bacterial disease. Bacteriologists, and among them Robert Koch, after discovery of Weils disease tried to isolate etiologic agent with no success. Etiologic agent was first isolated in 1915 by Inada and Ido.

The microorganisms responsible for leptospirosis belong to genus *Leptospira* of the family Leptospiraceae that traditionally consists of two species; L.interrogans and L.biflexa. The former includes all pathogenic serovars and the latter the saprophytic strains. Leptospira strains have been divided into 23 serogroups of which two belong to saprophytic leptospires. Each serogroup consists of several strains designated as serovar that is basically taxon.

6.3   EPIDEMIOLOGY

6.3.1 Global Scenario

Leptospirosis, an acute bacterial infection, that affects humans and a wide range of animals occurs more commonly in tropical countries, and man is invariably infected from animal sources.

The history of leptospirosis in the world is very old. The prototype of the clinical illness due to leptospiral infection; Weil's disease, was known even during the last part of the previous century. Human leptospirosis was recognized in Europe in the 1880s but the causative organisms called leptospires were first grown in Japan in 1914. By around 1930s the dynamics of the infection including the portal of entry of the bacteria, reservoirs of infection, vehicles of transmission, mode of transmission etc. were known.

Although Leptospirosis occurs worldwide, its impact on human health is biggest in humid tropical countries.

6.3.2 Indian Scenario

The history of leptospirosis and leptospiral research in India closely followed that in the rest of the world. There had been documented case series of clinical syndromes resembling Weil's disease in many parts of the country even during the last decades of the 19th century itself. In India it has been documented from all over Southern India, Maharashtra, Gujarat and the Andamans.

The first isolate of leptospites from human patients in India was recovered from the Andaman Islands in 1929. Even before this there had been cases of Infectious Jaundice with clinical features akin to Weil's syndrome in the islands. But it was in 1929 that Taylor and Goyle did a systematic search for the bacteria in Andamans. During the 6-month period of their investigation the observed 60 cases of leptospirosis confirmed either by culture or serology. They obtained 26 isolates from these patients. It was the first instance of successful isolation of leptospires in the country. Later there were reports coming from other parts of the country that established the fact that leptospires and leptospirosis were omnipresent in the country.

The 80s witnessed a sudden upsurge in the reports of leptospirosis in the country. The southern states were affected first. Then there was Andaman and the classical example of Andaman Hemorrhagic Fever in 1988. The so-called Andaman Hemorrhagic Fever was an acute hemorrhagic disease with predominant pulmonary involvement and high case fatality rate, which was later proved to be leptospirosis. Gujarat was another state that was affected by leptospirosis very badly in the recent years. Leptospisoris is a common causre of acute renal failure in South India. During the period 1997-99 several case series have occurred in the places like Assam, Pune, Bangalore and Manipal. In 1997 alone atleast four outbreaks have been reported from Mysore, Surat, North Andaman and Nagpur. Then a severe outbreak of leptospirosis hit Mumbai and Thane districts in May 2000, and in August 2000 in Middle Kerala, resulting into great morbidity and significant mortality.

Today, thousands of people are affected every year in this country and judging from the number of increasing cases from the various parts of the country, it can be said that leptospirosis is emerging as an important public health problem in the country.

Leptospirosis is perhaps the most widespread zoonoses in the world. It is caused by Leptospires, members of the order Spirotactales. Pathogenic leptospires belong to the species *Leptospira interrogans,* which is subdivided into serovars. Over 230 serovers have been identified and therefore the magnitude of this disease should be viewed in the broader perspective of more than 230 host adopted leptospiral serovars that are naturally carried by more than a dozen species of rodents, wildlife and domestic animals in moderate to high conducive eco-environmental

conditions. This amount to an abundantly available variety of hosts that perhaps outnumber humans and livestock put together, resulting in a very successful perpetuation of this organism.

Leptospira represents a group of highly successful organisms that effortlessly parasite kidneys of a variety of hosts for their survival, maintenance and perpetuation. In this process, they do not harm or cause significant biological problems to the carrier hosts (rodents, wildlife), but the accidental hosts like man and domestic animals do suffer from unapparent mild febrile conditions to fatal hepatic, renal and pulmonary complications.

Leptospirosis is indeed a successful culmination of the Leptospira, the reservoir hosts and the environment.

6.3.3 Leptospiral Serovars

"Two strains are said to belong to different serovars if after cross-absorption with adequate amounts of heterologous antigen more than 10% of the homologous titre regularly remains in at least one of the two antisera in repeated tests."

The first attempt to introduce some order into the growing number of serologically different strains of Leptospira was undertaken by Wolf and Broom in 1954. They formulated the first principles to differentiate serovars, previously designated as serotypes, on the basis of their serological characteristics using rabbit immune sera that was the only practical criteria available at that time.

The causative organism, *Leptospira interrogqans,* has over 230 serovars. The 230 or more leptospiral serovars have select global distribution and represent a highly successful parasitic pathogen of mans and animals. Theoretically any mammal is capable of being infected by any serovar of L *interrogans.* Practically, however, only a few serovars are enzootic in a given area. Each serovar is maintained on a long-term basis by one or more definitive hosts. Therefore, in a particular region a serovar will be adapted to infect one or more animal species. A serovar can also infect an accidental host such as man.

6.3.4 Reservoir Hosts

Perhaps no other viral or bacterial pathogen has such a wide range of natural rodent and non-rodent reservoir hosts. Some of the major reservoir hosts of leptospires include: rodents, foxes, wild cats and rabbits. The leptospira-host relationship is complex. Though the leptospires are not host specific, preferential hosts do exist. Rodents are believed to be infected for life and generally act as carriers of a particular serovar prevailing in the area. Rodents have an enormous ability to excrete large number of leptospirae in the urine, which is the main source of contamination incriminating human and animal leptospirosis. Therefore, the epidemiology of leptospirae of any country is best reflected by the serovars carried by the rodents in that country as it is universally accepted that rodents constitute the single largest group of reservoir hosts spreading leptospira infection to man and animals. Apart from rodents, both wild and domestic animals also serve as reservoirs of leptospires, which survive in the renal tubules of the infected animals and are excreted in their urine. Man, usually the dead-end host, gets infected when he comes in contact with the urine of rodents and/or infected animals directly or indirectly due to domestic, occupational or recreational activities. The bacteria enter the body through skin, oral or ocular routes.

Rat being the predominant natural carrier of leptospires, in India it is *Rattus rattus* that has been found reservoir for several serovars. In other regions, other wild life hosts such as mongoose have become important host carriers.

6.3.5 Ecological Interface

The endimicity of leptospirosis is directly related to the alkanality of the soil and water, which are required for their survival. Acid medium is detrimental to the survival of the organism. The optimal conditions for the survival of the organisms are a warm and wet environment. Because of the environmental conditions Leptospira infection in tropical and sub tropical regions occur throughout the year. Whereas, in temperate regions such infections are seasonal i.e. more common during rainy and warm months.

India is a huge bio-and-eco sphere that abundantly supports both natural reservoir hosts and the survival of leptospires following excretion through urine. A biological equilibrium is established between rodent species, leptospiral serovars and their survival in nature. Ecological disturbances such as earthquakes, floods etc. set off an imbalance between pathogen, host and environment. A geographical area, which is the natural home for a particular species of rodent, tends to carry a particular serovar. The intermixing of rodent species often results in the establishment of multiple lepto serovars. All these critical factors for maintenance and transmission of the leptospirosis are present in India.

6.3.6 Transmission

The natural reservoirs for leptospires are wild animals, which recover from the infection but continue to excrete organisms in their urine. Leptospires that are shed into the environment come into contact with the mucous membranes of a potential host or are ingested by small animals, and then the disease develops.

Leptospires can survive outside the host for up to six months in moist, warm conditions. Stagnant water is an ideal source of infection. On the other hand, high temperatures (greater than 36°C), freezing, drying, or an acid or alkaline environment rapidly kills the bacteria. For these reasons, leptospirosis is seen most frequently in areas of the world that have a temperate climate and a high rainfall. Leptospirosis usually gains access to a herd by means of an infected carrier animal or infected wildlife. Leptospires usually infect the kidney; thus urine from shedding animals can contaminate pens, corrals, pastures, drinking water, feed, etc. Alternatively, aborted fetuses, afterbirth or uterine discharges can also contaminate the environment. Infected bulls can also transmit the infection through semen to females in the herd. Infected swine have been known to pass the organism in their urine for up to two years. These long-term shedders are of particular importance because they transmit the disease to younger animals in the herd and the cycle goes on.

Dogs can acquire leptospirosis by coming in direct contact with soil or water contaminated with animal urine, or by consuming waste products from other animals. Although cattle and horses can contract, carry and pass the disease, the most likely transmissions come from wildlife such as opossums, raccoons, pigs, and skunks. It is extremely rare for cats to contract the disease. The carrying animal usually recovers from the disease, but it can continue to carry and excrete the leptospires for weeks or even up to years.

Humans can become infected by all these animals. The spirochetes invade mucous membranes and/or damaged skin and migrate to various organs in the body via direct or indirect contact with infected animal urine, or less frequently, from animal bites, handling infected animal tissues or swallowing contaminated food or water. Direct or person-to-person spread is exceptionally rare.

People can get leptospirosis by contact with fresh water, damp soil, or vegetation contaminated by the urine of infected animals. People who canoe, raft, wade, or swim in contaminated lakes, rivers, and streams can get leptospirosis. Leptospirosis can also afflict field workers who work in contaminated flood plains or wet agricultural settings. Mine workers are at particular risk because rats frequently spread the disease. Eating infected meat can also transmit the disease.

6.4 CLINICAL MANIFESTATIONS

Leptospirosis is an acute febrile systematic disease whose manifestations result from the effects of a general vasculitis. The symptoms are expressed depending upon the involvement of various systems and organs of the body and may also vary according to the serovars causing the infection. There is involvement of central nervous system, erythropoietic system, reproductive system, liver, kidney, lungs and eyes.

6.4.1 Cattle

In cattle, the age group most susceptible to the acute form of leptospirosis is calves up to one month of age. Affected calves will run a temperature as high as 41.5°C (107° F), are depressed and off feed, and their mucous membranes are often pale and show small hemorrhages. Jaundice is commonly observed, and blood colored urine is passed. The calves often develop anemia, which results in an increase in respiratory and heart rates. The death rate is frequently high, and any calves that do survive may undergo a long convalescence.

In older cattle, infection may cause abortion. An abortion storm can occur, particularly in groups of susceptible cattle that are pregnant and exposed to infection at the same time. Abortions are seen frequently in the last three months of pregnancy. In dairy cows, milk flow is considerably reduced, and the udder becomes flaccid. The milk may have a yellowish-orange appearance or even contain blood clots. Because of the high numbers of blood cells in the milk, it would appear that mastitis is present; in actual fact there is no true inflammation in the udder. Severe lameness has been described in some animals as a result of inflammation of the joints, whereas other animals may show evidence of photo- sensitization. This is a marked reaction to sunlight and is characterized by death of areas of unpigmented skin, particularly around the head. Jaundice mayor may not be present in adult cattle, although red colored urine is seen frequently. The latter results from breakdown of the red blood cells resulting in blood pigments in the urine.

6.4.2 Swine

Very few swine affected with leptospirosis show any signs of illness. Loss of appetite, fever and diarrhea may occur, but these signs last only from one to three days. At anyone time, there are probably only a small number of animals going through this acute stage, so it can easily be missed. The most important effect of the disease is seen in sows in which abortions occur generally within the last month of pregnancy. Alternatively, litters may be born at full term either dead or so weak that they fail to survive. An abortion storm can occur when the disease first

strikes a herd; however, the disease does tend to be self-limiting as natural immunity builds up in the herd.

6.4.3 Dogs

The most common manifestation of leptospirosis in dogs is kidney disease. Male dogs seem to be affected more than females. The majority of leptospira infections of dogs produce either no symptoms or symptoms so mild that they pass unnoticed. Dogs may exhibit signs of mild to moderate malaise and depression, low-grade fever, vomiting, and reddening of the mucous membranes and whites of the eyes. Blood analysis often reveals elevation of white blood cells and elevated levels of blood urea nitrogen.

6.4.4 Humans

The clinical spectrum of leptospirosis is very wide, with mild anicteric presentation at one end to severe leptospirosis with severe jaundice and multiple organ involvement on the other. Many infections may go unnoticed because of lack of significant clinical illness. Various clinical presentations of leptospirosis are as follows-

6.4.4.1 Anicteric leptospirosis
It is the milder form of the disease. Patients present with-
- Fever - Patients have remittent fever with chills. It may be moderate to severe.
- Myalgia - It is characteristic finding in leptospirosis. Calf, abdominal & lumbosacral muscles are very painful & severely tender. This symptom is very useful in differentiating leptospirosis from other diseases causing fever. This is associated with increase in serum Creatinine Phosphokinase (C.P.K.) which helps in differentiating leptospirosis from other illnesses.
- Conjunctival suffusion - There is reddish coloration of conjunctiva which is a very predominant sign in leptospirosis. It is usually bilateral and is most marked on palpebral conjunctiva. It may be associated with unilateral or bilateral conjunctival hemorrhage.
- Headache - Usually intense, sometimes throbbing, commonly in frontal region. It is often not relieved by analgesics
- Renal manifestations - Some form of renal involvement is invariable in leptospirosis. It usually occurs as asymptomatic urinary abnormality in the form of mild proteinuria with few casts and cells in the urine. Severe renal involvement in the form of acute renal failure, (which occurs in icteric leptospirosis) is rare
- Pulmonary manifestations –Cough and chest pain are primary manifestations and in few cases haemoptysis may occur. Severe involvement leading to respiratory failure does not occur in anicteric leptospirosis.
- Hemorrhage - Hemorrhagic tendencies are also present in some cases

Note: All the clinical features either decrease or disappear within two to three .days and then they reappear and may progress to severe disease. In endemic area all cases of fever with myalgia and conjunctival suffusion should be considered as suspected cases of leptospirosis.

6.4.4.2 Icteric leptospirosis
This is the more severe form of leptospirosis. As the name suggests, patients present with jaundice. Others features are-

- Fever – Same as in anicteric leptospirosis but may be more severe and prolonged.
- Myalgia – Calf muscle tenderness becomes more evident. Severe myalgia may force the patient to stop walking and may even be mistaken as paraplegia. Muscle pain may occur due to myositits, myonecrosis or bleeding into the muscles.
- Headache- 50 % of the patients present with diffuse headache which is seldom
- severe.
- Conjunctival suffusion – Many patients will have reddish yellow discoloration,caused by icterus and congested block vessels or sub conjunctival hemorrhage.
- Acute renal failure manifests as oliguria/anuria and/or proteinuria
- Nausea, vomiting, diarrhea, abdominal pain
- Hypotension and circulatory collapse.

6.4.4.3 Severe leptospirosis

The more severe form of disease with severe liver and kidney involvement is known as Weil's disease. Salient features of the organ involvements are described below.

- *Hepatic:* Jaundice is the most important clinical feature. It may be mild to severe. It starts after 4 to 7 days of illness. Hepatic encephalopathy or death due to hepatic failure is rare. Hepatomegaly & tenderness in right hypochondrium are usually detected.

- *Renal:* Renal involvement is almost invariably present in Leptospirosis. It presents as acute tubular necrosis (ATN) andinterstitial nephritis. Hematuria with complaints of Cola colored urine and RBC casts in urine microscopy is common. In severe cases patients have acute renal failure and present with:
    o decreased urine output (oliguria or even anuria)
    o edemaon face and feet.
    o featuresof uremia like breathlessness, convulsion, delirium and altered
    o level of consciousness in very severe cases.
  The renal dysfunction worsens during the first week to the end of 2nd week, after which it starts improving and complete recovery occurs by the end of the 4th week if the patients is maintained on renal support. Severe acute failure cases will need dialysis to tide over the acute phase .There is usually no residual renal dysfunction.

Pulmonary: In mild illness patient presents with only cough, chest pain and blood tinged sputum. In severe cases patients have cough, hemoptysis, rapidly increasing breathlessness which may lead to respiratory failure and death. On examination, these patients have increased respiratory rate with crepitation in the basal region, which rapidly spread upwards to middle and upper lobes.X-ray shows basal and mid zone opacity in severe cases. It may be normal in mild cases. Hemorrhagic pneumonitis with interstitial and intra alveolar hemorrhage surrounded by focal capillary injury are common pathologic changes. Death can occur within hours to two days due to pulmonary hemorrhage and severe respiratory distress. There are wide variations in pulmonary presentation. It is the commonest cause of death due to Leptospirosis.Case fatality rate in leptospirosis is 0-15% and more than ninety percent (90%) of deaths due to leptospirosis occur due to pulmonary alveolar hemorrhage and renal complications. Cardiovascular system involvement: Patients present with one or more of the following features:
Shock: Patient develop severe hypotension, cold clammy extremities, and tachycardia. Echocardiography reveals normal systolic function of left ventricle hence hypotension is due to either dehydration or peripheral vasodilatation.

Arrhythmias: Patient presents with palpitations, syncope and irregular pulse. Common arrhythmias seen are supraventricular tachyarrhythmia and various degrees of A.V. blocks. Ventricular tachy-arrhythmias are infrequent. Segment depression and T wave inversion may be present in some patients.

- **Central nervous system:** CNS involvement in leptospirosis commonly present as meningitis. Headache may be the only manifestation or irritability, restlessness, seizures and coma can occur. Encephalitis, focal deficits, spasticity, paralysis, nystagmus, peripheral neuropathies, nerve palsies, radiculitis, myelitis all have been reported.

- **Skin:**Macular, maculopapular erythematous skin eruptions are seen in the face, trunks and / or extremities in many patients with occasional cases of purpura. It may be noted that bleeding manifestations in leptospirosis are not directly related to the level of thrombocytopenia. They resolve in two to three days without any specific intervention.

- **Leptospirosis in Pregnancy:** Leptospirosis during pregnancy has a bad prognosis and fetal loss had been reported to be high in the first trimester and near term mothers.

All patients with severe, multiple organ involvement should be referred to tertiary care centre

6.5 LABORATORY DIAGNOSIS

Diagnosis of leptospirosis is often difficult to make. Diagnosis is determined by detection of antibodies to leptospires in the patient's blood, or more rarely by growing the bacteria from blood and other body fluids. Diagnosis is difficult because the bacteria are only found in the blood for 4-5 days. The serologic test for antibodies in the blood must be done at that time.

To confirm the diagnosis, it is necessary to demonstrate that leptospires are present in either urine or tissues. The most frequently used technique is immunofluorescence, which employs a special staining technique and examination of material under ultraviolet light. Silver staining, an older and more time-consuming method, is rarely used nowadays because it is much less specific. Culture of the organism from urine, blood or milk may be attempted using special media. Alternatively, leptospir'es can be cultured in guinea pigs or hamsters by injecting them with infected material provided that it is fresh.

Blood tests are also available but, because of inaccuracies, should be used on a herd basis rather than an individual animal basis. In an infected herd, not all the animals will react to an equal degree, and ideally a selection of animals from each age group should be tested. The results must be interpreted carefully and in conjunction with the herd history, exposure to wildlife and clinical findings. It should also be pointed out that, with some serovars, i.e., L.*hardjo,* the titers (blood reactions) do not remain high for a long time. In such cases the titer will rise at the time of infection but may have declined considerably by the time the animals abort.

The definitive diagnosis of leptospirosis depends on seroconversion or greater rise in antibody titer, or isolation ofleptospires from any clinical specimen. However, the etiological confinnation is absolutely unnecessary for the start of therapeutics; the exclusion of other important diseases (meningococcemia, malaria, Japanese Enchephalitis, septicemia etc.) is essential.

Presumptive test

- A positive result in IgM based immune- assays, slide agglutination test or latex agglutination test or immunochromatographic test.
- A Microscopic Agglutination Test (MAT)titre of 100/200/400 or above in single sample based on endemicity.
- Demonstration of leptospires directly or by staining methods

Confirmatory test

- Isolation of leptospires from clinical specimen
- Four fold or greater rise in the MAT titer between acute and convalescent
- phase serum specimens run in parallel.
- Positive by any two different type of rapid test.
- Sero-conversion.
- PCR test.

Supplementary Laboratory findings

- Total WBC count slightly elevated with neutrophilia
- Increased erythrocyte sedimentation rate (about 60 mm);
- Decreased blood platelet counts
- Increased BUN and serum creatinine;
- Normo/Hypokalemia
- Urinalysis with proteinuria, hematuria and casts;
- Increase in serum bilirubin (predominantly direct) levels;
- Alkaline phosphatase as well as SGOT and SGPT levels moderately elevated;
- Marked elevation in serum creatinine phosphokinase (CK) and MB variant, this
- second one in case of myocarditis.

6.6   TREATMENT

Once the diagnosis is established, immediate treatment should be started.

6.6.1 Mild to moderate cases

- Adults: Doxycycline 100 mg twice a day for seven days. Pregnant & lactating mothers should be given capsule ampicillin 500 mg every 6 hourly.
- Children< 8 years: Amoxycillin/ Ampicillin 30-50 mg/kg/day in divided doses for 7 days.

6.6.2 Severe cases

- Adults: Inj. Crystalline penicillin 20 lacs IU IV every 6 hourly after sensitivity test.(For the individuals who are sensitive to penicillin group of drugs following alternative regimes maybe used)
  Ceftriaxone 1 gm IV x 6 hourly for 7 days OR
  Cefotaxime 1 gm IV x 6 hourly for 7 days OR
  Erythromycin 500 mg IV x 6 hourly for 7 days
  Pregnant & lactating mothers should be given capsule ampicillin 500 mg every 6 hourly.

- Children< 8 years: Inj. Crystalline penicillin should be given 2–4 lacs IU/kg/ day for 7 days. (For individuals who are sensitive to penicillin group of drugs following alternative regimes may be used)

    Ceftriaxone 50-75 IV mg/kg/day for 7 days OR

    Cefotaxime50-100 IV mg/kg/day for 7 days OR

    Erythromycin 30-50mg/kg/day in divided dose for 7 days

In disease progression multiple organs such as kidney, liver, lungs , CVS and CNS may be involved. All suspected leptospirosis cases whether positive or negative with rapid immunodiagnostic test having feature of organ dysfunction as follows should be IMMEDIATELY shifted to higher centre.

- Renal :
    - Decreased urine output (< 400 ml per day)
    - High blood urea (> 60 mg. %)
    - High S. Creatinine (> 2.5 mg%)
    - Clinical features of uremia,breathlessness,convulsion,delirium,and / or altered level of consciousness
- Hepatic :
    - Jaundice
    - High S. Bilirubin(>3.0m.g. %)
- Pulmonary :
    - Breathlessness
    - Haemoptysis
    - Increased respiratory rate
    - X- ray chest showing opacities
- Blood :
    - Bleeding tendency
    - Low platelet count

The unfavorable indices are age (>50 years) and development of Jaundice. The more severe cases however are associated with a high mortality. So, prompt diagnosis and treatment help in terminating most of the fatal effect.The management of organ involvement does not differ then that of non-leptospirosis causes.

6.7 PREVENTION AND CONTROL

6.7.1 Personal protection

Hygienic methods such as avoidance of direct and indirect human contact with animal urine are recommended as preventive measures. Workers in flooded fields should be cautioned against direct contact with contaminated water or mud and should be advised to use rubber shoes and gloves. In case of any cuts or abrasion on the lower extremities of the body, the worker should apply an antiseptic ointment e.g. betadine, before entering the field and after exit. Those at risk should cover all cuts with waterproof plasters and wear protective clothing or other materials. Immersion in natural waters such as rivers, fakes and canals should be avoided, and individuals should wear footwear and wash or shower after canoeing, windsurfing or swimming.

Low lying or swampy areas should be drained or fenced, and access to stagnant water or runoff from corrals should be prevented. Contact between domestic animals and wildlife should be

controlled, if possible. Inside buildings, pens should be cleaned and disinfected after occupancy by infected animals. In hog barns, the pen partitions should be solid for at least part of their height to prevent urine splashing from one pen to the next. Pits rather than common manure alleys should be used wherever possible to prevent contamination from pen to pen. And finally, a minimized contact with fresh water, mud, and vegetation that might be contaminated with the urine of infected animals, especially rodents is sensible

6.7.2 Health education

The main preventive measure for leptospirosis is to create awareness about the disease and its prevention. This has to be conceptualized through intensive educational campaign, IEC templates/software for audio visual, print, press, outdoor outreach modes, new and emerging electronic media.

6.7.3 Chemoprophylaxis

Antimicrobial prevention is only indicated *for* those people who are under risk of exposure_- except on epidemics - to leptospires (doxycilcine 200mg/week) and *for* those ones with known exposure (doxycycline 100mg *for* 7 days) such as agricultural workers (eg. paddy field workers, canal cleaning workers in endemic areas) from where clustering of cases has been reported. The chemoprophylaxis should be for six weeks and never to be extended for more than eight weeks.

6.7.4 Rodent control

It is established beyond doubt that rodents are the major reservoirs of bacterium Leptospira interogans. Four species of rodents Rattus rattus (House rat), Rattus norvegicus (Norway rat), Bandicota bengalensis (Lesser bandicoot) and Bandicota indica (Larger bandicoot) are so far found to be reservoirs for this bacterium in India. Hence controlling these reservoir species with proper strategy planning and management planning will reduce the incidence of the disease in the affected areas. The strategic planning should cover the following:

   1. Identifying the reservoir species of affected area
   2. Delineating areas for anti rodent activities
   3. Completion of activities in pre monsoon months.
   4. Adopting appropriate technology for anti rodent operations. This includes correct inputs and appropriate application technology.
   5. Capacity building
   6. Creating awareness in general community and community participation.

6.7.5 Mapping of water bodies for establishing a proper drainage system.

The mapping of water bodies and human activities in water logged areas should be carried out. This will help to identify the high risk population. Farmers may be educated to drain out the urine from the cattle shed into a pit, instead of letting it flow and mix with water bodies (rivers, ponds etc.)

6.7.6 Health impact assessment of developmental projects

Health impact assessment should be made mandatory for all developmental projects

along with environmental assessment

6.7.7 Vaccination of animals

Leptospiral vaccines confer a limited duration of immunity. Boosters are needed every one to two years. Vaccination should however be very selective and used only in endemic situations having high incidence of leptospirosis. The vaccine must contain the dominant local serovars. While this prevents illness, it does not necessarily protect from infection and renal shedding.
 Vaccines consist of inactivated bacteria containing the leptospires *Leptospira canicola* and *Leptospira icterohaemorrhagiae.* Vaccines will neither prevent urinary shedding in infected animals nor eliminate the organism from these animals. Therefore, the vaccines do not prevent infection, but rather are effective in reducing the occurrence and severity of disease. Further, duration of protection following vaccination is relatively short, usually no more than 6 months and the immunity imparted is specific to the individual leptospires contained in the vaccine.Immunisation of cattle will reduce the excretion of leptospires in their urine. The rat population needs to be reduced, with destruction of their habitats and rat proofing buildings.

6.8 REFERRAL LABORATORIES

- Regional Medical Research Center (ICMR), Port Blair (A&N)
- National Centre for Disease Control, Delhi,
- National Institute of Epidemiology, Chennai
- Government Medical College, Surat
- BJ Medical College, Ahmedabad
- Madurai Medical College, Madurai
- National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bengaluru,
- Bacteriology & Mycology Division, IVRI, Izatnagar, (UP)
- DRDE, Gwalior (MP)
- Tamil Nadu Vetrinary & Animal Science University, Chennai,

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ECHINOCOCCOSIS

7.1   INTRODUCTION

Echinococcosis is a zoonotic infection caused by the adult and larval stages (matacestodes) of the cestode genus echinococcus (family Taeniidae). The term hydatidosis or hydatid disease is restricted to infection with the metacestode, while echinococcosis is applied in a general way to both adult and larval infections.

7.2   CAUSATIVE AGENTS

7.2.1 General description of the parasite

Echinococcus spp. are small tapeworms, rarely more than 7 mm in length. The scolex bears four suckers, and there are two rows of hooks, one small and one lage on the rostellum, the number and length of which may vary according to species. The number of proglottids of the body varies from two to six. The genital pore opens laterally and its position depends on species. The cirrus sac is horizontal or titled anteriorly and the vitellarium is globular.

The eggs are ovoid (diameter about 30-40mm) consisting of a hexacanth embryo (oncosphere) surrounded by several envelopes the most noticeable one being the embryophore, which gives the egg a dark striated appearance.

The metacestode basically consists of a bladder with an acellular laminated layer and an inner nucleated terminal layer, which may rise by asexual budding to brood capsules. Protoscolices arise from the inner wall of the brood capsules or from the germinal layer.

7.2.2 Species of the genus echinococcus

At present four species of the genus Eechinococcus are regarded as valid taxonomically. These are *Echinococcus granulosus, Echinococcosus multilocularis, Echinococcus oligarthrus* and *Echinococcus vogeli*. These four species are morphologically distinct in both adult and larval stages.

7.2.3 Morphology And Host Range

7.2.3.1 Echinococcus granulosus

7.2.3.1.1 Morphology
The adult worm varies between 2-7 mm in length and usually possesses three or four segments (rarely upto six). The penultimate segment is a mature, and the genital pore normally opens posterior to the middle of both mature and gravid segments.

The gravid uterus is character9ized by well-developed lateral sacculations. The larval stage is a fluid-filled bladder usually unilocular but communicating chambers also occur. Growth is expansive and endogenous daughter cysts may be produced. Occasionally, individual bladders may reach up to 30 cm in diameter.

7.2.3.1.2 Host range

Echinococcus granulosus has the least intermediate host-specificity of nay of any four species, and this contributes to its cosmopolitan distribution. The larval stage has been found in a wide range of mammals. The metacestode causes "cystic" hydatid disease in man. The parasite is perpetuated primarily in a domestic cycle involving the dog (*Canis familaris*) as the definitive host and domestic ungulates (e.g. sheep, cattle, pigs, goats, horses and camels) as intermediate hosts. However, in some areas wild animals are involved.

7.2.3.2 Echinococcus multilocularis

7.2.3.2.1 Morphology

The adult worm varies between 1.2-3.7 mm in length and usually possesses four to five segments. The antepenultimate segment is characteristically mature, and the genital pore is anterior to the middle of both mature and gravid segments. The gravid uterus is sac-like.

The metacestode is a multivesicular structure consisting of conglomerates of small vesicles (diameter usually not exceeding a few millimeters). Unlike the metacestode of E. granulosus, the larval mass often contains a semi-solid matrix rather than fluid. It proliferates by budding and this results in infiltration of tissues. It is commonly referred to as the alveolar hydatid.

7.2.3.2.2 Host range

Intermediate host specificity is relatively high and infection with the metacestode isconfirmed to rodents, particularly members of the family Cricetidae (Arvicolidae). Natural infections in soricid insectivores have also been reported. The metacestode causes "alveolar " hydatid disease in man.

Echinococcus multilocularis is mainly perpetuated in a sylvatic eycle involving foxes and cricetid (arvicolid) rodents. Domestic dogs and occasionally cats may also entr into this cycle when they eat infected wild rodents or rodents commonly associate with human dwellings such as house mice.

7.2.3.3 Echinococcus oligarthrus

7.2.3.3.1 Morphology

The adult worm varies between 1.9-2.9 mm in length and normally possesses three segments, the penultimate of which is mature. The genital pore is anterior to the middle in mature segments and approximately at the middle in gravid segments. The gravid uterus is sac-like.

The metacestode is polycystic and fluid-filled with a tendency to become septate and multichambered. The single cyst may reach a diameter of about 5 cm. It has been reported that predilection sites of this metacestode are internal organs and muscles.

7.2.3.3.2 Host range

Echinococcus oligarthrus characteristically use wild felids including the puma (Felis concolor), jagur (Felis onca), jaguarundi (Felis yagouaroundi) and Geoffroy's cat (Felis geoffroy) as definitive hosts. The agouti (Dasyprocta) and possibly other rodents serve as intermediate hosts. So far, no human infections due to E. oligarthrus have been confirmed.

7.2.3.4 Echinococcus vogeli

7.2.3.4.1 Morphology

The adult worm varies between 3.9-5.6 mm in length, and usually has three segments. The penultimate segment is mature and the genital pore is situated posterior to the middle of both the mature and gravid segment. The gravid uterus has no lateral branches or saccuulations, and is characterized by being relatively long and tubular in form.

The metacestode is similar to that of E.oligarthrus. It has been reported that the two species can be distinguished by comparing difference in the dimensions and proportions of the rostellar hooks on the protoscolex.

7.2.3.4.2 Host range

Echinococcus vogeli uses the bush dog (Speothos venaticus) and the domestic dog as definitive hosts, and pacas (*Cuniculus paca)* and possibly other rodents as intermediate hosts. The larval stage does develop in man causing a polycystic form of hydatid disease, predominantly in the liver.

7.2.4 General life cycle

Echinococcus spp. requires two mammalian hosts for completion of their life cycles. Proglottids containing eggs or free eggs are passed in the faeces of the definitive host, a carnivore. The eggs are highly resistant to physical factors and can remain infective for a long period in a suitable environment.

The intermediate host, represented by a wide range of mammals acquires infection by the ingestion of eggs. Following the action of enzymes in the stomach and small intestine, the oncosphere is released from the keratinized embryophore. Bile assists in activation the oncosphere, which penetrates the wall of the small intestine. Penetration is them aided by the hook movement and possibly by secretions of the oncosphere. Upon gaining access to a venule or lacteal, the oncosphere is passively transported to the liver, where some are retained. Others reach the lungs and a few may be transported further to the kidneys, spleen, muscles, brain or other organs.

Once the oncosphere has reached its final location it develops into the metacestode (hydatid larva) stage. Time of development is variable and it may take several months before protoscolices are produced (fertile metacestode). Not all metacestodes produce protoscolices (sterile metacestode). When protoscolices are ingested by a suitable definitive host, following the action of pepsin in the stomach, they evaginate in the upper duodenum in response to a change in pH and exposure to bile. They then develop to the sexually mature tapeworms, approximately four to six weeks after infection, depending on the species and on the susceptibility of the host.

The life cycle of *Echinococcus* species is complex involving two hosts and a free-living egg stage. The dynamics of transmission of the parasite are determined by the interaction of factors associated with these two hosts and with the external environment. An understanding of these interrelationships and the way in which they influence the dynamics of the system, in particular its stability in the face of perturbation, is central for the planning and assessment of control programme.

7.2.4.1 The adult worm within the definitive host

The number of worms haboured by dogs is determined primarily by the number of protoscolices ingested. Since asexual reproduction occurs within thee intermediate host, extreme clustering can occur and worm burdens in the thousands are not uncommon. Dogs show a variable degree of natural resistance to infection and there is evidence that weak acquired immunity develops slowly. this immunity may affect both the numbers of worms that establish themselves in the host and their size. The biotic potential of *Echinococcus* spp. is low. Individual proglottids contain 200-800 eggs and based on the growth rate of the worm it is estimated that one proglottid is shed approximately every 14 days.

7.2.4.2 Proglottids and eggs in the external environment

Under favourable conditions, detached proglottids may remain active for a few days after being expelled in the faeces. They performed rhythmic contractions and relaxations that assist egg-expulsion and may disperse the proglottids considerable distances from the faecal mass. Since sheep generally avoid grazing areas contaminated by dog gaeces, this dispersal enhances the chances of eggs being ingested. it also has important epidemiological implications because it means that a single heavily infected dog can be responsible for infecting intermediate hosts over a wide area.

Agents responsible for this dispersion have not yet been identified, although a variety of possibilities have been suggested. Wind may account for some movement of eggs, but the observed radial uniformity of the dispersion and the fact that the eggs are intolerant of desiccation suggests that this is not a major dispersion mechanism. An animal agent must, therefore, be suspected. However, birds, flies, beetles and ants would seem to be the most likely to be involved.

Eggs deposited on the apsture are subjected to the microclimate of the environment. They tolerate a wide temperature range. Heat damage does not appear to occur until temperatures reach 38^0C and the eggs withstand temperature as low as -30^0C. In contrast, they are very susceptible to desiccation and this factor probrbly dominates all others in restricting the survival of eggs of *Echinococcus* spp. in the natural environment. As a principle applying to all taeniids, population of eggs deposited by the definitive host appear to consist of eggs at various stages of maturity.

They may mature under suitable conditions. Under favourable conditions mature eggs age and their life span is largely determined by the environmental temperature. This life span may vary from about three days to one year. Within certain limits the longevity of the eggs decreases as the temperature increases. The aging of the eggs is characterized by a gradual loss of the ability of the oncosphere to activate *in vitro* and this is associated with a progressive decline in infectivity to the intermediate host.

7.2.4.3 The larval parasites within the intermediate host

The density, infectivity and availability of the eggs in the environment and the feeding behaviour of the intermidate host together determine the number of infective organisms entering the host. B However, the number of these that become established is strictly controlled by the host regulatory mechanisms. These consist of both a natural and acquired resistance to infection. Natural resistance causes parasite mortality to all stages during development and its extent may

be related to the strain, age sex and physiological condition of the host. Little is known of naturally acquired protective immunity in echinococcosis, but assumptions can be made from the extensive studies on other taeniid species. With these, acquired resistance develops after the ingestion of an initial immunizing dose of eggs. This response develops within two weeks of the ingestion of a few as 10-50 eggs, and prevents any further infection. The resistance is maintained throughout the life of the host by the copntinlued ingestion of eggs, but may wane within 6-12 months in the absence of reinfection. Immune mothers may transfer some degree of immunity to their offsring (e.g. via colostrum) but animals do not appear to become fully immunologically competent to cestode infections until several weeks after birth. The rapid development of immunity in competent animals means that the length of time that the intermediate host is susceptible to super-infection following the ingestion of the first eggs, is short (about two weeks). Thus, the events, which occur during this period, determine the larval worm burden for life. This means that grazing management can have a major influence on the level of infection. This immunity also means that the majority of eggs deposited by the definitive host do not initiate infections. Thus, considerable regulation of parasite numbers by the host at this stage.

In common with most host/ parasite systems that have been observed, the hydatid organisms show an overdispersed or aggregated distribution within their hosts. The majority of hosts, therefore, contain only few parasites while a small number contain many. This distribution is generated by heterogenecity in the system and has been shown to have a vital role in determining the dynamics of transmission.

7.3 EPIDEMIOLOGY

The perpetuation of echinococcosis disease depends upon the common presence of the parasite, the definitive and intermediate hosts. The continued existence of host and parasite populations depends upon the fine balance of various interacting regulatory forces. The ability of the populations to survive perturbation, in the form of, for example, environmental change, without either becoming extinct or reaching plague proportions is termed stability. Theoretical work on the dynamics o host/parasite systems has indicated that such characteristics as overdispersion of parasite numbers within the host population and the development of host immunity act as important stabilizing influences. Recently it has been recognized as global public health problem. It has been found in all sheep raising countries including India. The highest prevalence is reported in Andhra Pradesh and Tamil Nadu.

7.4   PATHOGENESIS AND PATHOLOGY

7.4.1 Cystic Hydatid Disease - (Echinococcus granulosus)

After their ingestion, cestode eggs hatch to release embryos in the small intestine. Penetration through the mucosa leads to blood-borne distribution to the liver and other sites, in which development of the hydatid cyst begins. Although most primary infections in humans consist of a single cyst. 20 to 40% of patients have multiple cysts or multiple organ involvement. The liver is the most common site for hydatid cysts *(50* to 70%), followed by the lungs *(25%)* and, less frequently the spleen, kidneys, heart, bones, central nervous system, and elsewhere. The fully developed hydatid cyst is filled with fluid and typically unilocular; however, multilocular or chambered cysts are not uncommon. Structurally, the cyst consists of an inner germinative layer

of cells supported by an acellular laminated membrane of mucopolysaccharide material (endocyst). Small secondary cysts, called brood capsules, develop internally from the germinative layer and produce multiple protoscolices by asexual budding. A protoscolex is a scolex in which the rostellum and suckers are deeply withdrawn into the post-sucker region. Surrounding the hydatid cyst is connective tissue adventitial reaction of variable intensity (ectocyst.)

Growth is by concentric enlargement. Rates of growth of hydtid cysts are highly variable and range from 1 to *5* cm in diameter per year. The slowly growing hydatid cyst is often well tolerated until if causes dysfunction because of its size. In humans the cyst may attain a volume of many liters and contain many thousands of protoscolices. Rupture or leakage of cysts may precipitate allergic reactions as a consequence of sensitization to echinococcal antigens. At the same time, seeding of the peritoneum or pleura with protoscolices may result in extensive seconday cyst formation.

7.4.2 Alveolar Hydatid Disease - (Echinococcus inultiloculoris)

Compared with *E. granulosus,* larval *E. multilocularis* is more progressive and damaging to the host. Human disease caused by E. *multilocularis* has many of the characteristics of malignant neoplasia. Growth in humans is different from that in natural rodent hosts in that the larval mass is inhibited from completing its development and remains in the proliferative stage indefinitely, it then continues to invade and destroy the hepatic parenchyma, and retrogressive stages within the mass result in necrosis of the central portion. Macroscopically the hepatic lesion usually appears as one or more firm to solid, whitish, rounded masses slightly elevated above the surrounding tissue of the surface of the liver. When transected, it appears to consist of a central cavity surrounded by dense, pale tissue that lacks a clearly defined border with respect to the adjacent hepatic tissue. In advanced cases the cavity usually contains turbid yellowish to brown fluid with particles or fragments of necrotic tissue. Microscopically, cysts of various sizes are scattered or closely aggregated in a matrix of connective tissue. Only rarely are brood capsules and calcalreous corpuscles present. Metastasis by a hematogenous route is not uncommon, and secondary foci usually occur in the brain and lungs.

7.5 CLINICAL MANIFESTATIONS

7.5.1 Cystic Hydatid Disease

Clinical manifestations of cystic hydatid disease may be absent or result from the mass effect and anatomic position of the slowly growing cyst(s). The interval between first infection and clinical manifestations is variable and often prolonged for many years. Most infections are diagnosed in patients between 10 and 50 years of age. The signs and symptoms of hepatic hydatid disease may include hepatic enlargement (with or without a palpable mass in the right upper quadrant), right epigastric pain, nausea and vomiting. Rupture or leakage usually results in acute or intermittent allergic manifestations. Complications that may exist at the time of initial presentation include traumatic or spontaneous rupture, thoracobilia and biliarv fistula. Twenty five percent of patients with hepatic cysts also have cysts in their lungs.

Leakage or rupture of hydatid cysts in the lungs causes chest pain, coughing, dyspnea and hemoptysis. Hydatid membranes may be expectorated, sometimes resulting in spontaneous cure. Emergency complications that may exist at the time of presentation include cyst rupture and

secondary bacterial infection. Nearly 40% of patients with pulmonary hydatidosis have liver involvement as well.

The first symptom of cerebral cysts may be raised intracranial pressure or focal epilepsy, whereas kidney cysts may be manifested by loin pain or hematuria. Bone cysts are often asymptomatic until pathologic fractures occur, and, because of their resemblance, they are often misdiagnosed as tuberculosis lesions.

7.5.2 Alveolar Hydatid Disease

The insidious and progressive nature of alveolar hydatid disease usually results in delayed onset of symptoms and diagnosis, by which time the lesions have invaded extensively or metastasized and are inoperable. The clinical signs of the disease usually do not become evident until middle age. Initial symptoms are generally vague. Mild upper quadrant and epigastric pain with hepatomegaly may progress to obstructive jaundice. Occasionally the initial manifestations are caused by metastases in the lungs or brain. Patients eventually succumb to hepatic failure or invasion of contiguous structures. The proportions of surgically resectable cases have varied from 20 to 60°. Of the nonresectable cases, 90% of patients died within 10 years.

7.6 LABORATORY DIAGNOSIS

The diversity of clinical manifestations and the difficulty of demonstrating the deeply located cysts often make diagnosis very difficult. Hydatid disease usually comes to the attention of the clinician for four major reasons: when a large cyst has some mechanical effect on body function; when allergic phenomena or other symptoms such as eosinophilia occur; following the accidental traumatic rupture of a cyst with consequent acute allergic reactions; and on the incidental finding cysts during roentgenography, body scanning or surgery for other clinical reasons.

7.6.1 Radiological methods

7.6.1.1 Cystic Hydatid Disease
Sensitive methods are now available for identifying and characterizing mass lesions in internal organs. Plain-film roentgenography permits the detection of hydatid cysts in the lungs, but usually only calcified cysts can be demonstrated in other sites, Radioisotopic and ultrasonic scanning, computerized tomography are useful for visualizing the avascular cyst(s) in many organs. Of these techniques, computerized axial tomography is the most specific in that it allows a more detailed characterization of the nature of space occupying lesions diagnosis typically provided by other scanning methods. Nevertheless, some cysts, particularly those in extrahepatic abdominal locations. may not be demonstrated by computed axial tomographic scans. Ultrasound imagings techniques are almost as useful as computed axial tomography and have the added advantages of avoiding radiation exposure and relatively low cost. The availability of portable generator powered ultrasound devices permits their use in fields studies. Hydatid cysts must be differentiated from cavitary tuberculosis, abscess, and benign or malignant neoplasms.

Closed aspiration of hydatid cysts should not be attempted because accidental spilling of the contents could cause secondary spread or anaphylaxis. Cyst membranes, protoscolices. or hooklets may sometimes be demonstrated in the sputum or in bronchial washings: identification is facilitated by stainingthehooklets. An intravenous pyelograme is helpful in visualizing renal cysts. Eosionophilia is present in one third of fewer of cyst carriers.

Hydatid cysts removed at surgery are frequently misidentified by inexperienced persons, particularly when protoscolices are lacking or the larva is otherwise degenerated or anomalous. Nevertheless, most lesions can be identified and differentiated from other larval cestodes (i.e. cysticerci or coenuri) and nonparasitic cysts by the characteristic appearance of the laminated membranes.

7.6.1.2 Alveolar Hydatid Disease

Alveolar hydatid disease caused *by E. miultilocularis* is typically observed in elderly persons and mimics hepatic carcinoma and cirrhosis, with which it is commonly confused. Plain-film roentgenography shows hepatomegaly and characteristic scatted radiolucencies outlined by calcified rings measuring 2 to 4 mm.

In Germany and other areas where both cystic and alveolar hydatid disease occur, the two can be distinguished by selective angiography and by computerized axial tomography. The characteristic computerized tomographic image in cystic hydatid disease shows sharply contoured cyst(s) sometimes with internal daughter cyst(s) and marginal calcifications. In contrast, the computerized tomographic image of alveolar hydatid disease reveals indistinct solid tumor with a central necrotic area and perinecrotic plaque like calcifications.

Exploratory laparotomy is often performed in order to diagnose and delineate the extent to which the lesion has invaded. Needle biopsy confirms the diagnosis if larval elements can be demonstrated. Even in the absence of protoscolices, the larval membranes can be readily demonstrated using periodic acid-Schiff staining or the sterile larva can be reared to the adult stage after secondary passage in susceptible rodents.

7.6.2 Immunodiagnosis

7.6.2.1 Blood sampling

Although the serological methods described below only require small amount of serum, at least 1— 2 ml should be obtained, as examination may have to be repeated. Moreover, it is advisable to retain a serum sample until the case is fully clarified.

Blood samples are collected under antiseptic precautions and are allowed to set at room temperature. Clotted samples are kept overnight in a refrigerator or cold room (2-4° C). After centrifugation at 700 g for 15 minutes, sera are carefully removed and stored in a refrigerator (for a few days) or deep frozen at -20°C (longer periods) until tested. When sera have to be sent to distant laboratories, precautions have to be taken to preserve them. If facilities are available sera should be lyophilized, if not, merthiolate should be added as a preservative.

Many inmunodiagnostic techniques have been evaluated for hydatid disease. Although results of most serodiagnostic tests agree fairly well, sera from some patients are negative in one or more tests but positive in others. On the basis of recent experience it is now widely accepted that the sensitivity and the specificity of immunodiagnosis can be improved by the simultaneous application of two or more methods (partly measuring different classes of antibodies). They include, for example the indirect haemagglutination (IHA) test and other particle agglutination tests (latex and bentonite), immunoelectrophoresis (IEP) and double diffusion (DD) tests the indirect immunofluorescent (1IF) test, and the enzyme-linked-immunosorbent assay (ELISA).

An important consideration is that tests based on the detection of antibody to the Echinococcus antigen *5* have the highest degree of specificity. However, because antibody to antigen 5 is sometimes absent in the serum of patients with hydatid disease who have antibodies reactive with other echinococcal antigens, serum specimens should be screened initially with one or more highly sensitive procedures and then tested for antibody to antigen *5*. Tests such as IHA, IIF. LA and ELISA are highly sensitive in detecting circulating antibodies in sera from patients with hydatid disease. The IHA and LA tests are recommended for initial screening because they have been shown to be highly efficient for detecting sera with antigen *5* antibodies and both are relatively simple procedures.

Antigen *5* is one of 10 or more distinct antigens of parasite origin present in hydatid fluid and somatic tissues of the metacestode. This antigen corresponds to antigen 'A" and antigen 4". Immunoelectrophoretic studies have shown that the presence of antibody to this antigen in patient's serum is diagnostic for hydatid disease.

The intradermal Casoni's test is less specific, but when properly controlled, it compares favorably in sensitivity with most serologic tests. Nevertheless, even with partially purified antigen solutions used at low nitrogen concentration, high rates of false-positive reactions have been observed in persons with other parasitic diseases, as well as in some non-parasitic pathologic conditions, especially when antigens with a high protein content are use. Further, skin testing may stimulate the production of circulating antibodies. Therefore, it has been suggested that the ID test should be replaced by serological procedures wherever possible.

Interpretation of immunodiagnostic results

In the interpretation of serological results many factors have to be considered. The performance of any serological test requires experience and careful and continuous control. Even minor technical errors may influence the results. Negative serological results do not rule out an Echinococcus infection because some of the individuals do not have detectable antibodies.

Immune responses have been associated with organ localization, the integrity of the metacestode and the size of the parasite biomass. Hepatic and peritoneal echinococcosis generally elicits stronger antibody reactions than infections of the lung, brain and eye. Children between three and 15 years old were found to produce weaker serological reactions than adults. Leakage or rupture of the cyst or surgical intervention leading to antigen release may be followed by an abrupt stimulation of antibodies as early as 10 days after the event. Dead metacestodes may cease to stimulate the host and carriers of such parasites may become seronegative. The host parasite relationships may also influence the immunodiagnostic results.

7.6.2.2 Indirect Haemagglutination (IHA) Test

Principle: If specific antibodies are present in the serum sample, they affect a cross linkage of erythrocyte. If no antibodies are present. the cells are deposited on the bottom of the reaction vessel in the form of buttons or rings.

Criteria for positivity: Diagnostically significant titres are present in a serum dilution of 1:32 to 1:64 or higher. Titers between 1:512 and 1:2048 are regarded as mean serum titres in patient with vstic echnococcosis. Low positive titres (1:32 to 1:128) should only be assessed together with the positive result of a second serological method (IFA/ELISA).

Sensitivity and specificity
Researchers have found a sensitivity of 71% and 95% in pulmonary and hepatic *E.granulosis* infection respectively with overall sensitivity was *83%.* If the diagnostic titre in the area has been correctly determined, non-specific reaction should be well below this level.

7.6.2.3 Indirect Immunofluorescent (IFA) Test

Principle: Antibodies reacting with antigen in sections of protoscolices are detected by fluorescein labelled anti-immunoglobulin labelled sera. The test was adapted for hydatid serodiagonosis by Azevedo and Rombert (1965). Whole or sectioned protoscolesces and hydatid fluid adsorbed onto solid matrices have been used as antigen.

Reading and criteria for positivity
Examinations are carried out with the low dry system (ocular: l0x objectives l0x) on a fluorescent microscope equipped as described above. Positive reactions are characterized by a distinct bright-green fluorescence. Negative samples should not exhibit fluorescence but may be slightly "hay-green". The antibody titre is defined as the highest serum dilution with a distinct fluorescence. The hooks of the protoscolices exhibit auto-fluorescence.

Sensitivity and specificity
The sensitivity of the test is relatively high, but this depends on the quality of antigen, organ localization of the parasite, the parasite species and other factors.

7.6.2.4 Enzyme linked immunosorbent assay
Principle: ELISA for the diagnosis of human hydatiosis involved the sensitization of polystyrene surfaces (tubes or plates) by allowing hydatid fluid antigens to be absorbed on to them. When a serum containing antibodies against these antigens is added, the antibodies bind to the absorbed antigen, giving an immunocomplex attached to the solid phase. After incubation with an enzyme - conjugated anti-human IgG rabbit immunoglobulin, the subsequent addition of the specific substrate will result in a colour change, which is proportional to the amount of antibody in the test serum. ELISA test has clearly improved immunodiagnosis and is being used routinely for screening surveys and for epidemiological studies in endemic areas.

Reading and criteria for positivity
In the absence of a generally agreed criterion, it is suggested that an absorbance reading of more than two standard deviations above the mean value for the negative control sera from healthy donors should be considered positive.

Sensitivity and specificity
The ELISA has similar or slightly higher sensitivity than IHA, when used with purified antigen *5.*

7.6.3 Examination of the Cyst Fluid: Diagnostic puncture should be avoided because of risk of secondary echinocococcosis due to spillage of viable protoscolices. The surgically removed cyst should be examined for diagnosis of echinococcosis and cyst fluid may reveal scolices, brood capsules and hooklets

7.7 TREATMENT

7.7.1 Cystic Hydatid Disease

Although the disease may not progress or may resolve spontaneously, the risks of anaphylactic reactions, dissemination following rupture, and relentless growth, which renders subsequent therapy more difficult or impossible provides a strong argument for early therapy.

Surgical extirpation of the hydatid cyst remains the most effective treatment. Extirpation is usually accomplished by using generous surgical exposure and aspiration of part of the cyst contents, followed by injection of a protoscolicidal agent (hypertonic saline or silver nitrate) and complete removal of the endocyst. The residual cavity is then treated by suture plication, marsupialization, closure after filling with saline, or omentoplasty. An alternative method of removal is en bloc excision, a procedure particularly applicable to splenic cysts and, to a lesser extent, to hepatic lobectomy. Diagnosis and surgical removal of intact cysts before the onset of complications result in high cure rates with low surgical mortality (1 to *4%).* In contrast, secondary or recurrent disease often requires repeated surgical intervention, with mortality reaching 20%.

Until recently, there has been little to offer patients with anatomically inesectable lesions e.g.those in whom the spine is involved or in whom spontaneous rupture, operative spillage, or wide spread metastatic cyst formation has occurred. Under such conditions, treatment with mebendazole or albendazole may be attempted, although the results are unpredictable and adverse reactions have been reported and liver cysts of *E.granulosus* appear to respond relatively well, but cysts in other locations, particularly in the brain, bone, and eye, respond poorly if at all. Mebendazole (50 mg to 150 mg/kg of body weight per day, as a single oral dose for 3 months) or albendazole (10 to 15 mg/kg per day, in divided oral doses for 1 month) are recommended for small sized cyst situated in inoperable sites; many patients require repeated courses. Albendazole is also recommended for prevention of accidental cyst rupture with spillage of contents. Reported side effects have included neutropenia, liver toxicity, and hair loss. As long-term prognosis in individual patients is difficult to predict, prolonged follow-up is required to determine the eventual outcome of treated cases.

7.7.2 Alveolar Hydatid Disease

 Resection of the entire larval mass is the aim of surgery for this otherwise progressive and fatal disease. Usually the entire affected lobe(s) must be removed. When involvement is more extensive wedge resections of the lesions may be attempted. Because alveolar hydatid disease is often not diagnosed until parasitic invasion is well advanced, the lesion is frequently not resectable; thus partial resections and biliodigestive and hepatodigestive anastomoses are performed as palliative measures, predominantly to ensure bile passage. For treatment ofnonresectable cases, continuous mebendazole therapy (40 mg/kg of body weight per day) results in clinical improvement and prolonged survival time: however the effect is inhibitory rather than lethal to the parasite. Recent reports on therapy with albendazole suggest similar effectiveness.

7.8 PREVENTION AND CONTROL

It is evident that the stability of the host/parasite system determines to a major extent the effort required for the control of Echinococcosis. Evidence from the several control programmes against echinococcosis indicate that the stability of this system is quite fragile. This is a contrast to similar or concurrent programmes against other taeniid tapeworms. It would seem that although these parasites have similar life cycles, important differences exist that markedly influence their stability. These differences may include the low egg production of Echinococcus spp. and the occurrence of asexual reproduction within the intermediate host.

7.8.1 Cystic Hvdatid Disease

For individual in endemic areas, preventive measures include careful personal hygiene, strict dietary regulation of pet dogs to preclude ingestion of sheep offal, and avoidance of dogs that are not so regulated. Prophylactic treatment of dogs with praziquantel at intervals just short of the prepatent period (e.g. 45 to 50 days) prevents egg excretion in dogs so treated.

The principal objective of control of cystic hydatid disease is reducing parasite prevalence in definitive and intermediate hosts to levels below those necessary for continued transmission. Measures that have been used in various circumstances include health education, control of livestock slaughtering in abattoirs and on farms, safe disposal of infected viscera, dog control, and periodic diagnostic testing and mass treatment of dogs with praziquantel. The results achieved by control programs in New Zealand, Tasmania, Cyprus, Falkland Islands, Argentina and Chile have been spectacular. Marked reductions in prevalence of cysts in sheep following the initiation of control methods, as well as in the numbers of new diagnosed human cases, indicate that the fundamental requirements for effective control are now tried and proved.

7.8.2 Alveolar Hydatid Disease

 Personal preventive measures include avoidance of contact with foxes and other potentially infected final hosts. Control of pet dogs and cats that are liable to eat infected rodents can he prevented by monthly prophylactic treatments with praziquantel (5 mg/kg of body weight). Potentially exposed human populations should be educated about the dangers of the disease to promote better personal hygiene and sanitation and to motivate them to take effective measures to prevent their pets from eating rodents.

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RICKETTSIAL DISEASES

The rickettsiae are gram negative, obligate intracellular bacteria. They are grouped into genera; *Rickettsiae, Ehrlichia, Orientia*, and *Coxiella*. Every species is associated with an arthropod vector (lice, ticks, fleas, or mites) at some stage in its life cycle. Except for louse borne typusrickettsiae*(Rickettsia provazekii),* humans are accidental hosts in the life cycles of the microorganisms. All rickettsiae are included in the family of rickttsiaceae in the order Ricekttsiales. At least 10 species are pathogenic for humans.

8.1 ANTIGENIC CHARACTERIZATION

The pathogenic rickettsiae have been subdivided into five groups of antigenically related microorganisms. This antigenic grouping follows the generic separation, except that the genus Rickettsia is subdivided into spotted fever, typhus, and scrub typhus serogroups.

The spotted fever and typhus groups include multiple species, whereas the scrub typhus group consists of different strains of only one species, *Orientiatsutsugarnushi.* Convalescent phase serum samples from patients infected with any given species of Rickettsia react with other species in the same serogroup, but they usually do not react with species in the other serogroups. The species within the various serogroups have other properties in common, including vector association, optimal growth temperatures and pathogenicity for animals. Because of the many common features of member species, the five different serogroups of ricketsiae are sometimes referred to as biogroups.

8.2 MORPHOLOGY, REPLICATION, PHYSICOCHEMICAL PROPERTIES AND STABILITY

8.2.1 Rickettsia

Members of the genus Rickettsia have morphologic and biochemical properties typical of gram-negative bacteria. They are short, rod-shaped or cocoobacillary organisms, ranging from 0.8 to 2.0 µm long and 0.3 to 0.5 µm wide. They multiply by transverse binary fission. Elongated forms frequently develop under suboptimal growth conditions in which cell division is impaired. Rickettsia do not have flagella.

The Rickettsia have a five-layered outer envelope complex. A microcapsular layer is attached to the outer leaflet, and it is covered by a polysaccharide slime layer. Lipopolysaccharide (LPS) has been detected in Rickettsia species. All Rickettsia are exceptionally unstable outside the host cell and are readily inactivated at temperatures *56°C* and by standard disinfectants. Rickettsia enter host cells by induced phagocytosis. Entry depends on both the metabolic activity of the rickettsiae and the phagocytic ability of the host cell. The rickettsiae escape the phagosome, however, and multiply in the cytoplasm of the host cell. The infection cycle of R. *rickettsii,* however, is somewhat more complex. *R.rickettsii*occasionally migrated unidirectionally across the nuclear membrane and infect cell nuclei. The infection cycle of *O.tsutsuganiushi*differs substantially from that of spotted fever and typhus- group rickettsiae. Growth of *O.tsutsugamushi*occurs primarily in the perinuclear region, mature microorganisms are extruded from   the   host   cell   and   surrounded   by   host   cell   membranes.

Although members of the genus Rickettsia grow only in the presence of eukaryotic host cells, they possess considerably synthetic capabilities and generate their own ATP via the tricarboxylic acid cycle. Additionally, Rickettsia possesses an ADP/ATP translocator system that allows them to exchange their own AIDP with the ATP in the host cell. Presumably the rickettsiae use host cell ATP as long as it is available and produce their own supply when the host's becomes depleted.

8.2.2 Coxiella

The genus Coxiella is composed of a single species, C.burnetti. it is a short, rod shaped microorganism, 0.2 to 0.4 μm wide and 0.4 to 1 ,0μm long. Like the Rickettsia, C. burnetiiis an obligate intracellular bacterium that enters the host cell by phagocytosis. Unlike Rickettsia, however, C.burnetiidoes not escape from the phagosorne. Instead, it multiplies efficiently there (by binary fission) until the host cells eventually lyse and release microorganisms into the environment. The low pH, optimum (4.5) of C. burnettiiIenzymes evidently allows this microorganism to survive and multiply in the phagosome. C.burnetiiis resistant to inactivation by physical and chemical treatment. It can survive for a year or more when attached to wool or other fomites, but it is incompletely inactivated when held at 63°C for 30 mm. It can be inactivated by 0.05% hypochlorite, 5% H202, or a 1:100 dilution of Lysol. C.burnetiidisplays an antigen phase variation that is unique among the rickettsiae. The organism exists in two phases (I & II), analogous to the smooth to rough transition that occurs in some species of enteric bacteria. C.burnetliis in antigenic phase I in nature, but it changes to phase II after multiple passages in tissue cultures or embryonated eggs.

8.3 PATHOGENESIS, PATHOLOGY, AND PATHOPHYSIOLOGY

8.3.1   General

Members of the genus Rickettsia display aunique tropism for the endothelial cells of the microcirculatory system, especially the capillaries, and the consequent damage to the vasculature is the basis for similarities in the pathogenic, pathologic, and pathophysiologic features of rickettsial diseases.

8.3.2   Rickettsia

Rickettsiaare transmitted by the bite of infected ticks and mites or by contamination of the skin or mucous membranes with louse or flea feces. Regardless of their mode of entry, rickettsiae invade the capillary vascular endothelium and frequently cause focal swelling and a mixed inflammatory infiltration at the site of inoculation. Rickettsiae then proliferate locally at the site of infection. A result is the primary lesion or eschar that frequently appears during the incubation period of scrub typus, rickettsial pox and Boutonneuse fever.

After local proliferation in the endothelial cells the rickettsiae become disseminated in the vasculature of many organ systems. The route of this dissemination has not been elucidated, but is presumed to be hematogenous or lvmphangitic. Focal involvement of small blood vessels of the skin and other organ is seen, and this correlates closely with many of the clinical manifestations of typhus and spotted fever group infections. The disseminated lesions of rickettsial infections directly reflect rickettsia induced focal swelling and proliferation of

endothelial cells as well as the acute inflammatory infiltration. As the endothelial cells die, necrosis of the intima and media of the blood vessels leads to the formation of hyaline thrombi composed of fibrin and cellular debris. These hyaline thrombi cause microinfarcts and extravasation of blood, and they are manifested grossly by the petechial lesions that are the hallmark of rickettsial disease. Gross and microscopic lesions are found in the brain, kidneys. lungs, and heart. The clinical consequences of infarcts in these tissues are much greater than those of the vasculitis.

Unlike other rickettsiae, *R. rickettsii* is not confined to capillary endothelium; it also invades and destroys vascular smooth muscle cells and the vascular endothelium of larger vessels. Typhus and spotted fever group infections may cause interstitial pneumonitis; there is considerable variation in the frequency of this finding. The consequences of vasculitis, vascular damage and increased perrneabilinvinculde alveolar edema.fibrin formation, macrophage accumulation and hemorrhage: and interlobar, septal. and pleural effusion. The pulmonary dysfunctions (e.g., coughing. pneumonia, and pulmonary edema) that sometimes accompany Rockey Mountain Spotted Fever (RMSF) are the clinical consequence of this involvement. Lesions in the central nervous system (CNS) play a prominent part in the clinical complications of RMSF and typhus. All portions of the brain and spinal cord may be involved, but the rickettsiae display a special proclivity for the midbrain and inferior olivary nucleus. Focal proliferations of endothelial and neuroglial (predominantly oligodendroglial) cells have been called 'typhus nodules". Other pathogenic manifestation of CNS involvement in epidemic and scrub typus includemononuLlear cell meningitis, perivascular cuffing of arteries, focal hemorrhages and degeneration of ganglion cells. Unlike epidemic and scrub typhus, the neurologic findings of RMSF are the result of microinfarcts in the CNS and frequently involve the white matter.

A discrepancy is found between the severity of myocarditis and the severity of cardiac insufficiency. The hypotension that often accompanies RMSF and typhus is not associated with left ventricular dilatation; this strongly suggests that myocardium is notinvolved in this complication. Shock is primarily attributable to an increase in capillary permeability that leads to a decrease in intravascular volume and systemic vascular resistance. In epidemic typhus, interstitial myocarditis, epicarditis, and mural endocarditis spare the heart valves and are secondary to capillary injury. Cardiac involvement in epidemic typhus is generally infiltrative and primarily involves small blood vessels. The urinary tract is commonly involved in RMSF and typhus, but this infrequently leads to clinical consequences; the remote effects of decreased intravascular volume are primarily responsible for the abnormalities of renal function. Although the nodular proliferative reactions seen in the CNS and skin are uncommon in the kidneys, multifocal perivascular interstitial nephritis is frequent.

In epidemic typhus, scrub typhus, and RMSF, histopathologic studies have shown that testes, epididymis, scrotal skin, and adrenals also are involved. Lesions attributable to typhus also have been found in most parts of the alimentary tract (with the possible exception of the colon and salivary glands), but there is general agreement that the gastrointestinal tract is neither frequently nor markedly involved.

8.3.3 Coxiella

Microscopically the pathology of Q-fever resembles that of bacterial pneumonia. There is a severe interalveolar, focally necrotizing hemorrhagic pneumonia that is patchy in distribution, involves primarily the alveolar lining cells, and is associated with a necrotizing bronchitis and bronchiolitis. *C.burnetii*is found in the histiocytes of the alveolar exudate. Histiocytic hyperplasia is found in the mediastinal lymph nodes, spleen, and adrenals.

Hepatocellular damage in acute Q-fever is not widespread: the serum transaminase levels generally are moderately elevated. Liver biopsies in patients with Q-fever almost all showed granulomatous changes of the lobules. The granulomas consisted of a nondistinctive focal histiocytic and mixed inflammatory cell infiltrate with varying numbers of multinucleated giant cells. In the late stages of disease, there is often necrosis in the center of the granulomas. Morphologic abnormalities may persist much longer than the clinical illness.

8.4   EPIDEMIOLOGY

Except for epidemic typhus and scrub typhus, the rickettsioses were not really recognized as distinct clinical entities until the 20th century. Only when specific rickettsial serologic testing was introduced in the 1940s could the precise etiologies of various rickettsial diseases be determined with certainty. Thus, there is little information about the epidemiology of most rickettsioses before World War II. Even now, there is paucity for surveillance for Boutonneuse fever, scrub typhus, rickettsiaI pox, or Q-fever on a global scale, data on their prevalence, distribution, and movement compiled primarily from scattered reports.

8.4.1   EpidemicTyphus

Epidemic typhus has historically been one of the most devastating infectious diseases. Outbreaks have been reported on all continents except Australia. Globally, louse borne typhus remains endemic in mountainous regions of Mexico, Central and South America (especially Peru and Bolivia), Africa (Ethiopia, Rwanda, Burundi, and other countries that border the Sahara), and numerous countries of Asia. The first authentic report of epidemic typhus in India came from Gooty in 1808. Outbreaks of epidemic typhus have been restricted entirely along the northern frontier of the country running from Baluchistan across the Himalayan hills to Kulu valley. The incidence of typhus is greatly underreported because of the lack of adequate surveillance systems in endemic areas,

In classic epidemic typhus, the human body louse, which spends its entire existence in the clothing of humans, becomes infected by feeding on a rickettsernic human. The rickettsiae multiply in the epithelial cells of the louse's midgut and are shed in its feces. Lice infected with *R.prowazekii*die within 14 days. but apparently their feces can remain infectious for several months if temperature and humidity are low. Humans are infected when louse feces or crushed lice contaminate broken skin or the mucous membranes of the eyes or respiratory tract.

Sporadic cases of *R.prowazekii*infection have been reported in the United States in the past 10 years in association with flying squirrels. The flying squirrels are a zoonotic reservoir of *R.prowaekfii.* The exact mechanism by which flying squirrels transmit *R.prowazekii*to humans is unknown.

8.4.2 MurineTyphus

Like epidemic typhus, murine typhus has occurred since ancient times, but it was not distinguished from epidemic typhus until the 1 920s. The geographic distribution is much broader than that of epidemic typhus. Murine typhus is endemic in at least some part of every continent except Antarctica, and occurs most conmon1v here rats, rat fleas, and humans are found together. The disease is usually transmitted to humans when crushed fleas or flea feces contact broken skin or mucous membranes. In India sporadic cases of murine typhus were reported from Lucknow, Mysore, Calcutta and Golconda. Studies in Pune and Bombay yielded 14 and 5 strains of R. typhirespectively from humans, rats and fleas.

Rats, mice and possible other small mammals serve as the reservoir for murine typhus rickettsiae. The tropical rat flea *(Xenopsyllacheopsis)* is the primary vector. *R. lyphi*multiplies in the cells of the flea gut, but does not kill the flea. The extend of the rickettsial multiplication depends on temperature: this may explain the higher incidence of disease in warmer climates. The microorganism is shed in flea feces, where it can remain infectious for up to 100 days. Although rodents are considered the reservoir of R. *typhi*and fleas merely vectors, recent data indicate that tansovarial transmission of R. *tjphi*occurs to a limited extent *in Xenopsyllacheopsis.*

8.4.3 ScrubTyphus

Scrub typhus is widely distributed in central, eastern and southeastern Asia and the southeastern Pacific. Outbreaks of scrub typhus usually occur when susceptible humans enter an endemic area for military or vegetation clearing operations. Scrub typhus accounts for a substantial proportion of febrile illnesses in some endemic areas. Scrub typhus came into prominence as a war disease in India, especially in the eastern regions. A comparatively mild outbreak of scrub typhus in South India was reported first time in 1945. Serological surveys by VRC have revealed the prevalence of scrub typhus in Jammu & Kashmir and Rajasthan.

Disease is transmitted to humans when they intrude into "typhus islands", the innumerable and sometimes very small foci where the agent *(O. tsutsugamushi),* the host/vector *(Leptotrombidiumdelienese),* and wild rats *(Rattus*sp.) coexist. The foci are found in such diverse settings as alpine meadows, disturbed rain forests, and seashores. Chiggers (the larval stage of mites) are both vectors and reservoirs; females transmit *O. tsutsugamushi*transovarially to their progeny. Many species of murids, insectovores, marsupials and domestic animals have been infected with *R. tsutsugamushi.* Small mammals have no role in the dissemination of rickettsiae; they only serve as hosts for the development of chiggers.

8.4.4 Spotted Fever Group

Tick-borne rickettsioses of the spotted fever group can be separated into two categories: RMSF in the western hemisphere and the various forms of tick typhus (Boutonneuse fever. Siberian tick typhus, Indian tick typhus) in the eastern hemisphere. Rocky Mountain spotted fever is caused by a single species of rickettsiae*(R. rickettsii),* which is found in western Canada, the United States, Mexico, Central America. Colombia, and Brazil. The other types of tick typhus can be attributed to three different rickettsial species, each of which occurs over a broad but distinct geographic area: *R. conorii* (responsible for Boutonneuse fever) is found in the Meditteranean basin, including southern France and Italy, parts of Africa, the Middle East, India, and Pakistan: *R.*

sibirica (Siberian tick typhus) occurs in broad band that crosses the Soviet Union and parts of the Indo-Pakistan subcontinent and *R. australis*Queensland tick typhus) is found only in Eastern Australia.

Various species of ixodid ticks are both reservoirs and vectors of these rickettsiae. The microorganisms are maintained in the ticks for several generations by ovarial passage, but the rickettsiae may ultimately be deleterious to the tick host. Many rodents and other mammals are susceptible to spotted fever-group rickettsiae and amplify the infection in a particular tick host ecosystem. Humans are infected by the ticks when they intrude on these cycles of infection.

8.4.5 Q-Fever

Q-fever has been reported on all continents except Antarctica and is endemic in almost all countries except for certain ones in Scandinavia. However, even considering possible under diagnosis, this disease is very rare in the United States. Serologic evidence of Q-fever has been found in a large number of wild and domestic animals, but infection is almost always subclinical. The primary animal reservoir of C. *burnetii*varies from area to area. *C.burnetii*is carried by many species of tick, and although ticks may serve as a vector between animals, they probably do not transmit the disease to humans. Humans are usually infected when they inhale aerosols generated by infected livestock. Infection occurs most frequently in abbatoirs, sheep research facilities, dairies, and in animal husbandary operations.

8.5 MODE OF TRANSIMISSION

Spotted fever-group rickettsiae are present in the salivary secretions of ticks and mites and are injected into the body when the ectoparasites feed on humans. Typhus-group rickettsiae, on the other hand, enter the body when broken skin is contaminated by the infeced feces of lice or fleas. C.burnetiiorganisms are usually transmitted when the host inhales infectious aerosols. They enter the host's cells by passive phagocytosis, preferentially infecting histiocytes and Kupffer cells.

8.6 INCUBATION PERIOD

The incubation period of rickettsial diseases is generally from 7 to 21 days but can range from ito 32 days. Q-fever has the longest incubation period usually 18 to 21 days.

8.7 SYMPTOMS, SIGNS AND CLNICAL COURSE

8.7.1 Initial Symptoms

Most patients with rickettsial illnesses initially have non specific systemic symptoms and signs. Fever and headache are most commonly reported, but chills, myalgias, arthralgias, malaise, and anorexia also are noted. Onset of disease is sudden in about half of the cases. The severity of symptoms and organ system involvement vary greatly, depending on the etiologic agent, host factors (especially age), inoculum size, and possibly strain differences. Fever increases during the first week of illness often reaching 104°F or higher. Nonspecific systemic symptoms and signs become common during the first week of illness. Mucous membrane involvement (e.g., conjunctivitis and pharyngitis) may be noted. In scrub typhus, early in the course of infection,

regional lymphadenopathy proximal to the eschar is found in about 20% of the patients. Later, generalised lymphadenopathy that may be mistaken for mononucleosis is seen in about 80%.

8.7.2 Cutaneous Manifestations

Although rash is considered a hallmark of rickettsial infection, it usually follows systemic symptoms. Its absence should not rule out a possible rickettsial etiology, especially during the first week of illness in only *50%* of patients with RMSF and 10% of patients with scrub typhus. As many as two-thirds of the patients with scrub typhus and 10% of patients with RMSF never develop a rash, and, another 5% of RMSF patients do not develop rash until after the 10th day of illness. In RMSF, the rash begins on the wrists and ankles in about *50%* of the patients and on the palms and soles in another *25%.* It is often macular and blanching during the early stages: later, the rash spreads centripetally and becomes petechial, ultimately involving the palms and soles in up to 80% of patients. Boutonneuse fever is almost always distinguishable epidemiolgoically and by the presence of the eschar that is found in two-thirds of patients.

In epidemic typhus, faint pink to red blanching macules appear first, usually on the anterior trunk and axillary folds on the 5th to 7th day of illness. Depending on the severity of the illness, the rash may remain on the upper thorax and abdomen, or it may spread rapidly to the extremities. Except in very ill patients, it spares the face, palms, and soles. In mild cases the lesions fade over the course of a few days: in more severe cases they become maculo-papular and petechial and fade during the next few weeks. The rash of scrub typhus is similar to that of epidemic typhus, beginning on the trunk, axillary folds, and proximal extremities as non-confluent pink to red macules that fade on pressure. The initial lesions are larger than those seen in epidemic typhus. The rash spread centrifugally, involving most of the body except the face, palms and soles. It becomes maculo-papular and petechial. then fixed and confluent, and in severe cases, hemorrhagic. It is evanescent or absent in 15 to 65% of cases in some areas.

8.7.3 Organ System Involvement

Headache is a very common accompaniment of most rickettsial diseases. Photophobia is also common, but evidence of more serious CNS impairment (confusion, stupor, delirium, seizures, and coma) is found only in about 25% of patients with RMSF and typhus who receive proper treatment and is virtually never seen in the other rickettsial diseases. Abnormalities of the cerebrospinal fluid, especially pleocytosis and increased protein, tend to parallel other signs of CNS involvement. Mild pulmonary involvement, manifested by cough and infiltrates on the chest roentgenogram, is common in epidemic typhus and is found in about half of Q-fever and scrub typhus patients and in about one-third of patients with RMSF. Gastro-intestinal symptoms, especially nausea or vomiting, are reported in about one-half of patients with RMSF and in most other rickettsial diseases (except rickettsial pox). Constipation is reported by two thirds of patients with RMSF and epidemic typhus. Diarrhoea is reported by about 10% of patients with rickettsal diseases. Hepatomegaly and/or splenomegaly is found only in about 20% of patients with rickettsioses, except for Q-fever where hepatomegaly and hepatitis may dominate the clinical picture in as many as half of the patients. The symptoms of murine typhus and flying squirrel associated typhus fever are similar but milder than those of epidemic typhus.

8.7.4 Course and Outcome

The course and outcome of rickettsial diseases are quite variable. The primary determinants are the specific infectious agents and the rapidity with which effective antibiotic treatment is initiated. Other factors include the patient's age and the virulence of the infecting strain. Epidemics of louse-borne typhus have had attack rates as high as 96% and often have involved millions of people with fatality rates of 10 to 66%. Poor nutrition and other underlying health problems undoubtedly contribute to the high fatality rates of some epidemics. The case fatality rate of RMSF was 23 to 70% in the preantibiotic era. With the advent of antibiotics the fatality rates quickly fell below 10%. In World War II, before tetracycline was available, the fatality rate for American soldiers with scrub typhus was *8.5%.* Servicemen in Vietnam in the 1 960s and 1 970s with scrub typhus were treated with tetracycline, and no fatalities were attributable to this disease. Boutonneuse fever is generally a much milder disease.

8.7.5 Infectivity

Patients with rickettsial disease develop rickettsemias of varying durations at which time blood and tissues, including pathologic specimens obtained antemortem and post mortem, are potentially infectious. However, there are no well-documented cases of human to human transmission of any rickettsial disease. In some Q-fever patients, however, the illness did appear to be attributable to human contagion.

8.7.6 Clinical Laboratory Data

Routine laboratory tests are unlikely to be diagnostic for any rickettsial diseases with the possible exception of Q-fever. Mild abnormalities of liver function are found in the majority of patients with Q-fever (and therefore make this disease a part of the differential diagnosis of non-A, non-B, hepatitis) and in about one-third of patients with RMSF and Boutonneuse fever. However, jaundice is rare in all these illnesses. Mild azotemia is common in RMSF but rare in Boutonneuse fever; sever abnormalities of renal function are seen frequently in epidemic typhus and occasionally in RMSF. Thrombocytopenia is observed in more than half of the patients with epidemic typhus, in about 40% of those with R±MSF and Boutonneuse fever, and less commonly with scrub typhus.

8.8 DIAGNOSIS

Techniques for the diagnosis of rickettsial diseases have improved considerably in recent years, but unfortunately they have not improved the outcome of most rickettsial infections. Attempts to directly detect rickettsiae or rickettsial antigens in clinical specimens have only been marginally successful, and even with the most sensitive serologic techniques one usually cannot detect rickettsial antibodies until a week or more after the onset of symptoms. Similarly, the lengthy generation time of rickettsiae usually produce their isolation and identification in less than a week. These time frames are unsatisfactory because many rickettsioses have high fatality rates unless specific antibiotic therapy is administered within 3 to 5 days after the onset of illness.
Despite these shortcomings, techniques for the diagnosis of rickettsioses have inherent value and can contribute indirectly to a reduction in rickettsial morbidity and mortality when used as part of a detailed surveillance system.

8.8.1 Specimen Collection

Rickettsiae are hazardous (Biosafety Level {BSL} 3) microorganisms that have been responsible for numerous laboratory infections over the years, usually in research laboratories where rickettsiae are propagated. Rickettsialtitres in blood specimens begin to fall off dramatically after several hours at room temperature. Processing freshly drawn blood to obtain serum does not pose a serious threat to the careful laboratory worker, provided that aerosolization is minimized and the clot is discarded carefully and then auto claved. Surgical gloves should be worn when handling blood specimens.

8.8.2 Serological Diagnosis

Diagnosis of the etiology of rickettsial diseases can be accomplished most easily and rapidly by demonstrating a significant increase in specific antibodies in the serum of the patient during the course of infection and convalescence. Several serological tests are currently available for the diagnosis of rickettsial diseases like Weil-Felix Test (WFT), IIF, ELISA, RIA, etc. Although many techniques have been used successfully for rickettsialsero diagnosis, relatively few are used regularly by most laboratories.

8.8.2.1 Weil- Felix Test
The Weil - Felix test is helpful in establishing presumptive diagnosis in diseases caused by members of typhus and spotted fever groups of rickettsiae. In rickettsial infections the Weil-Felix reaction depends upon the development of antibodies that agglutinate certain strains of non motile Proteus organisms, i.e. *P vulgaris* 0X19*and* 0X2 *and P.mirabilis*OXK

Procedure

A macroscopic agglutination test is performed by thoroughly mixing 0.5 ml of serum dilution and 0.5 ml of antigen suspension. Serum dilutions of 1:10 through 1:610 (final dilution 1:20 through 1:1280) usually suffice. A control tube containing 0. 5m1 of saline solution should be included and a positive serum control as well. Tests and controls are incubated in the water bath at 37°C for 2 hours followed by storage overnight in the refrigerator. Complete agglutination is indicated by absolute clearing of the supernatant fluid and by settling of the organisms in large white particles at the bottom of the tube. Partial agglutination is indicated by incomplete clearing of the supernatant fluid and diminution in size of bacterial clumps. When the tubes are shaken, granular agglutinated masses of bacteria are seen. To facilitate reading, the tubes may be held in front of a concave mirror and the image examined.

Interpretation of results

The typical responses obtained with suspensions of 0X19, 0X2 and OXK, Proteus organisms and convalescent sera from patients with rickettsial diseases are given in Table 8.1

Table 8.1. Interpretation of results

	OX19	OX2	OXK
Epidemic typhus	++++	+	0
Murine typhus	++++	+	0
Scrub typhus	0	0	+++
RMSF	++++	+	0
Other Tick brone SF infection	+	++++	0
Rickettsial pox	0	0	0
Q-fever	0	0	0

The classical findings of agglutination of the OX1 9 organism by sera from cases from epidemic and murine typhus and of OXK suspensions by sera from patients with scrub typhus are indicated. The Weil-Felix response in Rocky Mountain spotted fever may be of several types - a high 0X19 and a low 0X2, or elevation of both 0X19 and OX2, or, occasionally, a low 0X19 and a high OX2. Sera of patients with rickettsial pox or Q fever fail to agglutinate any of the three organisms. The Weil-Felix agglutinins may appear as early as the *5th* or 6th day after onset of fever in those diseases in which the reaction becomes positive, and they are almost always present by the 12th day. These antibodies generally reach their maximum in early convalescence and then decline rather rapidly to non-diagnostic levels in 1 to several months. Occasional patients with epidemic typhus or spotted fever do not develop OX agglutinins. About 15 per cent of vaccinated persons who subsequently contract epidemic typhus fail to show this reaction and negative results are the rule in Brill's disease. Moreover, in scrub typhus 15 to 50 percent of cases may fail to develop significant levels of OXK antibodies. A rise in antibody titre, demonstrated in a series of two or mroe sera is essential for the presumptive diagnosis of rickettsial disease when the Weil-Felix test is employed. Interpretation of results on the basis of tests on a single serum is not justified unless the titer is high, that is, well above 1:160. The Weil-Felix test is of no value in differentiating epidemic and murine typhus and frequently it fails to provide even presumptive evidence for separating spotted fever from murine typhus. Patients with urinary tract infection attributable to the Proteus organism or recurrent fever caused by spirochetes of the genus Borrelia also develop antibodies that react with the Weil - Felix antigen. The importance of maintaining a supply of known positive human sera for rechecking the bacterial suspensions at frequent intervals should be emphasized. Aging sometimes render such antigens hyperagglutinable; this should be suspected if a number of unexplained positive results are found.

8.8.2.2 Indirect Immunofluorescent Antibody (1FA) test

The IFA technique for detection of rickettsial antibodies is a standard two - stage antibody assay. In the first stage rickettsial antigens are overlaid with dilutions of human serum. In the second stage the antigens (along with antibodies i.e. antigen-antibody complex) are overlaid with fluorescein isothiocyanate (FITC) -- labelled antisera to human immunoglobulin. In this manner, the rickettsiae are rendered fluorescent by immune sera, which are not themselves labelled. The IFA technique remains the most popular procedure for serodiagnosis of rickettsial diseases,

primarily because of the ease and economy with which it can be incorporated into existing antibody screening system. In addition, it readily detects 1gM antibodies; this feature is quite valuable for serodiagnosis during the early stages of rickettsial infections. The TEA procedure for rickettsiae is similar to conventional techniques, where inactivated yolk sac or tissue culture suspensions of rickettsiae are normally used as antigens. When infected yolk sacs are used as antigens, sera are diluted in a 3% suspension of normal yolk sac to adsorb antibodies to the egg substrate that might be present in the patient's serum.

8.8.2.3 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA techniques, particularly immunoglobulin M (IgM) capture assays, probably the most sensitive tests available for rickettsial diagnosis, but they require large quantities of purified antigens that are commercially unavailable. Although ELISA tests can readily screen multiple serum specimens, this is often not useful for rickettsial diseases, because, with the exception of epidemic typhus and Q-fever, rickettsioses usually occur sporadically at a relatively low frequency. However, the sensitivity of the ELISA test could be exploited and economy of antigen used maintained if the appropriate rickettsial antigens were included in a battery of antigens designed to test for several possible causes of febrile illnesses of unknown origin.

8.8.3 Molecular techniques

Molecular techniques may be used for detection of *Orientiatsutsugamushi, Rickettsia prowazeki, Rickettsia conorii, Ricketsiatyphi.*DNA extracted from clinical specimens (eschar, blood) can be used to diagnose rickettsial infections.

PCR

PCR targeting the specific target sequence for 56 KDa outer membrane protein of *Orientiatsutsugamushi*.

Real time PCR

These are fluorogenic real-time PCR assays that use a fluorescently labeled probe to detect the O tsutsugamushi specific double-stranded product that is produced. The fluorogenic 5' nuclease assay (TaqMan assay) use a fluorogenic probe, consisting of an oligonucleotide with a reporter dye (FAM) attached to the 5' end and a quencher dye attached at or near the 3' end. The probe anneals to an O. tsutsugamushi specific target sequence (47 KDa) located between the specific forward and reverse primers.

8.9 PREVENTION AND CONTROL

The prevention and control of the rickettsial diseases are primarily accomplished by interrupting transmission of the infective agent from its vector or reservoir to humans. This can often be achieved by simply avoiding vector-infested areas (or, in the case of Q-fever, areas where infected animals are kept), by vector control, or by wearing protective clothing. At the present time immunization plays a minimal role in the prevention of rickettsial diseases, because, with the exceptions noted beloi safe and effective vaccines are generally unavailable. In addition, with the possible exception of scrub typhus, chemoprophylaxis is generally both impractical and ineffective.

8.9.1 Epidemic Typhus

Delousing remains the cornerstone of the control of epidemic typhus. A residual insecticide is applied to the patient and his contact, their clothing, bedding and living quarters. If facilities for washing clothes and bathing cannot be provided, patients should be isolated and their contacts kept under surveillance for 2 weeks. In an epidemic, the entire community may be treated. Residual insecticide including organochiorides has to be sprayed. Increased resistance to these agents, however, has necessitated the use of the more expensive, but less efficient, organophosphates (e.g., 1% malathion). Immunization was widely used during World War II, and is still recommended by some organizations for persons working in close contact with infected patines in typhus-endemic areas. However, commercial production of a killed epidemic typhus vaccine was discontinued in 1980 because of questions regarding the vaccine's efficacy and the need for safer production facilities. An attenuated vaccine prepared from the Madrid E. Strain of *R.prowazekii*is available under investigational new drug (1ND) status in the United States, but is not routinely administered. Immunization is not currently required for entry into any country. Protective measures for persons occupationally at risk include wearing clothes that are either treated with repellent or storing them overnight with a decorous strip in an airtight bag.

8.9.2 Murine Typhus

Murine Typhus usually occurs sporadically or in small outbreaks. Persons can prevent infection by avoiding areas where flea-infested rats are found or by wearing repellent treated clothing. Rat populations can be controlled by preventing their access to food. In murine typhus-endemic areas, vector control with insecticide powders should precede rodent control measures to prevent increased exposure of humans to fleas. No vaccine is available to murine typhus.

8.9.3 Scrub Typhus

The chigger vectors of scrub typhus are especially amenable to control because they often are found in distinct areas (typhus island). These foci can be eliminated by treating the ground and vegetation with residual insecticides (of the organophosphate, organochiorine, or carbomate groups), reducing rodent populations, and destroying limited amounts of local vegetation. Persons who cannot avoid infested terrain should wear protective clothing, impregnate their clothing and bedding with a mitocide (e.g. benzyl benzoate) and apply a mite repellent to exposed skin. Chemo prophylaxis should also be considered. In a controlled trial, the weekly administration of 200mg doxycycline decreased the incidence of clinical illnesses but not of in apparent infection. Prophylaxis with chioramphenicol resulted in an unacceptable number of relapses when the drug was discontinued.

8.9.4 Spotted Fever Group

Rocky Mountain spotted fever and the other tick borne rickettsioses are prevented by avoiding tick infested areas, carefully searching the body for ticks and removing them, wearing clothing impregnated with a tick repellent. Integrated tick control measures are generally not economically feasible. Ticks should be removed from the body by grasping them with tweezers as closely as possible to the point of attachment and pulling slowly and steadily until they are dislodged. The tick bite wound should then be washed with soap and water. Other methods, such as the application of nail polish remover or a lighted cigarette, do not work nearly as well.

Because of the potential danger of becoming infected when crushing ticks using fingers for this procedure is discouraged. The hands should always be washed thoroughly after removing a tick. Education about the early symptoms and signs of RMSF and the need for prompt empiric therapy if symptoms develop is also very important to control.

8.9.5 Q-Fever

In the 1 970sand 1 980s, most reported outbreaks of Q-fever in various countries were associated with work related exposures to sheep and their birth products. Therefore, prevention measures should be directed primarily at (1) avoiding exposure in institutions where sheep are used for research and (2) educating personnel in agricultural settings about disinfection and disposal of the products of conception. Vaccines made from killed organism have been tested ever since *C. burnetii*was discovered. A formal inactivated phase I vaccine, evaluated in a large comparative study in Australian abattoir workers, provided complete protection to vaccinated subjects. However, vaccination of individuals with pre-existing immunity has frequently caused erythema, in duration and sterile abscesses. Most of these adverse affects can be avoided if immune individuals are identified by skin tests before vaccination. Vaccination should be considered when contact with infected sheep is unavoidable.

8.10 THERAPY

Prompt institution of effective antibiotic therapy against rickettsiae is the single most effective measure for preventing morbidity and mortality due to rickettsial diseases. Antirickettsial therapy improves the outcome of all ricketsioses, with the occasional exception of fulminate or complicated cases of RMSF, epidemic typhus, and scrub typhus, where the illness is no longer susceptible to intervention. If the illness is severe, the cardiac, pulmonary, renal, and central nervous systems should be assessed and additional measures instituted to prevent complications. Even in the less serious rickettsial diseases, such as Q-fever and rickettsialpox, treatment during the first few days appears to shorten the duration and lessen the severity of the illness. Although they are rickettsio static rather than rickettsiacidal, tetracyclines and chloramphenical remain the only proven therapy for the rickettsial diseases. Clinical experience and limited comparative studies suggest that the responses of patients treated with either antibiotic are similar, but other have found a slightly more rapid response with tetracycline than with chloramphenicol. Tetracyclines are also less likely to cause hematologic complications, but they may cause discoloration of teeth, hypoplasia of the enamel, and depression of skeletal growth in children; the extent of discoloration is directly related to the number of courses of tetracycline therapy received. Therefore, chlorarnphenicol should be used for children under 8 years of age and for pregnant women. The high blood and tissue levels and long half-life of the lipophilic tetracyclines give them a theoretic advantage. In a small controlled study of epidemic typhus patients, the response to a single dose of 100mg doxycycline was comparable to that of 10-day course of chioramphenicol or tetracycline. Similar results were obtained in larger trials with doxycycline in scrub typhus patients. Although, occasional relapses have been reported in patients receiving single dose doxycycline therapy. Doxycycline is excreted in the gastrointestinal tract and it is the tetracycline of choice in patients with renal failure. Doxycycline may cause dental changes less frequently because it binds less with calcium than do other tetracyclines, and it is preferred if tetracyclines must be used in children.

Further Reading

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TOXOPLASMOSIS

9.1   INTRODUCTION

Toxoplasmosis is one of the important parasitic zoonoses. Its causative agent Toxoplasma gondii is an intestinal coccidian of felids. It has an unusually wide range of intermediate hosts. It is widely distributed in man and animals all over the world.

Toxoplasma gondii was first discovered in two different species of animals - a rodent, Ctenodactylus gondii in North America and a rabbit in Brazil. Its relation to human disease was noticed only in 1923 when Janaku described the parasite in the retinal sections from an infant who had apparently died of congenital toxoplasmosis. Its role in human pathology was confirmed by sub-inoculation of animals with infected human tissues from cases of congenital and acquired toxoplasmosis.

9.2 CAUSATIVE AGENT

The name Toxoplasma alludes to its curved shape and is derived from the Greek word Toxon meaning arc. Toxoplasma is an obligate intracellular parasite. The organism exists in different forms depending on the stage in its life cycle. The prototype is the rapidly dividing form and is referred to as the tachyzoite. This is a crescent shaped structure *5* - 7m in length and 2 - 3m in breadth with pointed anterior and rounded posterior ends. Giemsa stained preparations show a delicate azure coloured cytoplasm and reddish ovoid or spherical nucleus placed near the posterior end. Cysts, which originally develop within a host cell, eventually usurp the entire cell, become an intercellular structure. Bradyzoites develop slowly in the cyst.

Life - cycle consists of two phases:
a) Asexual stage -Occurs in intermediate and definitive host
b) Sexual stage-Occurs in definitive host

In man the infection may be acquired either by swallowing mature oocysts present in the contaminated soil or from raw/undercooked meat containing tissue cysts. Tachyzoites are not usually infective when ingested as they are destroyed by the acid in the stomach. However, infections due to laboratory accidents, transplacental infection and blood transfusions are documented.

The infection in man is dead end host. In man the life cycle of Tgondii stops at this stage. But in nature the cycle is maintained by members of the feuds when they prey on infected rodents and other infected animals.
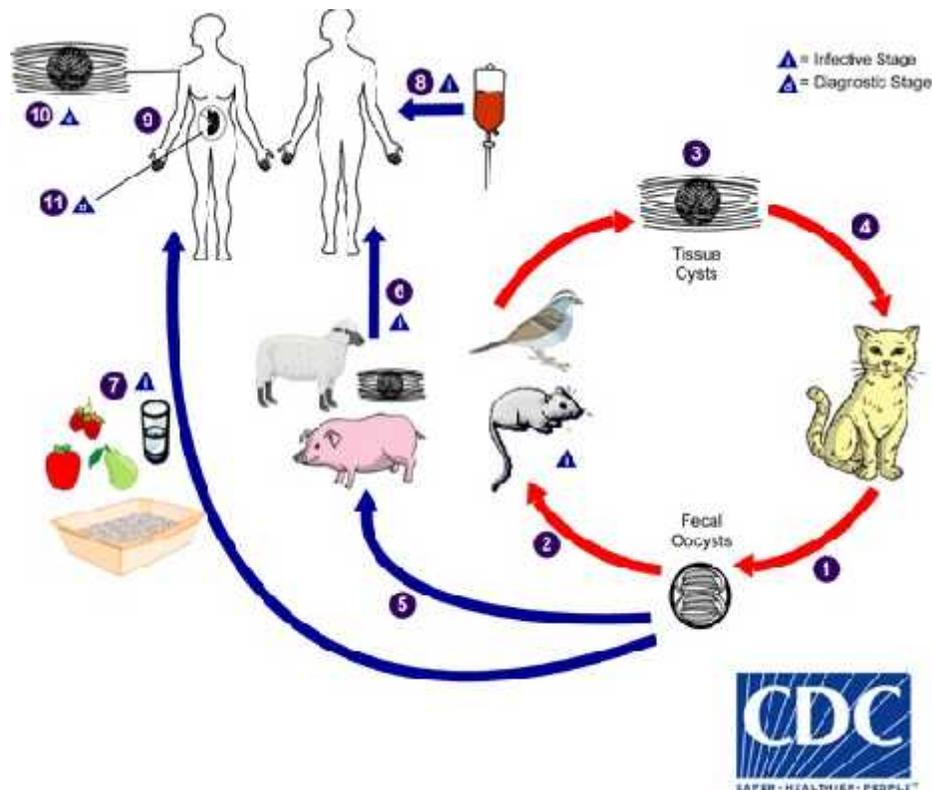
Fig 9.1. Life cycle of *Toxoplasma* parasite. Humans can become infected by any of several routes such as; eating undercooked meat of animals harboring tissue cysts, consuming food or water contaminated with cat feces or by contaminated environmental samples (such as fecal-contaminated soil or changing the litter box of a pet cat), blood transfusion or organ transplantation transplacentally from mother to fetus. (available at: http://www.cdc.gov/dpdx/toxoplasmosis/index.html

9.3 EPIDEMIOLOGY

Toxoplasma infection is widespread throughout the world but its incidence in animals and man varies in different geographical areas. Environmental conditions, climate, animal fauna and cultural habits of the people may contribute to the natural spread of Toxoplasma.

The majority of congenital infections in man occur when the mother acquires infection during pregnancy. However, the role of chronic toxoplasmosis of the mother in producing congenital infection is controversial.

Unlike man repeated congenital infections can occur in mice, rats, guinea-pigs and hamsters. Researcher have reported atleast ten generations of congenitally infected mice from a congenitally infected mother. In sheep on the other hand, like man, congenital infection occurs only when the ewe acquires infection during pregnancy.

Post-natally toxoplasmosis can be acquired either by ingestion of food contaminated by mature oocysts or by eating undercooked or raw infected meat (toxoplasma infection being common in many animals like cattle, sheep, rabbits and pigs which are used for food).

Cats and other members of felidae family are the key animals in the epidemiology of post-natal toxoplasmosis. Oocyst formation is greatest in domestic cats. After a primary infection a cat can shed oocysts for about two weeks and millions of these can be present in a single sample of stool. Oocysts are resistant to most ordinary environmental conditions and can survive under moist conditions for months and even years. Coprophagous invertebrates like cockroaches and flies can spread oocysts mechanically.

The tachyzoites are very susceptible to external environment and are readily destroyed by gastric juice when ingested.

However, infections due to laboratory and autopsy accidents and handling of infected meat in homes and slaughterhouses have been reported. Other possible means of transmission are venereal, ingestion of milk, from eggs and by organ transplant.

9.4 CLINICAL FEATURES

Toxoplasmosis in man may occur as a congenital or an acquired infection and in both forms the number of asymptomatic individuals far out number those with manifestations of disease. Congenital infection occurs by the transplacental transfer of Toxoplasma and the outcome of infection is varied. There may be severe fulminating infection resulting in abortion, miscarriage, stillbirth or the birth of a baby with manifestations of acute toxoplasmosis. In the benign forms the disease may become manifest in infancy, childhood or even adult life. There is evidence that the severity of congenital toxoplasmosis differs with the duration of infection of the foetus. Infections in the first trimester are associated with more severe lesions.

Two types of congenital infections have been described. In the generalised form anemia, jaundice, hepatosplenomegaly, lymphadenitis and retinochoroiditis are common. In the neurological form the tetrad of hydrocephalus; retinochoroiditis, convulsions and intracranial calcification are well recognized.

Acquired infection in man is usually less severe and can present with a varied clinical picture and is therefore often misdiagnosed. Lymphadenopathy is the commonest manifestation and maybe accompanied with fever, malaise and rash. It is also a common cause of chorioretinitis. Acute generalised toxoplasmosis with involvement of the brain may result, especially in persons undergoing immunosuppressive therapy. As many as 70-80% of patients with AIDS have been diagnosed as having toxoplasmic encephalitis. Toxoplasmosis can also occur from seropositive donors of bone marrow and solid organ transplants to seronegative recipients.

9.5 LABORATORY DIAGNOSIS

9.5.1 Collection and Transportation of Specimen

Tissue specimen for culture or histology should be be obtained and transported as quickly as possible to laboratory because of the poor viability of Toxoplasma outside the host and its somewhat fragile nature. Tissue specimen should be neither frozen nor allowed to dessicate. If there is delay in transportation, keep in refrigerator at +4°C. The diagnosis of toxoplasmosis consists of demonstration and isolation of the organism and demonstration of specific antibodies in the serum.

For the above tests the following clinical specimens have to be collected.

   a)   Enlarged lymph node and/or biopsy from the affected organ for histopathology and culture.
   b)   Whole blood or CSF in acute phase for culture.
   c)   Blood in plain vial for antibody detection. It should be allowed to clot and serum is separated by centrifugation.

9.5.2 Demonstration and Isolation of the parasite

Biopsy material (e.g. lymph node, muscles) or impression smears can be used for detection of the parasite by
   a) Special staining with Giemsa staining, PAS, H&E, GMS. Due to sparse distribution of organisms, it is usually difficult to find organisms by this method.
   b) Fluorescent antibody staining of tissue sections helps in detection of the organism.
   c) Indirect fluorescent antibody staining techniques using anti human gamma globulins is being used. It confirms toxoplasmosis

9.5.3 Isolation of the Organism

Parasite can be isolated by inoculating suspected tissue into following:
   a) Cell cultures
   b) Vero cell lines are inoculated. The cell lines can show cyopathology effect. Tachyzoites of typical morphology either free or in clusters or rosettes can be visualized.
   c) Mice inoculation

Tissue samples are ground with physiologic saline in a mortar and pestle and inoculated intraperitoneally in young albino mice. Whole blood or cerebrospinal fluid can also be inoculated. When biopsy material is contaminated it can be treated with antibiotics prior to inoculation.

When toxoplasmas are present the animals show the presence of peritoneal exudate in 7- 10 days and trophozoites can be readily identified by examination of the fluid under the microscope.

Several blind passages in mice may be required before the organism becomes apparent.

Mice surviving more than 4 weeks should be tested for antibody and, if positive, should undergo necropsy to demonstrate the organism in the tissues using Giemsa stain.

9.5.4 Specific Antigen Detection

Techniques for detecting specific toxoplasma antigen in tissue and body fluids are important in determining acute infection. A peroxidase - antiperoxidase technique has been used to identify antigen and whole tachyzoites in tissues. An Enzyme linked Immunosorbent Assay has been developed and has been found to be sensitive and specific for detecting antigenemia (stage specific 98 kDa Cytoplasmic antigen or nonspecific 15-45 kDa membrane antigen).

Immunodiagnosis
The isolation of the parasite is time consuming, cumbersome and is not always successful. Therefore, immunologic procedures are the primary methods for diagnosing toxoplasmosis.

Serum Antibody Tests

There are two types of tests available: those that are qualitative or semi quantitative and measure the presence of antibody, which implies immunity; and those that are quantitative and may or may not differentiate between IgG and IgM antibody to determine the likelihood of active infection. Researchers have developed ELISA test incorporating a recombinant protein (rP35 antigen of *Toxoplasma gondii*) that detects Ig G antibodies produced only during acute stage of infection thereby serving as serological marker to differentiate between recently acquired and latent old infection.

9.5.4.1 Qualitative Tests

Designed only to detect the presence or absence of antibody. In general, these tests are simple, rapid and inexpensive.

9.5.4.1 Direct Agglutination Test

In this test a suspension of toxoplasma organism is reacted with patients serum, resulting in agglutination if antibody is present. Some false positive results are reported, but its simplicity and low cost make direct agglutination test a prime candidate for screening test.

9.5.4.2 Latex agglutination

In the latex agglutination test, latex particles are coated with an extract of *Tgondii* and reacted with a patient's serum. Preliminary results are available in 90 minutes with final results after overnight incubation. The test is simple and economical and has been found to be effective in screening adults.

9.5.4.3 Enzyme Immuno Assay (EIA)

Three types of EIA are available:
  i.   Solid phases EIA test are performed on plates or beads on which Toxoplasma antigen has been affixed.
  ii. Ig M Immunosorbent agglutination assays latex particles are coated with antigen, reacted with patient serum, and then entrapped on a filter.
  iii. In the third type, the antigen is attached to the filter, where the entire test is performed.

All these tests are rapid, easy to perform but are strictly the screening procedures and should never be used for the positive diagnosis of toxoplamosis.

9.5.4.4 Quantitative Tests

The diagnosis of toxoplasmosis depends to a large extent on detecting an increase in antibody titres, the persistence of unusually high titres or the persistence of 1gM antibody. The quantitative procedures are more time consuming, more difficult to perform and more expensive but are designed to measure titre levels which aid in establishing a diagnosis.

9.5.4.4.1 Sabin Feldman Dye Test

This is the first successful quantitative serological procedure for toxoplasmosis, is now performed only in specialised laboratories and has for the most part been superceded by newer methods. This test is highly specific and sensitive and has been thoroughly studied for its clinical significance. It primarily measures IgG antibody and for many years has remained the standard against which other tests are measured. The drawbacks of the test are need for specialised training and special reagents including live Toxoplasma organism.

9.5.4.4.2 Indirect haemagglutination Test (IHA)

It is simple to perform, inexpensive and reasonably rapid. The IHA test becomes positive much later than other serologic procedures and as a consequence may be negative in documented cases especially congenitally acquired cases. The test has been used successfully for population studies and could be used as screening test to determine immunity in pregnant women. It should not be used as a diagnostic procedure when recent infection is suspected. The test is quantitative and reproducible and can be used in conjunction with other serologic tests to identify recency of infection.

9.5.4.4.3 Indirect Immunofluorescence Test (IIF)

The Indirect Immunofluorescence Test (IIF) is the most frequently used procedure in the clinical laboratory for the serologic diagnosis of toxoplasmosis. Studies have shown a direct positive correlation between the results of IIF and those of dye test. IIF has three major advantages over the dye test: it uses a more or less permanent antigen of formalin killed Toxoplasma organisms fixed on a slide; all special reagents are commercially available; and both IgG and 1gM antibodies can be measured independently. Its only major disadvantage compared with the dye test is the requirement for a fluorescent microscope with an ultraviolet light source. IIF test is considered to be sensitive, accurate and reproducible. For performing the test, following procedure can be used :Multispot slides are coated with formalin killed Toxoplasma tachyzoites and fixed with chilled acetone.Add 10 ul of diluted patient serum. (Serial doubling dilutions of the serum starting from 1:8 dilutions are used). For detection of specific antibodies, FITC conjugate antihuman immunoglobulin is added. And the slides are examined under the fluorescent microscope.

9.5.4.4.4 Enzyme Immunoassay

Technically, Enzyme Immunoassay procedure is similar to IIF with an enzyme substituted for the fluorescein label and the reaction read by colour development rather than microscope fluorescence. The major advantages of ETA are that soluble antigen can be used and that the tests tend to be more sensitive than ITF. ETA correlates well with IIF, but is technically much more difficult to perform and should be monitored by a more gent quality control system. For detection of IgM antibodies, a double sandwich Immunoglobulin - M enzyme linked immunosorbent assay (DS-IgM-ELISA), in which a labeled anti Toxoplasma antibody is used as the detecting system; and an enzyme labeled antigen or reversed ELISA in which labeled antigen is the detector. The sensitivity and specificity of both these tests have been found to be superior to IIF. Ig M antibodies may not always differentiate between primary infection, reinfection and reactivation. Persistence of Ig M antibodies for more than 6 months and sometimes even for years has been shown by most commercial available ELISA test system. However, an IgM ELISA titre of 1:256 or higher may be diagnostic of recent infection. Ig G avidity ELISA, that determines net antigen binding force to the population of the specific Ig G antibodies. It is initially low after primary antigenic challenge and increases during the subsequent weeks or months. Low avidity IgG ELISA suggests recent infection and high avidity IgG ELISA suggest past infection.

9.5.4.3 Other Tests

9.5.4.3.1 Complement Fixation Test
Once very popular, the Complement Fixation Test is used only in few laboratories. Complement fixing antibodies appear somewhat later than those measured in other tests and disappear much sooner. Due to lack of standardization and the relative poor sensitivity, complement fixation test has limited use.

9.5.4.3.2 Polymerase chain reaction
Detection of *T.gondii* DNA in body fluids (amniotic fluid, CSF) by use of Polymerase chain reaction (PCR) has added new dimensions in the diagnosis of fetal infection earlier in pregnancy and CNS infection in AIDS patients. In doubtful cases, test of aqueous humor of eye is also suggested to confirm presence of Toxoplasma DNA using Polymerase chain reaction. The most frequent PCR target is amplification of gene B1.

9.6 TREATMENT

Sulfonamides and Pyrimethamine are two drugs widely used for the therapy of toxoplasmosis. They have a synergistic effect and are usually well tolerated but occasionally thrombocytopaenia and leucopoenia may develop, Trimethoprim and sulfamethoxazole have been tried as alternative drugs but they don't have a synergistic effect. Spiramycine has also been used successfully in man. It produces high concentrations in the placenta without crossing the placental barrier and is therefore useful in treating infected pregnant patients.

9.7 PREVENTION AND CONTROL

Man is infected by Toxoplasma from tissue cysts in meat, the oocysts in soil and by tachyzoites ransplacentally. In view of the prevalence of Toxoplasma infection in domestic animals proper cooking of meat is essential. Heating meat to 66°C kills the organisms. Freezing causes reduction of organisms but is not adequate. The hazards inherent in handling raw meat as in slaughterhouses and in the home are well recognized and measures such as hand washing or wearing of rubber gloves should be adopted.

Freshly passed oocysts in cat's faeces are non-sporulated and therefore non-infective. Proper disposal at this stage by incineration or flushing down the toilet obviates the risk of infection. However, sporulated occysts can remain infective for about a year in moist soil. As cats are usually infected by ingestion of raw meat, wild birds and rodents, effective prevention can be achieved by control of the diet of pet cats and elimination of stray. Sand and soiled represent a durable source of infection; therefore, children's play areas should be made inaccessble to cats. Pet cats shedding oocysts can be temporarily isolated and treated with sulfadiazine and pyrimethamine. Other drugs used for cats are 2 suiphameyl 1 -4 diaminodiphenyl sulfone (SDDS) and clindamycin but these do not completely stop the shedding of oocysts. Immunoprophylaxis has been attempted in animals using phenol killed toxoplasma and irradiated toxoplasma with some success.

As the foetus and neonate represent the most vulnerable section avoidance of infection by pregnant women is very important. Routine serological check - up in antenatal clinics and treatment of infected mothers would be a valuable step in protecting the foetus.

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TAENIASIS AND CYSTICERCOSIS

10.1 INTRODUCTION

Taeniasis and cysticercosis are diseases caused by the adult and larval stages of the cestode or tapeworm parasites *Taenia saginata* and *Taenia solium* in their definitive host (humans) and intermediate hosts (cattle, pigs, humans). These intestinal infections, termed taeniasis, normally produce only mild symptoms. Eggs passed in the feces of human carriers can cause further disease if ingested by cattle, pigs, or humans. In these intermediate hosts, the egg develops to the larval (cysticercus) stage, and the disease is termed cysticercosis.

10.2 CAUSATIVE AGENTS

The larval stage of T.saginata infects cattle, whereas T.solium larvae can infect both pigs and humans. Although larvae invade mainly skeletal muscles. T.soliurn larvae frequently invade the central nervous system of humans, and is, consequently a serious public health problem**.**

10.2.1 Life Cycle

The life cycle of *T.saginata* and *T.solium* is given below

Development in Definitive Host
The adult stages of *T.saginata* and *T.solium* are flat tapeworms consisting of **a** scolex (head), and strobila (chain) ofproglottids (segments), *Taenia saginata* can contain 1000 to 2000 proglottids and can vary in length from 4 to 12 m: T. Solium usually contains fewer than 1000 proglottids and is usually less than 3m in length. Mature gravid proglottids, containing a uterus filled with as many as 80, 000 eggs, detach from the strobila either singly or in multiples and pass out of the intestine through the anus.

Development in Intermediate Hosts
Ingestion of the *T.saginata* egg by cattle or the *T.solium* egg by pigs or humans results in the hatching of the larval stage (onchosphere). Activated by host gastric juices, the onchosphere penetrates the host's intestinal mucosa and enters the blood or lymphatic system. The primary site in cattle for development of the *Tsaginata* onchosphere to the infective cysticercus stage is the dilated lymphatic spaces in skeletal muscles: some also reach full development in the heart. In pigs and humans, the cysticerci of *Tsoliurn* develop in skeletal muscle, brain, myocardium, and the eye. In humans, localisation in the central nervous system is frequent. The cysticercus is composed of a scolex invaginated within a fluid filled vesicle or bladder. *Taenia saginata* cysticerci are 7 to 10 x 4 to 6 mm, and *Tsoliurn* cysticerci measure 5 to 20 mm in diameter. The cysticerci in cattle and pigs become infective for humans in 10 to 12 weeks after ingestion of eggs, and the life cycle is completed when these cysticerci are ingested via raw or inadequately cooked beef or pork and contaminated vegetables. The life span of the cysticerci in cattle and pigs is reported to be as short as a few months to as long as 3 years.
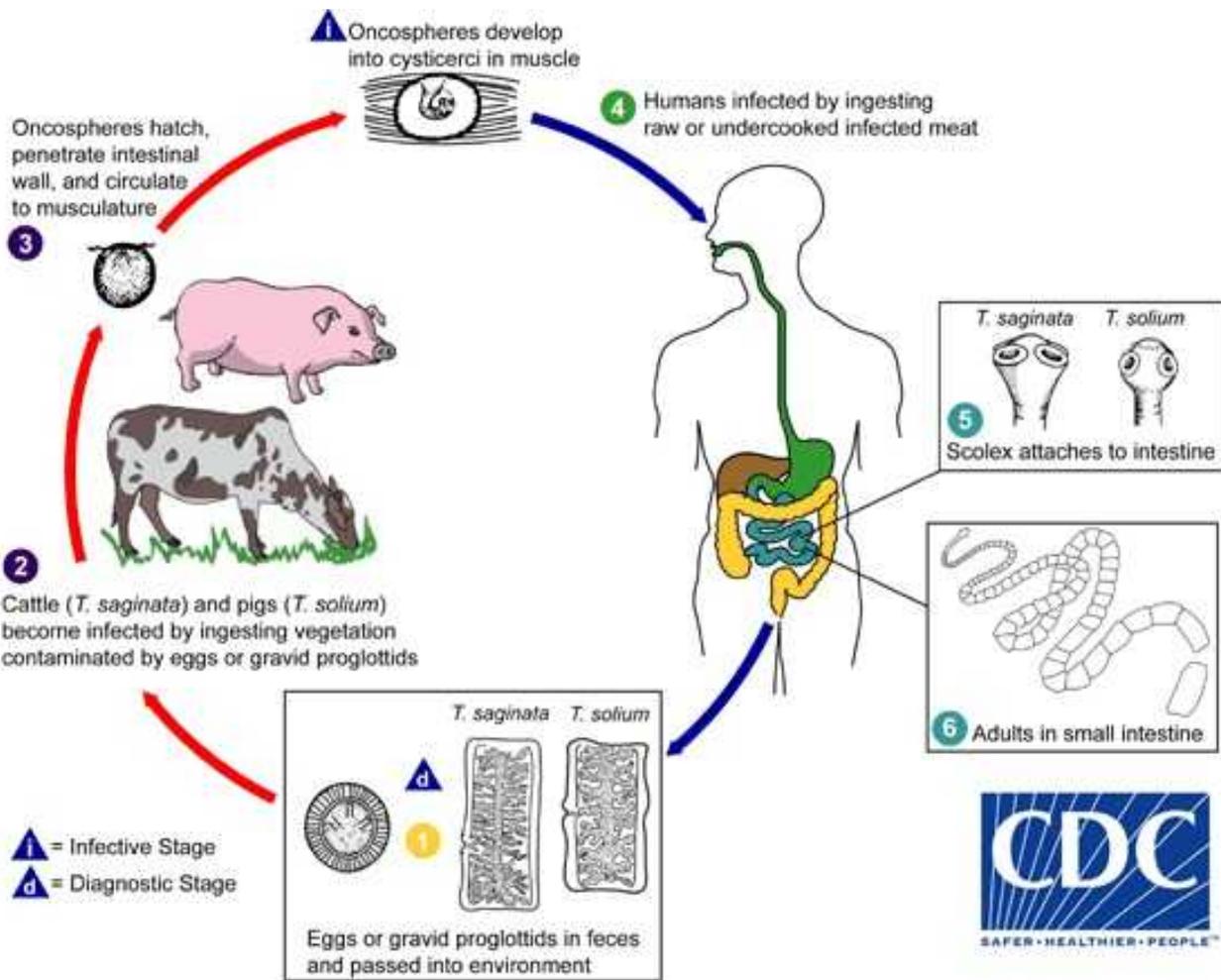
Fig 10.1: Life cycle of *T. saginata* and *T. solium. (*available at http://www.cdc.gov/dpdx/taeniasis/index.html*)*

10.3 CLINICAL SYMPTOMS

10.3.1 Taeniasis

Infections of adult T.saginata and T.solizim in humans produce only mild symptoms. These might include discharge of proglottid, abdominal pain, nausea, loss of weight, change in appetite, and headache.

10.3.2 Human Cysticercosis

Taenia soliurn cysticerci that develop in muscle tissue present few symptoms, although pain results if muscle cysts encroach on nerve endings. The death and calcification of muscle cysts is a more important cause of discomfort as is eye involvement. The most common locations of cysts in the eye are vitreous humor and subretina, and the inflammatory response to the parasite can lead to retinal detachment or atrophy, iridocyclitis, and chorioiditis. Myocardial cysticercosis frequently occurs in massive infections, yet clinical signs rarely result. Involvement of the

central nervous system can cause serious clinical disease resulting from either an inflammatory response (to dead or dying cysticerci) or an obstruction (involving the foramina and ventricular system of the brain). Symptoms include seizures, hydrocephalus, headaches, dizziness, arterial thrombosis, loss of vision and nausea.

10.4 EPIDEMIOLOGY

10.4.1 Animal to Human Transmission

The cysticerci of *Tsaginata* and *Tsolium* are transmitted to humans through the ingestion of raw or inadequately cooked beef or pork and contaminated vegetables.

10.4.2 Human to Animal Transmission

Humans harboring tapeworms can contaminate the environment with large number of eggs (250, 000 to *500,* 000 eggs per day per individual). These eggs can survive for long periods and are resistant to moderate dessication, disinfectants, and low temperature. Tapeworm eggs can be transmitted directly to pigs and cattle by the handling and feeding of these animals by farm workers with egg contaminated hands. Infected farm workers also can cause transmission by contaminating hayfields or hay, sillage, and other feeds, as well as irrigation water used on field crops, through indiscriminate defecation. A source of indirect contamination of the environment with eggs is the effluent from sewage treatment plants used for the fertilization of agricultural lands. Taeniid eggs can survive many sewage treatment plants used for the fertilisation of agricultural lands. Taeniid eggs can survive many sewage treatment processes. Transmission can also occur by birds, earthworms, or beetles and vegetables that have fed on raw sewage sludge because eggs can survive passage through their digestive tracts and can then contaminate pastures. Eggs can also be dispersed by wind and sewage water.

10.4.3 Human to Human Transmission

Autoinfection of humans can occur with *Tsoliurn* eggs transmitted directly from anus to mouth, or transmission can occur between individuals through contaminated hands or food. The development of human cysticercosis by internal autoinfection (whereby gravid proglottids enter the stomach via vomiting or reverse peristalsis). However, the frequency of this occurrence is questioned, and it would appear that humans most commonly become infected like pigs through the ingestion of *Tsolium* eggs contaminating the environment (i.e., drinking water contaminated by raw or inadequately treated sewage or eating vegetables fertilized with raw sewage).

10.5 LABORATORY DIAGNOSIS

10.5.1 Taeniasis

Eggs of T**.** Saginata and *Tsoliurn* can be detected in fecal smears or anal swabs. Eggs are not distributed uniformly throughout the stool, and fecal smears are accurate for diagnosis in only about 77% of cases involving a single examination; the anal swab method is about *85%* accurate. Tapeworm species can be determined by examination of proglottid morphology (if a proglottid is recovered) but not by eggs alone. The proglottid is pressed between glass slides and the number of uterine branches counted. For *T saginata* the number of branches is >18; for T. Solium, it is <12. Specific diagnosis also can be done by an experienced observer through examination of a scolex, which is usually voided after drug treatment.

10.5.2 Human Cysticercosis

10.5.2.1 Biopsy
Definitive diagnosis can be accomplished by surgical removal and biopsy of nodules located on the surface of skin or in subcutaneous tissues and muscles.

10.5.2.2 Radiology
Calcified intramuscular cysts can be detected by X-Ray analysis. Ocular cysts can be detected by opthalmoscopy. Computed axial tomography (CAT scan) can detect cysticercus in the central nervous system, but this procedure is not totally specific. Although the detection of multiple cystic or calcified lesions is considered highly suggestive ofcysticercosis, the detection of single lesions or non-specific findings such as hydrocephalus are less diagnostically certain. Also, cysts located in certain regions of the brain (subarachnoids or intraventricles) often are not detectable by this procedure.

10.5.2.3 Serology
Immunodiagnostic tests include complement fixation immunofluorescence, indirect hemagglutination *assay,* and enzyme linked immunosorbent assay (ELISA). These tests exhibit varying degrees of specificity and sensitivity. Currently, the ELISA shows the highest accuracy. ELISA kits are commercially available, Using either a crude extract of pig cysticerci as antigen or a purified fraction thereof (antigen B), antibodies were detected by ELISA in sera or cerebrospinal fluid (CSF) in 70 to 80% of clinically diagnosed cases. False positive reactions were eliminated by combining ELISA with an immunoblotting technique. The complementary use of an ELISA designed for the detection of larval *T solium* antigens and one designed for the detection of anti *T solium* antibodies in CSF was effective in detecting 69% of patients with a clinical diagnosis of cysticercosis and 100**%** of patients for whom cysticercosis was confirmed surgically or histologically; no false positive reactions were found. Recently, an ELISA using anyone of three purified proteins isolated from the scolex of *T. solium* metacestodes by monoclonal antibody-immunoaffinity chromatography was able to detect 100% of patients with cysticercosis, with no false positive reactions. These procedures are of greatest value when complemented and correlated with other diagnostic procedures. Enzyme linked Immunoelectrotransfer blot is a recent technique using *T. solium* metacestode antigen to demonstrate anticysticercus antibodies.

10.5.2.4 Molecular methods:
DNA probes have been developed for rapid and sensitive diagnostic test for detection of T. solium ova.

10.6 TREATMENT

10.6.1 Taeniasis

Niclosamide (reported efficacy between 85 and 95%); and praziquantel (95% efficacy with single dose of 10mg per kg body weight) are effective for human taeniasis. Praziquantel has the advantage in that it effectively removes the scolex and causes destruction of the strobila without release of gravid proglottids and it has minimal side effects.

10.6.2 Cysticercosis

Praziquanel has been shown also to be effective in humans for *Tsoliuni* cysticerci. Previously, the only treatment available was the surgical removal of cysts. Traditional supportive therapy includes anti seizure and steroid medication and decompression for h) drocephalus. Praziquantel is effective also against cysticerci in cattle and pigs.

10.7 PREVENTION AND CONTROL

Prevention and Control centres on improved public health measures. Man is the sole definite host; therefore health education is of paramount importance; indiscriminate human defaecation must be strongly discouraged and sewage should be treated to kill *Tsoliurn* eggs. Husbandry practices must also be improved so that pigs do not wander widely and consume human faeces. The source of infection with adult *Tsoliuin* is infected pork. Cysticerci in meat can be killed by freezing at - 20°C for 12 hours or by cooking at 50°C. Careful inspection of pork is obviously of value. Whenever a *Tsoiiurn* infection is diagnosed in man, it should be treated with appropriate antiparasite agent(s); furthermore family members and close contacts should be investigated for the presence of infection, and treated accordingly.

Further Reading

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BRUCELLOSIS

11.1 INTRODUCTION

Brucellosis is a zoonotic infection caused by the bacterial genus Brucella. The bacteria are transmitted from animals to humans by ingestion through infected food products, direct contact with an infected animal, or inhalation of aerosols. The disease is an old one that has been known by various names, including Mediterranean fever, Malta fever, gastric remittent fever, and undulant fever. Humans are accidental hosts, but brucellosis continues to be a major public health concern worldwide and is the most common zoonotic infection.As a global problem brucellosis commands all attention because of its significant health and economic implications. In man it may take the form of a prolonged, often vague, illness requiring expensive therapy and may produce varying degrees of incapacitation even when the patient is ambulatory. In animals, serious losses occur due to abortions, still births premature birth of weaklings, infertility, loss of meat and milk.

11.2 EPIDEMIOLOGY

11.2.1 Magnitude

Global :

The global burden of human brucellosis remains enormous. The infection causes more than 500,000 infections per year worldwide. The disease occurs worldwide, except in those countries where bovine brucellosis (*B. abortus*) has been eradicated. This is usually defined as the absence of any reported cases for at least five years. These countries include Australia, Canada, Cyprus, Denmark, Finland, the Netherlands, New Zealand, Norway, Sweden and the United Kingdom. The Mediterranean countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico and Central and South America are especially affected. While *B. melitensis* has never been detected in some countries, there are no reliable reports that it has ever been eradicated from small ruminants.

India:

The presence of brucellosis in India was first established early in the previous century and since then has been reported from almost all states.Epidemiological evidence shows that in India brucellosis is present in different species of mammalian farm animals including cattle, goats, buffalo, yaks, camel, horses and pigs. Brucellosis in cattle seems to be associated primarily with intensive farming practices in large organised dairy farms. Risk behaviours such as unrestricted trade and movement of animals, use of local cattle yards and fairs for trading, sending dry animals back to villages for maintenance, use of semen from unscreened bulls for artificial insemination and poor farm hygiene probably all contribute to the spread and transmission of the infection. Free grazing and movement with frequent mixing of flocks of sheep and goats also contribute to the high prevalence and wide distribution of brucellosis in these animals in India. Increasing demand for dairy products and protein, changing agricultural methods, and increased

trade and movement of animals have caused concerns that the prevalence may increase. Therefore, there is an urgent need for the strict implementation of a control policy not only for cattle but also for small ruminants. Given the potentially huge economic and medical impact a control policy could be cost-effective. India has already a policy for the control of brucellosis in dairy cattle .

11.2.2 Agent

Brucella organisms, which are small aerobic intracellular coccobacilli, localize in the reproductive organs of host animals, causing abortions and sterility. They are shed in large numbers in the animal's urine, milk, placental fluid, and other fluids. To date, 8 species have been identified, named primarily for the source animal or features of infection. Of these, the following 4 have moderate-to-significant human pathogenicity:
1. Brucella melitensis (from sheep; highest pathogenicity)
2. Brucella suis (from pigs; high pathogenicity)
3. Brucella abortus (from cattle; moderate pathogenicity)
4. Brucella canis (from dogs; moderate pathogenicity)

11.2.3 Source of Infection

The animals that are commonly known to serve as source of human infection are goats, sheep, cattle, buffalo & swine. Infection of reindeer, caribou, camels, and yaks is of epidemiological importance in some parts of the world. Dogs have long been known as carriers of brucella, and the newly recognized species *Br canis* may be transmitted from dogs to man.

11.2.4 Transmission:

The modes of transmission to man are ingestion, contact, inhalation and accidental inoculation.
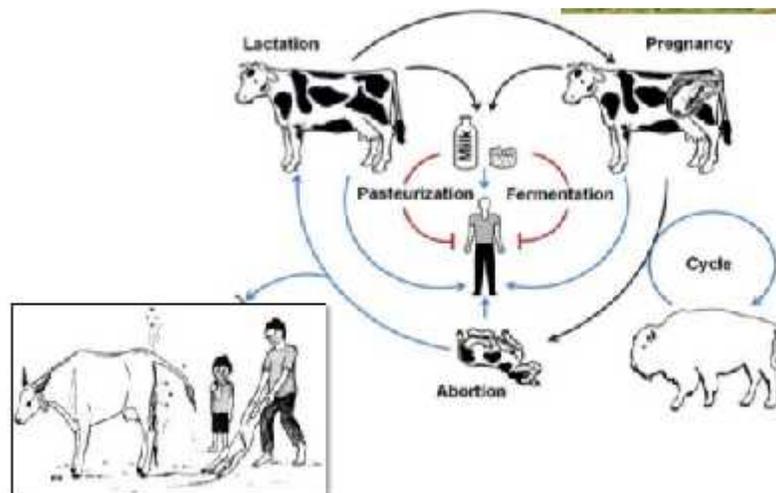
Fig. 11.1: Infection by ingestion may occur via the gastrointestinal tract or by penetration through the mucous membrane of the throat. The usual vehicles of infection for man are: untreated food products of raw milk from infected animals; viscera, bone marrow, and lymph nodes in muscle tissue from infected carcasses, which may retain viable brucellae for more than a month after slaughter, and for much longer in the case of frozen or chilled meats; water supplies, and raw vegetables contaminated by infected animal urine or excreta.

As knowledge accumulates concerning the dietary habits of various peoples, new chains of transmission to man are revealed. For example, observations from the arctic and subarctic regions have shown that both children and adults may become infected with *Br.suis* biotype 4 as a consequence of feeding upon raw bone marrow and raw meat from infected animals. Contact with brucellae in vaginal discharges, foetuses, placentas, urine, manure, caracasses, and salvaged animals cause a large proportion of human cases. The skin and mucous membranes, including the conjunctivae, provide the portals of entry. The contact route of transmission is especially important among veterinarians, farmers, rendering-plant employees, packinghouse workers, animal handlers, factory workers engaged in the primary processing of wool, and laboratory workers. The chances of infection by contact are particularly high during the season in which abortions occur, mainly because of the massive contamination of the premises. Infections by contact play an important role among those who, because of climatic conditions, bring their animals into the human habitation, where transmission of brucellae from animals to humans occurs, especially among children who use the animals as pets. Water shortages in summer may prevent adequate personal hygiene and thus tend to increase the possibility of the infection being transmitted to man, In winter, extremely cold temperatures may have similar results by bringing infected animals into closer contact with shepherds. Infection occurs when man inhales infected dried materials of animal origin, such as the dust from sheep wool, railway trucks and lorries that have transported infected animals, abattoirs, infected farm premises, and brucella laboratories. Infection by accidental inoculation is not frequent among veterinarians and laboratory workers. Those involved in the large-scale production of brucella vaccines and diagnostic antigens are also at special risk of infection and/or sensitization reactions.

Prevention of infection by the routes described above would therefore, include, firstly, special care and supervision in the handling of carcasses of infected animals, and, secondly, adequate personal hygiene and environmental sanitation. Personal hygiene, such as washing of exposed body surfaces and removal, washing, and disinfection of soiled clothing and footwear, is essential. Environmental sanitation, which is of particular importance in preventing airborne and contact infections, requires that the surroundings be kept as free as possible from contaminated substances. The persistence of Br *abortus* in liquid manure, in numbers of 100-1000 per ml for 4 months after the last date of contamination, stresses the need for attention to the extraordinary powers of resistance of Brucella, which possibly exceed those of other genera of gram negative bacilli.

Spread of infection to urban areas may occur when urban dwellers purchase goats that have aborted and which are therefore, sold at reduced prices. In some countries, *Br melitensis* infections may occur especially in summer during periods of bovine milk shortages, since the milk of cows and buffaloes may then be mixed with the less popular, and frequently infected, milk of goats and sheep and consumed as raw milk or ice cream. The transmission of brucella infection to man and its prevalence in different areas of the world depend upon local food habits, methods of processing milk for cream, butter, and cheese, social customs, types of animal husbandary practices, species ofbrucella prevalent in the region, climatic conditions, and standards of personal and environmental hygiene.

11.2.5 Reservoir of Infection:

Wild animal reservoirs and localization in nature:
A very wide host-range is one of the important characteristics of the genus Brucella, although individual biotypes may have a very limited host-range in natural infection.
Natural infections have been reported from a large number of species of vertebrates, mammals, and birds. Four different situations apparently occur:

1. Infections that are derived from domesticated animals and that disappear when the domesticated foci are eliminated. Carnivorus are more readily infected than herbivorous wild animals in enzootic bucellosis areas, probably through ingestion of aborted fetuses and membranes (e.g., fox infection by swine foetus and wolf infection by reindeer meat).
2. Infections that are derived from domesticated animals and that can persist independently and for a long time in the wild host.
3. Foci of infection that appear to have existed independently of the infection in domesticated animals have been found in wild animal populations (rodents, herbivora, and carnivora) in Africa, the USA, and the USSR. The causative organisms of the infection have not been observed so far in domestic animals. Examples where this situation applies are: Neotoma lepida Thomas, harbouring *Br neotonme, Rattus assimilis, Melomys cervinipes,* and *M lutillus* in Queensland, Australia, contaminating distinctive biotypes that remain unclassified; Arvicanthis niolticus and Mastomys natalensis in Kenya, harbouring *Br suis* biotype 3; and *Mus musculus,* harbouring distinctive biotypes, the cultures of which appear to be unstable and difficult to classify.
4. Foci of infection in wild animals that are a source of infection in domestic animals (e.g., *Br suis* biotype 2 in hares *(lepus europaeus),* which spreads to pigs).
5. Infection is transmitted among animals directly as well as by blood sucking arthropods (insects, ticks, and mites). It has been shown experimentally that the organisms multiply and persist much longer in ticks than in insects and maintain their virulence for mammals. Ticks transmit the infection through bites and also excrete the organisms in the coxal fluid. However, only a small percentage of ticks collected in a focus yield the organism and they may not play an important epidemiological role in transmission.

Brucellosis of wild animal origin is mostly transmitted to man indirectly through domesticated animals, but a few cases of direct transmission through ingestion, contact, or blood-sucking arthropods are known. Man and sledge dogs share a common source of infection with *Br suis* biotype 4 in the meat and offal of infected caribou. Attention must also be drawn to the possibility of human infection through fur animals (mink, foxes) fed with viscera from infected animals. Care should be taken to feed fur- bearing animals with cooked meat or with meat from Brucella-negative domestic animals.

11.2.6 Brucella in food products

Important sources of infection with brucellosis are infected raw milk and freshly prepared cheese, cream, and butter, produced form unheated milk of infected animals. Raw meat and certain meat products may also be sources of infection. In a few cases the ingestion of raw vegetables contaminated with animal urine and faeces has been found to produce human infection.

1. **Milk and milk products**

The principal animal products conveying the disease are the milk of goats, sheep, and caves, and products prepared therefrom when not adequately heat-treated. Brucella may also be transmitted through the milk of water buffaloes, camels, and yaks. The three principal species of brucella are all excreted in the milk. The transmission of brucellosis by milk and milk products can be prevented by adequate heat treatment. Legislation should be enacted to require pasteurization of milk and milk products. Brucellae are killed by a lower time-temperature combination than that required for *Mycobacterium tuberculosis* or *Mycobacterium bovis,* the killing of which has always set the standard for pasteurization, so that milk pasteurized in accordance with the official standard does not contain living brucellae, Where milk cannot be pasteurized it is, in fact, sufficient merely to raise the temperature of the milk to boiling point and then to cool it immediately. The cream of infected milk is usually more heavily infected than the rest of the milk, because the fat globules rising to the surface carry the organisms with them. A high time-temperature combination should be used for pasteurization of cream derived from unpasteurized infected milk, in order to make it safe, than for the rest of the milk. Souring of the milk inhibits brucellae but may not eliminate them completely for several days. Koumiss (fermented mare's milk) has been suspected as a source of infection, but this is unlikely to be common since the low pH reduces the probability of viable organisms being present. It is believed that matured fermented cheese is safe from brucellosis, but little accurate information is available on the length of aging needed to ensure safety. In this connection, some evidence has been advanced that a period of 3 months is sufficient for hard fermented cheese to become non- infected. However, no categorical statement can be made on this point because of the great variety of methods used for making cheese in different countries. Soft cheeses made from the milk of sheep are especially hazardous, as brucellae may be present in large numbers during the period after lambing and the cheese are eaten-crude after preparation. In some countries soft cheese are stored in 10% salt solutions, but *Br. melitensis* survives for at least 100 days under these conditions. Poor sanitary conditions in cheese making may lead to environmental contamination with brucella. Another possible source of contamination with brucella during cheese making is the use of the stomach of an infected animal. Butter made from milk that has been well soured is unlikely to contain viable brucellae. However, butter may harbour brucellae for several months when the product is manufactured from milk that has not been well soured and has not been pasteurized. During churning, brucellae find their way into the buttermilk and the water used for washing the butter, thus causing spread of the pathogen. Therefore, it is recommended that, in countries where milk is not processed, tests on various cheeses, butter, and other milk products should be carried out by inoculation into guinea pigs and that the results of these tests should be used as the basis for preventive measures concerning the products in question. In the interest of public health, strict measures concerning the delivery of milk originating from infected herds should be enforced and closely supervised. The containers holding such milk should b clearly marked as such, where practicable. If infected milk shows changes in its organoleptic properties, it should be disposed of in such a way as not to infect animals, man, or the surroundings.

2. **Meat and meat products**

Cases of human infection due to ingestion of meat or meat products are very few and very difficult to demonstrate. Nevertheless, the meat, organs, and blood of infected animals of all species may contain brucellae. Investigations have shown that the carcasses of sheep, cattle, and pigs may contain brucellae, not only during the acute phases of the disease, when its presence is revealed by clinical symptoms (abortion), but also when the disease has passed into the chronic

stage and its presence is apparently healthy animals is revealed only by diagnostic tests. It seems, however, that, in cattle at least, the number of organisms per gram of muscle is very small, so that muscle should be considered only weakly infective except for the lymph nodes it contains. Brucellae may be transmitted through untreated meat of reindeer, caribou, and infected wildlife. Besides primary brucella infection of meat, secondary contamination of the carcass may occur, particularly through milk. Milk from infected udders may find its way into, and infect the surface of carcasses at the time of udder skinning, when the teats are cut off the milk ducts fully opened. The transmission of brucellosis by meat and meat products can be prevented by subjecting them to adequate heat treatment. It has been shown that the organism can survive pickling and smoking as well as chilling and freezing.

In the interest of public health, the above considerations taken together justify the adoption of strict measures concerning the delivery of brucella infected animals to abattoirs, the conditions under which they are slaughtered, and the methods of inspecting and judging the merit. Personnel should be well protected against the risk of infection by wearing gloves, and should be kept under medical surveillance. Health education of abattoir personnel is important.

11.2.7 Immunoglobulins

An incomplete antibody has been found to persist longer than the agglutinating and complement-fixing antibody after vaccination with strain 19 or strain 45/20 and after infection in cattle. Studies have also been conducted on the immunoglobulins in beagle dogs naturally infected with *Br. canes.* After infection, IgM appeared in the serum, followed after 7-13 days by four IgGs, namely, 7S (1), 7S(2a), 7 S(2b) and 7S(2e). 1gM and the first three IgGs mentioned above caused agglutination; 7S(2e) however, did not appear to have any antibody activity in the tests used. Colostrum was found to contain only small amounts of IgA, which had no antibody activity, and an unusual 7S immunoglobulin that had antibody activity and was susceptible to cleavage, by 2- mercaptoethanol.

11. 3 CLINICAL FEATURES

A. Human brucellosis

Clinical manifestations
The clinical manifestations of human brucellosis are variable. In an endemic area, the clinician must consider brucellosis in the differential diagnosis of any febrile disease.

Acute and subacute brucellosis
Acute and subacute brucellosis are accompanied by fever and bacteremia. Some patients develop an acute illness of limited duration followed by apparent recovery; others have prolonged fever followed by frequent relapses. Either variety can be accompanied by various complications (articular, osseous, visceral, and neurological).

Chronic brucellosis
Chronic brucellosis, which is usually non-bacteraemic, occurs with or without demonstrable localized foci of infection. The symptoms are generally related to the state of hypersensitivity of the patient. Illness may persist for a number of years.

11.4 DIAGNOSIS

The diagnosis of human brucellosis is based on epidemiological information, clinical manifestations, and laboratory tests. Diagnostic methods include bacteriological, serological, and allergic tests.

11.4.1 Bacteriological tests

The tissues from which Brucella can most easily be isolated are blood and sternal marrow. If first attempts are unsuccessful they should be repeated. Other sources, such as lymph nodes, cerebrospinal fluid, urine, and any abscesses that maybe present, should be investigated. Brucellae have occasionally been isolated from sputum, placenta, mother's milk, vaginal discharges, seminal fluid, etc

11.4.2 Serological tests

These tests should be repeated when the first tests are suspicious or negative in cases of active disease.

11.4.2.1 Rose Bengal Test
The classical Rose Bengal test (RB) is often used, as a rapid screening test. RB is based on the agglutination of serum antibodies with a stained whole cell preparation of killed Brucella. RB is performed by mixing on a glass plate a drop of RB reagent with an equal volume of serum and agglutination is read after 2 to 4 min. The sensitivity of RB is very high (>99%) but the specificity is low. As a result, the positive predictive value of the test is low and a positive test result thus requires confirmation by a more specific test. The negative predictive value of RB though is high and a negative test results excludes active brucellosis with a high degree of certainty. To increase the specificity and the positive predictive value of RB the test may be applied to a serial dilution (1:2 through 1:64) of the serum sample. The specificity of the RB increases when higher dilutions agglutinate and titres of 1:8 or 1:16 and above may be regarded as positive. This approach however inevitably results in a lower sensitivity.

11.4.2.2 Serum Agglutination Test :
For confirmation of RB, serum agglutination test (SAT) or Enzyme Linked Immunosorbent Assay (ELISA) may be used. SAT is performed by mixing serial dilutions of serum, usually 1:20 through 1:2,560, with Brucella antigen in test tubes or in wells of an ELISA plate.

After overnight incubation agglutination is read either by the unaided eye or under a binocular. As a guidance agglutination at titres of 1:160 or above is considered of diagnostic value as long as the patient has signs and symptoms of disease. In endemic areas the diagnostic threshold value will have to be set at least one titre step higher (1:320) to provide a sufficiently high specificity as many asymptomatic individuals will have titres equal to the lower threshold level of 1:16056. The use of the higher threshold level however severely restricts the sensitivity and clinical importance of the test.

11.4.3 ELISA:

ELISA is used to discriminate between the presence of specific IgM and IgG antibodies and to roughly access the stage of illness.

11.4.4 Intradermal test

A positive intradermal test normally indicates a state of specific allergy for brucellosis. When possible, the test should be performed with an allergen that neither stimulates antibody formation nor produces a non-specific skin reaction. A positive intradermal test, in healthy persons with past exposure to infection, can result in a rise in agglutinins when certain skin-test allergens are used.

The intradermal test is only a complementary aid in the diagnosis ofbrucellosis. It can be helpftil: (a) in chronic brucellosis, when a positive test maybe the only objective indicator of infection; great caution should be exercised in making a diagnosis of chronic brucellosis based solely on a positive skin-test and vague clinical symptoms, since a positive brucella skin reaction is known to occur at various stages of other chronic diseases; (b) when it is persistently negative; this excludes brucellosis; (c) in areas with a low incidence of endemic brucellosis, when a positive reaction may have important significance; (d) in epidemiological surveys; it is important to bear in mind the fact that vaccinated individuals may have positive skin-tests.

11.5 TREATMENT

In the treatment of brucellosis, the acute and subacute forms must be considered separately from the chronic form of the disease.

Therapy of acute and subacute brucellosis

In view of the frequency of spontaneous recovery, the following points are of value in judging the effectiveness of any therapy:
a)   Clinical improvement within a week;
b)   The failure to recover brucella from the blood, or from other tissues or sites, when previous results were positive;
c)   A reduction in the frequency of complications;
d)   A reduction in the frequency of relapses;
e)   A drop in antibody titre.

11.5.1 General treatment

Supportive therapy is recommended, including bed rest and adequate diet, during the acute manifestations of the disease. Patients can recover without treatment, and this should be recognised in evaluating any new treatment.

11.5.2 Antibiotic therapy

The antibiotic most widely recommended is tetracycline, the daily dose for adults being 1-2 g orally. Treatment is continued for 3 weeks but be prolonged if necessary. If a relapse occurs, tetracycline therapy should be repeated further 2-3 weeks.

In severe cases, it may be necessary to administer tetracycline parenterally. In severe cases, with demonstrable localised lesions, such as those due to *Brsuis* or *Br melitensis,* tetracycline may be combined with streptomycin 1 g daily for 2 weeks.

11.5.3 Corticoestiroid therapy

Corticosteroids are not recommended in ordinary cases of brucellosis. Their use with antibiotics, however, may be justified in certain conditions. They should be used in severe septicaemic brucellosis to prevent toxic reactions, but should not be administered for more than a few days. Corticosteroids may also be used in certain visceral forms of brucellosis.

11.5.4 Therapy of chronic brucellosis

In cases of persistent localized infection, treatment with antibiotics and even surgical intervention may be useful. However, desensitization should be considered. Physiotherapy may be useful adjunct to treatment.

11.5.5 Vaccination

A vaccine produced from *Br abortus* strain 19-BA has been widely used for the past 30 years in the USSR, where it is given to population groups occupationally exposed to infection with *Br melitensis. The* vaccination, as a supplement to sanitary and hygienic measures, is a method of protecting human beings where there is a great danger of occupational infection, e.g., for owners and handlers of sheep and goats, for workers in abattoirs and the meat industry, and for veterinarians and laboratory workers. These vaccines, which may be killed or live, can cause postvaccinal sensitization and produce persistent antibodies, which are sometimes difficult to distinguish from post-infection antibodies. The decision on whether to carry out vaccination must be taken in agreement with the national or local health authorities.

11.5.5.1 Live vaccines

There is convincing evidence for the protective power of the 19-BA strain vaccine against *Br melitensis* infection. Observations during the last few years have shown that administration of a dose of 19-BA strain vaccine ten times as high as the established immunising dose for man may cause the disease. According to communications from a number of authors, Br. abortus strain 19 has caused severe clinical illness among veterinary workers who have been accidentally infected with it. A single epicutaneous immunisation (by scarification) with live 19-BA vaccine provokes slight sensitization and does not lead to illness. Annual epicutaneous re-vaccination over a period of 5 years, with half the original vaccination dose, may cause a high degree of cutaneous sensitization in over half of those to whom it is administered, and in some cases may cause pathological manifestations. Therefore, primary and booster vaccinations are given only to those occupational groups that are highly exposed to *Br melitensis* infection, and only if the intradermal test is negative. Thus the human vaccination is a temporary protective measure against brucellosis. An important protective role is played by the strict application of sanitary and hygienic measures. Authorities concerned with animal and human brucellosis should concentrate on brucellosis control in animals and its eventual eradication, which is the means whereby human brucellosis can eventually be eliminated.

11.6 NATIONAL CONTROL PROGRAMME ON BRUCELLOSIS

Indian Veterinary Reserch Insitutes has launched National Control Programme on Brucellosis with an objective to prevent and control Brucellosis in Live Stock .

The program has three componenets
1. Biannual village level screening of pooled milk samples. For differentiation in clean and non clean herds
2. Biannual B.abortus S-19 vaccination for female calves of 4-8 M age
3. Mass screening & castration of infected bulls

Expected outcome of NCPB:

- Reduce the impact of disease on human health and to reduce economic losses
- NCPB is a time bound 5-year intensive location targeted control program.
- Intends to involve village milk cooperatives in diagnosis and control through vaccination.
- Periodical surveillance using milk ring test for pooled milk and ELISA for random or herd screening.
- Targets B. abortus S19 vaccination for all the female calves of 4 to 8 months in infected villages.
- Program assures very high & sustained cost benefit ratio to farmer & dairy industry & helps to establish accredited herds/ villages.

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ARBOVIRAL INFECTIONS

Arboviruses are viruses, which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagus arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods and are passed on to new vertebrates by the bites of arthropod after a period of extrinsic incubation period. The amplifying vertebrate host develops viremia of sufficient titer and duration to infect subsequently feeding vectors. Infection of the vector is typically life long, with no apparent ill effect. Certain arboviruses may be transmitted transovarially and venereally by their arthropod vectors. In addition to biological transmission, some arboviruses occasionally may be transmitted mechanically by arthropods and other may be transmitted via throat secretions, milk or other excreta of the vertebrate host.

More than 530 viruses have been listed as arboviruses. Of these a few are not arthropod-borne but are antigenically related to known arboviruses. Vectors mainly responsible for transmission are mosquitoes, ticks, sandflies, midges and mites. Nearly 41 viruses have been isolated in India of which a few viruses of public health importance are Japanese Encephalitis (JE), Dengue, West Nile and Kyasanur forest disease (KFD) viruses.

12.1 PREVALENCE

Arboviruses are most prevalent in the tropical rain forest areas of different continents. It is due to the favourable climatic conditions and of the abundance in kind as well as in number of animal and arthropod species. As the arboviruses are maintained by cycles involving arthropods as well as vertebrates the tropics offer the most favourable conditions for these complex biologic cycles.

In India of the mosquito borne infections Japanese encephalitis has been responsible for outbreaks in most of the states with a case fatality rate of 6-60%. An antibody to JE has been detected in different species of animals. Dengue viruses have been responsible for many outbreaks in many cities and some rural areas in many parts of the country.

12.2 PATHOGENESIS

Arboviruses induce high titers of viremia in susceptible vertebrates 1-2 day after parenteral inoculation or following bites by infected arthropods; viremia persists for several days and serves as a source of infective blood meals for other biting arthropods. The primary site of virus replication is not known but is likely to be in the reticuloendothelial cells in lymph node, liver and spleen or endothelial cells of blood vessels. Release of virus from these sites may be associated with non-specific 'flu like' symptoms. During this period of viremia, usually at about 5 – 7 days after exposure, virus enters the target sites of the central nervous system, skin etc. There may be a brief period of relief after the initial symptoms i.e. before the onset of specific features. Thus, the presentation is a biphasic illness. Symptoms of encephalitis begin 7-10 days

after exposure to infection and persist 1 week or more, followed either by remission or by death. Wild birds and mammals regularly exhibit viremia without symptoms.

Antibodies are first detected when the fever subsides, usually within 2 days after the onset of encephalitis and persist for many years. Antibodies of the IgM class for may persist 1 – 7 weeks after infection, subsequently they are of IgG class.

It is important to note that although many subjects become infected with encephalitis viruses, relatively few develop illness manifested as meningitis or encephalitis. There is little information about the role of the immune system, although it may have a role in the pathogenesis of the dengue haemorrhagic shock syndrome, which is seen in young children who experience a second dengue virus infection. Antigen antibody complex formation has been thought to underlie the syndrome, which is associated with increased capillary permeability.

12.3 CLINICAL FEATURES

As might be expected with such a heterogeneous group of agents, clinical manifestations in humans are diverse & can be divided into fever (including fever with rash), encephalitis, haemorrhagic fever.

Fever

Little is known about the pathology of mild, undifferentiated fevers, because mortality is rare. The most common symptoms usually benign are characterized principally by mild, undifferentiated fevers with duration of 3 – 7 days. The onset is usually abrupt with fever, headache, and general malaise with vomiting or nausea and pain on moving the eyes-muscle or joint pains may be conspicuous; a macular or maculopapular rash or a series of rashes may be present. Leucopenia sometimes very marked, is quite common. For arboviruses like Chikungunya, Dengue and West Nile, fever is the major clinical symptom.

Encephalitis

The second most frequent symptom is encephalitis or meningoencephalitis. It typically comprises of the sudden onset of fever and headache, followed by neck stiffness, nausea or vomiting, drowsiness, and disorientation, frequently advancing into stupor or coma after an incubation period of 4 – 14 days. Convulsions may be an important feature of the acute illness. Rigidity or weakness of the limbs may occur, together with absent or irregular deep tendon reflexes and upgoing plantar reflexes. Symptoms are most severe 2 – 5 days after onset after which the patient may die or the fever and other symptoms may regress slowly during the next 2 – 3 weeks. During most outbreaks of arboviral encephalitis, a proportion of case develops aseptic meningitis only, without significant involvement.

Haemorrhagic Fever

A general and frequently massive erythrocytic diapedesis is seen in many skin lesions and other tissues. Edema and haemorrhage are usually conspicuous. A mild or severe thrombocytopenia is common. Most infections with hemorrhagic fever viruses result in mild fever rather than

severe diseases. Arboviruses that may cause hemorrhagic fever include Dengue, KFD and Yellow Fever (YF) etc.

Some of the medically important arboviruses

I. **Family _Togaviridae_**

    ▪ **Alphavirus**
    (i) _Encephalitis causing virus_

        ➢ Eastern Equine encephalitis
        ➢ Western Equine encephalitis
        ➢ Venezuelan Equine encephalitis
        ➢
    (ii) _Fever causing virus_
        ➢ chinkungunya
        ➢ Onyong-nyong
        ➢ Mayaro
        ➢ Sindbis
        ➢ Ross River

II. **Family _Flaviviridae_**

    ▪ **Flavivirus**
    a) **Mosquito borne**

    1. _Encephalitis virus_
        ➢ St. Louis encephalitis
        ➢ West Nile
        ➢ Japanese encephalitis
        ➢ Murray Valley encephalitis

    2. _Yellow fever virus_

    3. _Dengue virus types 1, 2, 3, 4_
    b) **Tick Borne**

    1. _Encephalitis virus_
        ➢ Russian spring summer encephalitis complex
        ➢ Louping III
        ➢
    2. _Haemorrhagic Fever_
        ➢ Kyasanur Forest Disease
        ➢ Omsk Haemorrhagic Fever

III. **Family _Bunyaviridae_**
    ➢ Bunyamwera Group
    ➢ Bwamba Group
    ➢ C Group
    ➢ California Group
    ➢ Simbu Group
    ➢ Turlock Group
    ➢ Crimean Congo Haemorrhagic Fever Group
        • CCHF
        • Hazara
    ➢ Phlebotomas Fever Group
        • Sandfly fever
        • Karimabad
    ➢ Nairobi sheep Disease Group
        • Nairobi Sheep Disease
        • Ganjam
    ➢ Phlebovirus group
        • Rift Valley Fever

IV. **Family _Reoviridae_**

    ▪ **Orbivirus**
    ➢ African Horse Sickness
    ➢ Colorado tick fever
    ➢ Playam
    ➢ Vellore

V. **Family _Rhabdoviridae_**
    ▪ **Vesiculovirus**
    ➢ Chandipura virus

12.4 TREATMENT

There are a few effective treatments for arbovirus infections. Treatment is often symptomatic such as administration of analgesics to reduce discomfort and antipyretics to reduce fever.

12.5 PREVENTION

Excellent vaccines are available for YF (live attenuated) and JE (inactivated) but there are no available vaccines for most arboviruses.

12.6 LABORATORY DIAGNOSIS

12.6.1 Collection and transport of specimen

Isolation of arboviruses from clinical or field collected specimen is difficult and frequently unproductive even when the appropriate specimens are obtained. Because of the lability of arboviruses proper collection, storage, shipment and processing of specimen to preserve virus infectivity are essential.

Specimen should be processed promptly after collection. They should be processed chilled in buffered isotonic diluent containing protein stabilizers. The specimen must be transported to laboratory frozen. If dry ice is used, the container should be tightly sealed to prevent infiltration of CO_2, which could inactivate the virus.

Arboviruses are only infrequently isolated from most patient specimen; infectious virus typically is no longer detectable by the time the patient seeks care. Viremia in most arboviral infections ceases 2 or 3 days after onset of symptoms. Antibody often complexed with antigen becomes demonstrable 4 or 5 days post onset. Therefore, an acute phase blood specimen for isolation should be collected immediately when arboviral aetiology is suspected to avoid the inhibitory effects of antibodies. Prompt specimen collection increases the probability for positive isolation.

Mosquito borne encephalitis viruses such as JE are rarely isolated from blood of patients. In contrast dengue viruses are frequently isolated from blood of patients during the first two days of illness and usually longer. In general isolates can be recovered from blood for several days to a week or more after onset for those viruses for which humans are the principal vertebrate host such as YF, dengue and chikungunya.

12.6.2 Samples for virus isolation and serological test

12.6.2.1 Human Specimen

1. Blood serum or Plasma-   Paired samples – one acute phase or immediately on reporting of case and second after 2-3 weeks of onset of fever.
2. Throat secretions
3. Cerebrospinal fluid
4. Brain in case of encephalitis
5. Other organs like spleen, lungs, liver etc.

12.6.2.2 Animal specimen

1. Blood
2. Tissues

12.6.2.3 Isolation of viruses

Arboviruses can be isolated by the use of animals, cell cultures or mosquitoes as below:

Animal inoculation
1. Suckling mice – by intracerebral and intraperitoneal routes
2. Adult mice
3. Other animals: Hamster, Click embryo etc.

To check for sickness in mice and confirm by immunofluoresence or haemagglutination (HA) & haemagglutination inhibition (HAI) test etc. or check for presence of antibodies in surviving mice.

Cell Cultures
1. Vertebrate cell lines – Vero, BHK-21, LLC-MK$_2$
   Primary cells-Hamster kidney, chick embryo, duck embryo etc.

2. Invertebrate cell lines - $C_{6/36}$
   To check for cytopathic effect, plaque formation and immunofluorescene etc.

Mosquito inoculation by i.c. and intrathoracic route.
- Confirm by immunofluorescence & HAI etc
- Strain confirmation by use of monoclonal antibodies e.g. Dengue

12.6.3 Detection of antigen in tissues by
   i) Immunofluorescene
   ii) Enzyme immunoassay
   iii) Polymerase chain reaction

12.6.4 Identification of virus

In addition to study of physical and chemical properties following tests are used in identification of virus:
   i. Haemagglutination & Haemagglutination inhibition test
   ii. Complement Fixation Test
   iii. Neutralization Test
       a. Mice – protection
       b. Plaque reduction in tissue culture
   iv. Immunofluorescence
   v. Enzymeimmunoassay
   vi. Molecular Techniques – Polymerase Chain Reaction

12.6.5   Serological Tests

Presence of antibodies to arboviruses can be detected by the following techniques:

i   Haemagglutination inhibition Test
ii.   Complement Fixation (CF) Test
iii.   Neutralization Test
iv.   Immunofluorescene Test
v.   Enzyme immunoassay

Above serological tests are briefly described as follows:

12.6.5.1   Haemagglutination and Haemagglutination Inhibition Test

Arboviruses have the property of agglutinating goose erythrocytes at different pH. This property is made use of in identification of an isolate: method of Clarke and Casals is used for preparation of antigens. Antibodies to a particular virus inhibit the HA.   This can be a monospecific reaction or group specific depending on the days post infection for collection of sample or cross reactivity in viruses with close antigenic relationship. The sera used in HI Test are treated to remove non-specific antibodies using Kaolin or acetone.A four fold or more rise in paired sera is considered a recent infection due to the virus for which the antigen has been used in the test. Secondary response i.e. high titres may be present in a patient with previous exposure to antigenically related viruses e.g. JE, WN or Dengue.

12.6.5.2   Complement Fixation Test

The CF Test may be used to identify an isolate by testing the unknown agent against a number of specific antisera or to establish a tentative diagnosis in the absence of virus isolation by screening acute and convalescent specimen for rise in antibody against several viruses considered to be likely etiological agents. CF test is less specific than the neutralization test and is frequently group specific.   A fourfold or greater difference in titre between acute and convalescent phase sera is considered diagnostic.

12.6.5.3   Neutralization test

Antibodies against a particular virus or group of arboviruses or present in a person coming in contact with such viruses can neutralize the virus.   The test is useful in demonstration of antibodies to a particular virus when virus used is a known virus or for identification virus when antisera are known.   The neutralization test can be performed using different assay systems a) animal e.g. suckling mice b) cell cultures i.e. demonstration of plaque reduction.

12.6.5.4   Immunofluorescence test

The tissues taken from patients or inoculated animals can be tested for the presence of antigen by specific conjugates; antibodies assessment can be made using cell infected with known viruses.

12.6.5.5   Enzyme immunoassays

Presence of IgG & IgM antibody or antigen can be detected by immobilizing a particular antibody on a solid phase and then capturing specific antigen or antibody. The reaction is made visible by use of anenzyme and its specific substrate.Most commonly employed test for establishing an infection is IgM capture ELISA used for JE, WN and Dengue viruses, which can detect IgM antibodies in both CSF and blood.

12.6.5.6   Polymerase chain reaction (PCR)

PCR can be used to detect many arnboviruses, provided at least part of the gene sequence is known for the virus under consideration. PCR is used to select a segment of the viral gene sequence, reverse transcribe it with reverse transcriptase, amplify the resulting complementary DNA (cDNA) and detect the amplified product.

12.6.5.7   Transport medium
Transport medium for virological specimen pH 7.2

Hanks balanced salt solution (HBSS)	87 ml
10 percent Bovine albumin solution	10ml
$NaHCO_3$ (4.4 percent)	2ml
Pencillin sodium	10,000 units
Streptomycin Sulphate	10 mg
Mycostain	2.5 mg

50 percent Phosphate Buffered Glycerol Saline

NaC1	8.00 gm
KC1	0.20 gm
KH_2PO_4	0.12 gm
Na_2HPO_4 (anhydrous)	0.91 gm
Add DDW to make it one litre pH	7.5

Mix with equal quantity of analytical grade glycerol
Sterlize at 18 lbs pressure for 30 minutes.

12.7   ARBOVIRUSES OF PUBLIC HEALTH IMPORTANCE

Some of the arboviruses of public health importance in India viz. Dengue fever, Japansese Encephalitis and Kyasanur Forest Disease are discussed in detail.

12.8   DENGUE FEVER AND DENGUE HAEMORRHAGIC FEVER

Dengue fever is the most important mosquito spread viral disease and a major international public health concern. Dengue fever is a self limiting disease found in tropical and sub-tropical regions around the world, predominantly in urban and semi-urban areas. Dengue Haemorrhagic Fever (DHF), a potentially lethal complication, was first recognized in 1950s during the dengue epidemic in Philippines and Thailand but today DHF affects most Asian countries and is a leading cause of childhood deaths. In India, first major outbreak associated with haemorrhagic manifestation occurred in Calcutta in 1963. Since then there has been a dramatic rise in the incidence of DHF cases.

12.8.1 Causative agent

DF/DHF is caused by dengue virus which belongs to genus Flavivirus family Flaviviridae and includes serotypes 1, 2, 3 and 4 (Den-1, Den-2, Den-3 and Den-4). When a person has had classic dengue (i.e. infection by one serotype), a second infection later by another serotype increases the likelihood of suffering from DHF.

12.8.2 Epidemiology

Geographical distribution

The global prevalence of dengue has grown significantly in recent decades. The disease is now endemic in more than 100 countries in South-east Asia, Western Pacific, Eastern Mediterranean, Africa, the Americas. South-east Asia and Western Pacific are most seriously affected. Before 1970 only nine countries had experienced DHF epidemic, a number that had increased more than four-fold by 1995.

Some 25000 lakhs people are now at risk from dengue. WHO currently estimates there may be 500 lakhs cases of dengue infection worldwide every year with around 24,000 deaths. During 1996, Delhi had a large outbreak of dengue and dengue haemorrhagic fever when 10,252 cases and 423 deaths were reported to the State Directorate. There was a decline in the number of cases.

Situation in India

India is also endemic for Dengue Fever (DF) and Dengue Haemorrhagic Fever (DHF). Every year cases of DF and/or DHF are reported. In 1996, there was a large outbreak of DF and DHF. Cases and deaths were reported from various parts of the country viz. Delhi 10,252 cases and 423 deaths, Haryana 1631 cases and 54 deaths and Maharashtra 3068 cases and 5 deaths. In total, 16517 cases and 545 deaths were reported from all over the country. Again in the year 2003 an outbreak of DF and DHF was reported from various parts of the country especially Delhi, Kerala, Karnataka, Punjab, Tamil Nadu, Uttar Pradesh & Maharashtra. In the past decade many outbreaks have been reported from different parts of the country (Table 12.1).

Table 12.1 Dengue Cases and Deaths in the Country since 2010 (C=Cases | D=Deaths)

Sl. No.	Affected States/UTs	2010		2011		2012		2013		2014		2015(Prov.)		2016*	
		C	D	C	D	C	D	C	D	C	D	C	D	C	D
1	Andhra Pradesh	776	3	1209	6	2299	2	910	1	1262	5	3159	2	212	0
2	Arunachal Pradesh	0	0	0	0	346	0	0	0	27	00	1933	1	0	0
3	Assam	237	2	0	0	1058	5	4526	2	85	0	1076	1	38	0
4	Bihar	510	0	21	0	872	3	1246	5	297	0	1771	0	3	0
5	Chattisgarh	4	0	313	11	45	0	83	2	440	9	384	1	53	0
6	Goa	242	0	26	0	39	0	198	2	168	1	293	0	53	0
7	Gujarat	2568	1	1693	9	3067	6	6272	15	2320	3	5590	9	621	0
8	Haryana	866	20	267	3	768	2	1784	5	214	2	9921	13	3	0
9	Himachal Pradesh	3	0	0	0	73	0	89	2	2	0	19	1	0	0
10	J & K	0	0	3	0	17	1	1837	3	1	0	153	0	0	0
11	Jharkhand	27	0	36	0	42	0	161	0	36	0	102	0	3	0
12	Karnataka	2285	7	405	5	3924	21	6408	12	3358	2	5077	9	1664	0
13	Kerala	2597	17	1304	10	4172	15	7938	29	2575	11	4075	25	2753	5
14	Madhya Pradesh	175	1	50	0	239	6	1255	9	2131	13	2108	8	131	0
15	Meghalaya	1	0	0	0	27	2	43	0	0	0	13	0	0	0
16	Maharashtra	1489	5	1138	25	2931	59	5610	48	8573	54	4936	23	973	2
17	Manipur	7	0	220	0	6	0	9	0	0	0	52	0	0	0
18	Mizoram	0	0	0	0	6	0	7	0	19	0	43	0	1	0
19	Nagaland	0	0	3	0	0	0	0	0	0	0	21	1	0	0
20	Orissa	29	5	1816	33	2255	6	7132	6	6433	9	2450	2	75	0
21	Punjab	4012	15	3921	33	770	9	4117	25	472	8	14128	18	10	0
22	Rajasthan	1823	9	1072	4	1295	10	4413	10	1243	7	4043	7	73	0
23	Sikkim	0	0	2	0	2	0	38	0	5	0	21	0	2	0
24	Tamil Nadu	2051	8	2501	9	12826	66	6122	0	2804	3	4535	12	982	3
25	Tripura	0	0	0	0	9	0	8	0	6	0	40	0	3	0
26	Telangana	0	0	0	0	0	0	0	0	704	1	1831	2	137	0
27	Uttar Pradesh	960	8	155	5	342	4	1414	5	200	0	2892	9	2	0
28	Uttrakhand	178	0	454	5	110	2	54	0	106	0	1655	1	0	0
29	West Bengal	805	1	510	0	6456	11	5920	6	3934	4	8516	14	257	0
30	A& N Island	25	0	6	0	24	0	67	0	139	0	153	0	21	0
31	Chandigarh	221	0	73	0	351	2	107	0	13	0	966	1	2	0
32	Delhi	6259	8	1131	8	2093	4	5574	6	995	3	15867	60	28	0
33	D&N Haveli	46	0	68	0	156	1	190	0	641	1	1154	0	15	0
34	Daman & Diu	0	0	0	0	96	0	61	0	46	0	165	0	6	0
35	Puduchery	96	0	463	3	3506	5	2215	0	1322	1	771	0	186	0
	Total	28292	110	18860	169	50222	242	75808	193	40571	137	99913	220	8307	10

* Provisional till 28th June 2016. (Available at: http://www.nvbdcp.gov.in/den-cd.html, cited on 19th July 2016.

Transmission

The infection is transmitted by the bite of an infected female mosquito – *Aedes Aegypti*. Mosquitoes generally acquire the virus while feeding on the blood of an infected person. After virus incubation for 8 – 10 days, an infected mosquito is capable of transmitting the virus to susceptible individuals for the rest of its life (i.e. 3 weeks).

Humans are the main amplifying host of the virus, although studies have shown that in some parts of the world monkeys may become infected and perhaps serve as a source of virus for uninfected. The virus circulates in the blood of infected humans for two to seven days.

Vectors of transmission

Aedes Aegypti is the main vector of dengue transmission in India. Dengue outbreaks have also been attributed to *Aedes albopictus*. The mosquito is a domestic breeder. Mosquito breeding can occur in any water-catching or water-storage containers, such as desert coolers, over head tanks, discarded buckets, tyres, utensils and large containers used for collecting rain water which are not emptied and cleaned periodically. The mosquitoes rest indoors, in closets and other dark places. Outside, they rest where it is cool and shady. *Aedes* mosquito can fly upto a limited distance of 400 metres but can spread over vast distances mechanically in various types of vehicles used by man. The outbreaks of DF/DHF are most likely to occur in post-monsoon period when the breeding of the mosquitoes is highest.

High risk areas

Usually urban areas, having high population density, poor sanitation and large number of desert coolers, overhead tanks, discarded buckets, tyres, utensils etc. which promote mosquito breeding, are at high risk. Dengue fever/DHF can also occur in rural areas where the environment is friendly for mosquito breeding like storage water for cattle feeding and drinking, discarded tins, tyre, bottles etc. which are not emptied and changed periodically.

12.8.3 Clinical manifestations

The incubation period of dengue fever is usually 5 – 6 days, but may vary from 3 to 10 days. Dengue fever is a severe form like illness that affects infants, young children and adults, but seldom causes death. The clinical features of dengue fever vary according to the age of the patient. Infants and young children may have a non-specific febrile illness with rash. Older children and adults may have either a mild febrile illness or classical disease with abrupt onset of high fever, severe headache, muscle and joint pain and rash.

DHF is a potentially deadly complication that is characterized by high fever, accompanied by headache, anorexia, vomiting and abdominal pain. A haemorrhagic diathesis is commonly demonstrated by scattered fine petechiae on the extremities, face, trunk and in the axillae. A positive tourniquet test is always present. Bleeding from nose, gums and gastrointestinal tract are also seen. The liver is usually enlarged, soft and tender.In moderate DHF cases, all signs and symptoms abate after the fever subsides. In severe cases patient's condition may suddenly deteriorate after a few days of fever, varying degree of circulatory disturbances occur and the patient may rapidly go into a critical state of shock (Dengue Shock Syndrome) and die within 12 – 24 hours, or quickly recover following appropriate volume replacement therapy. Without proper treatment, DHF case fatality rate can exceed 20%. With modern intensive supportive therapy such rate can be reduced to less than 1%. A person with dengue should never take aspirin as it may increase his tendency to bleed.

Danger signs in Dengue
- ➤ Minute spots on the skin suggesting bleeding within the skin
- ➤ Nose bleeds and gum bleeds
- ➤ Abdominal pains or passage of black and tan like stool
- ➤ Refusal to food or drink
- ➤ Abnormal behaviour or drowsiness
- ➤ Difficulty in breathing or cold hands and feet, reduced amount of urine being passed

12.8.4 Laboratory diagnosis

Serological diagnosis

The diagnosis of DF/DHF can be confirmed by serological tests. The tests include detection of IgM antibodies which appear around the end of first week of onset of symptoms and are detectable for 1 – 3 months after the acute episode. Demonstration of IgM antibody or a rising titre of IgG antibody in paired sera taken at an interval of ten days or more is confirmatory. High IgG antibodies early in the course of illness indicate previous infection and are useful for conducting sero epidemiological studies to determine the extent of silent infection and immunity levels in the local population.

Isolation/detection of virus/antigen

Virus isolation can be done by inoculation in suckling mice, tissue culture or mosquitoes and further identification by using fluorescent antibody test or other test.Viral genomic sequences can be detected in autopsy tissue, serum or CSF sample by using PCR (Polymerase Chain Reaction).

12.8.5 Treatment

Treatment of dengue fever is symptomatic with bed rest, antipyretic and analgesic. ORS solution are recommended for patient with excessive sweating, nausea, vomiting, or diarrhoea to prevent dehydration. Management of DHF: Management during febrile phase is similar to DF with antipyretic and analgesic. Besides this fluid and electrolyte replacement by IV fluids, plasma expanders, if clinically indicated results in favourable outcomes. In some cases fresh frozen plasma is indicated and in rare cases like patient with severe shock or massive bleeding, blood transfusion is required. Amount of fluid given should be constantly monitored. Any evidence of swelling, shortness of breath or puffiness may indicate fluid overload. Adoption of appropriate standardized clinical management practices can effectively reduce DHF case fatality rates.

12.8.6 Vaccine

No effective vaccine is available for dengue. Research into dengue vaccines focuses on the use of live attenuated or inactivated vaccines, infectious clone-derived vaccines, immunogens vector by various recombinant systems, sub unit immunogens and nucleic acid vaccine.Among these intensive and stringent laboratory studies conducted for live attenuated tetravalent vaccine in Thailand. This vaccine was evaluated in animal models and phase-1 clinical trial of this vaccine was recently completed in Thailand. After two doses, sero conversion to all four serotypes was

demonstrated in most vaccinated volunteers and antiviral activity remained quite stable for at least a year.

In order to promote the evaluation of live attenuated vaccines in clinical trials, a group of WHO experts has been developing guidelines for the safety of dengue vaccine. These guidelines could help public health officials to make decisions about conducting dengue vaccine trials in their countries.

12.8.7 Surveillance in DF/DHF

Surveillance is a pre-requisite for monitoring the dengue situation in the area and should be carried out regularly for early detection of an impending outbreak and to initiate timely preventive and control measures. Surveillance should include epidemiological, entomological and laboratory parameters.

12.8.8 Prevention and control of DF/DHF

The only method of controlling or preventing dengue fever and DHF is to combat the vector mosquitoes. In India, *Aedes Aegypti* breeds primarily in man made container like water cooler, earthenware jars, concrete cisterns used for domestic water storage, discarded plastic food containers, used automobile tyres and other items that collect rain water.Vector control can be implemented using environmental management and chemical methods. Proper solid waste disposal and improved water storage practices, including covering containers can prevent access by egg-laying female mosquitoes. These methods should be encouraged through community-based programmes.Chemical methods of control include application of appropriate insecticides to larval habitats, particularly those considered useful by householders, e.g., water-storage vessels. These prevent mosquito breeding for several weeks but must be re-applied periodically. During outbreaks, emergency control measures may also include the application of insecticides as space spray to kill adult mosquitoes using portable or truck mounted machine.However, the killing effect is only transient and variable in its effectiveness because the aerosol droplets may not penetrate indoor to microhabitats where adult mosquitoes are sequestered. Regular monitoring of vector's susceptibility to the most widely used insecticides is necessary to ensure the appropriate choice of chemicals. In biological method of control, larvivorous mosquito–eating fish, dragon fly larvae, copepods (mesocyclops), peppermint oil and a fungus lagenidium gigantum have been used with some success.Active monitoring and surveillance of the natural mosquito population should accompany control efforts in order to determine the impact of the dengue control programme.

12.8.9 Case definition of Dengue fever and Dengue Haemorrhagic Fever (DHF)

Case definition of Dengue fever
The clinical case description of Dengue fever is an acute febrile illness of 2-7 days duration with 2 or more of the following :
Headache, retro-orbital pain, myalgia, arthralgia, rash, haemorrhagic manifestation and leucopenia.
Suspect case: A case compatible with the clinical description

Probable case: A case compatible with clinical description with one or more of the following:
- Supportive serology
- Presence of confirmed case in the area during the same period

Confirmed case: A case compatible with clinical description and laboratory confirmed

Case definition of DHF

A probable or confirmed case of dengue fever with haemorrhagic tendencies evidenced by one or more of the following:

- Positive tourniquet test
- Petechiae, ecchymoses or purpura
- Bleeding from buccal mucosa, gastrointestinal tract, injection site or others
- Haematemesis, malaena
- Signs of plasma leakage (pleural effusion, ascites, hypoproteinaemis)

12.8.9 Laboratory Diagnosis of Dengue Fever/DHF

Hematological criteria for diagnosis

Thrombocytopenia (100,000 cells or less per mm^3)
Haemoconcentration (>20% rise in average haematrocrit for age and sex)

Microbiological diagnosis

A definite diagnosis of dengue infection can be made by:
i.   Isolation of the virus
ii. Demonstration of viral antigen or RNA in the tissue or serum
iii. Demonstration of IgM antibodies or a rising titre of IgG antibodies in paired sera against dengue virus i.e. serological diagnosis
iv. Molecular diagnosis (PCR) & genotyping.

(i)   Isolation of the virus

Virus isolation can be done by inoculation of clinical material in suckling mice, tissue culture or mosquitoes and further detection is performed using fluorescent antibody test or haemagglutination inhibition test.

(ii)   Demonstration of virus antigen or RNA

Viral antigen can be demonstrated by doing direct fluorescent antibody test using specific monoclonal antibodies for dengue virus.
Viral RNA or genomic sequence can also be detected in autopsy specimen, serum, CSF or culture supernatant by doing polymerase chain reaction and gene sequencing.

(iii)   Serological diagnosis

Detection of IgM antibodies: IgM antibodies against dengue virus appears around 5 days after onset of symptoms and are detectable for 1- 3 months after the acute episode. The tests employed are IgM capture ELISA test and Rapid IgM strip test. IgM capture ELISA test kit is available from NIV Pune and commercial sources and Rapid IgM Strip Test kit is available commercially.

Detection of IgG antibodies: IgG antibodies appear later than IgM antibodies in primary infection of dengue and persist at high level for 30 – 40 days before declining to levels found in past infection and persist for life. Detection of four fold or greater increase/fall in IgG titre in paired serum samples taken at an interval of 10 – 14 days confirms the diagnosis of dengue. Test employed are IgG ELISA for dengue and Haemagglutination Inhibition (HI) Test.

Collection, storage and transportation of samples for Dengue and DHF
Proper collection, processing, storage and transportation of the specimens is an essential aspect of the laboratory diagnosis.

Collection of samples for serology

Sample- Blood in plain vial/Serum

Time of collection
1st Sample: 5 days after onset of illness for IgM detection as these antibodies appear at this time
2nd Sample: At least 7 to 14 days after the first sample or, in the event of a fatality, at the time of death.

Collection of samples for isolation & molecular diagnosis

Samples-   Serum
                Plasma
                Whole blood (washed buffy coat)
 Autopsy tissues - liver, spleen, lymph nodes & thymus
Mosquitoes collected in nature
Time of collection- Within first five days of illness

Blood collection in tubes or vials
➢ Aseptically collect 4-5 ml of venous blood.
➢ Allow blood to clot at room temprature, centrifuge at 2000 rpm to separate serum. Collect the serum in clean dry vial.
➢ Fix the cap with adhesive tape, wax or other sealing material to prevent leakage during transport.
➢ Use adhesive tape marked with pencil, indelible ink, or a typewritten self adhesive label to identify the container. The name of the patient, identification number and date of collection must be indicated on the label.
➢ All clincal samples should accompany the clinical information as per proforma. (Annexure)

Transportation of samples

Transport specimens to the laboratory at $2 - 8\ ^0C$ (ice box) as soon as possible. Do not freeze whole blood, as haemolysis may interfere with serology test results.

If more than 24-hour delay is expected before specimens can be submitted to the laboratory, the serum should be separated from the red blood cells and stored at refrigerated temperature. Samples for isolation and molecular diagnosis should always be stored frozen.

12.8.10 Biosafety

Dengue virus is a Group 2 pathogen and hence all tests can be performed in BSL-2 laboratories. Requirements of BSL – 2 laboratory are as follows:

- Limited access
- Handwashing facilities
- Laboratory coats
- Gloves
- Autoclave for sterilization of waste
- Biohazard sign at the entarance

Dengue has become a major international public health concern in recent years. Dengue/ DHF is widely prevalent in India and all four dengue serotypes are known to exist. Dengue infections have the potential of rapid spread resulting in an acute public health problem. Therefore, special attention is required to be paid for its surveillance, prevention and control. Health authorities should take appropriate and anticipatory actions to prevent DF/DHF outbreak and should it occur, they must be geared up and be in a state of epidemic preparedness to minimize the impact of outbreak in terms of morbidity and mortality.

12.9 JAPANESE ENCEPHALITIS

Japanese Encephalitis (JE) is a zoonotic viral disease caused by group B arbovirus (flavivirus), involving the Central Nervous System. In nature, the virus is maintained in animal and birds, particularly pigs and ardied birds (e.g. cattle egrets, pond herons etc.). Although infection in human is incidental, the virus can cause serious neurological disease in human. The majority of infections are not apparent and only 1 in 300 to 1 in 1000 infections result in symptomatic illness. The disease occurs with sudden onset and the common symptoms are headache, high fever, stiff neck, abnormal movements (coarse tremor, convulsions in children), impaired consciousness and coma. Case fatality rate in JE is high, ranging from 20-50%.

JE occurs in a large number of countries/area of Asia. It is a disease of public health importance because of its epidemic potential and high case fatality rate. In patient who survive, complications may lead to life long sequelae.

12.9.1 Causative agent

The disease is caused by JE virus, which belongs to genus Flavivirus and family Flaviviridae.

12.9.2 Epidemiology

Geographical distribution

JE occurring in a large number of countries of Asia, including Cambodia, China, Indonesia, Japan, Laos, Malaysia, Myanmar, Philippines, Korea, Thailand, Vietnam, South-eastern Russian Federation and the Indian subcontinent. In recent decades, JE has gradually spread to previously non-affected Asian regions.

Occurrence of JE in India

In India, JE was first recorded in Vellore and Pudducherry in mid 1950s. The first major outbreak of JE occurred in 1973 in Bankura & Burdwan districts of West Bengal. In 1976, wide spread outbreaks were reported from Andhra Pradesh, Assam, Karnataka, Tamil Nadu, Uttar Pradesh and West Bengal. In 1978 JE cases were reported from 21 states and Union Territories. The Directorate of NAMP is monitoring JE incidence in the country since 1978. The worst ever recorded outbreak in India was reported from Uttar Pradesh during 1988 when 4485 cases with 1413 deaths were recorded from eight districts with case fatality rate of 31.5%. The highly affected states include Andhra Pradesh, Assam, Bihar, Goa, Karnataka, Manipur, Tamil Nadu, Uttar Pradesh and West Bengal. Outbreaks of JE usually coincide with monsoons and post-monsoon period when the vector density is high. However, in endemic areas, sporadic cases may occur throughout the year. Case fatality rate in newly affected areas ranged from 10 – 70%. However, with early detection and management of cases it has come down to an average of approximately 20%.

Transmission

The infection is transmitted through the bite of an infected culicine mosquito. The transmission cycle is maintained in animals and birds. Infection in man is the dead end of the transmission. Man to man transmission has not been documented.

Reservoir of infection

JE virus has its natural cycle in vertebrates and mosquitoes. The animal hosts include pigs, cattle and horses and amongst birds are the water birds e.g. pondsherons, cattle egrets, poultry birds and ducks play a significant role in the natural history of JE virus. Pigs are the major vertebrate hosts and are considered as amplifying hosts. Currently available evidences does not indicate major role of cattle and horses. Infection in man appears to be correlated with living in close proximity with animal reservoirs, especially pigs.

In India, birds particularly those belonging to family Ardeidae and pigs play important role in maintenance of JE virus in nature. Various studies conducted on detection of the presence of JE antibodies in the sera of birds belonging to different species have indicated that *Ardeola grayii* (pond heron) and *Bubulcus ibis* (cattle egret) play a definite role in maintenance of JE virus in nature. In different parts of the country, 12 to 44 per cent pig population has been found to be positive for JE antibodies particularly in JE endemic areas. Besides birds and pigs, bovines and

bats have also been found positive for JE antibodies but their role in maintenance of virus in nature is doubtful as the titres found in them are very low.

Vectors

Mosquitoes belonging to Culex vishnui group are most important vector species in India. 11 more species of mosquito have been incriminated as vector of JE. Culex mosquitoes generally breed in water bodies with luxurious vegetation like irrigated rice fields, shallow ditches and pools. Mosquitoes are zoophilic, feeding primarily on animal and wild birds. Epidemics usually coincide with monsoons and post-monsoon period when the vector density is high. Female mosquitoes get infected after feeding on a viraemic host and can transmit the virus to other hosts after an extrensic incubation period of 9 to 12 days.

In India, JE virus was first isolated from wild caught mosquito species at Vellore in 1956. Since then the virus has been isolated from 12 mosquito species in wild caught specimen from different parts of the country. Maximum isolations have been made from Culex vishnui group consisting of C.tritaeniorhynchus, C.vishnui and C.pseudovishnui.

Age and Sex Distribution

In various outbreak studies, majority of the cases were in younger age groups, although all age groups were affected. In areas where disease has become endemic, cases are mainly reported from age groups below 15 years. Various epidemiological studies conducted during investigation of outbreaks, observed that though both sexes are affected, males outnumber females.

12.9.3 Clinical manifestations

The incubation period in man, following mosquito bite varies from 5 to 15 days. The clinical features of JE are those of encephalitis. The patient will give a history of acute onset with fever and change in behaviour or sensorium lasting for more than 24 hours. Focal neurological deficits may or may not be present. In majority of the cases, however, the infection is mild with no overt clinical symptoms or mild fever with headache. Clinical laboratory finding in acute encephalitic stage shows moderate to high polymorph leucocytosis with neutrophilia (differential count 70% or greater). CSF findings are variable: fluid pressure is normal to mildly elevated, mononuclear cell pleocytosis is the rule, CSF glucose is normal to moderately elevated. Case fatality rate is high i.e. 20 to 40% in severe cases. Patient who recover from the acute episode may have neurological sequelae viz. mental impairment, severe emotional instability, personality changes, paralysis etc.

12.9.4 Case definition

Clinical case description

Japanese Encephalitis virus infection may result in a febrile illness of variable severity associated with neurological symptoms ranging from headache to meningitis or encephalitis. Symptoms can include:

Headache, fever, meningeal signs, stupor, disorientation, coma, tremors, paresis (generalised), hypertonia, loss of co-ordination. The encephalitis cannot be distinguished clinically from other central nervous system infections.

Suspect case
- High grade fever of acute onset with at least two of the following:
- Decrease in level of consciousness independent of convulsions
- Significant change in mental status either in behaviour or personality
- Convulsions

Probable case
- Suspected case of Japanese encephalitis, and
- Usually not more than a few cases (1-2) in one village
- Presence of animal hosts and high density of vector

Confirmed case
- IgM antibody in serum and/or CSF
- Rising IgG antibody titre in paired sera

12.9.5 Laboratory diagnosis of JE

Laboratory diagnosis of JE is done by following methods:
A.   Detection/isolation of antigen/virus
(i)   Demonstration of viral antigen in the autopsied brain tissue by the fluorescent antibody test
(ii)   Isolation and identification of the virus from CSF, occasionally from peripheral blood (within 3 to 4 days after onset of symptoms) or autopsied brain tissue.

B.   Detection of antibody
The detection of antibodies to JE virus can be done routinely by Haemagglutination Inhibition Test (HI) test, IgM Capture ELISA test. The antigen and reagents for both the tests are available from National Institute of Virology, Pune.

Collection, storage and transportation of specimens for Japanese Encephalitis
The laboratory diagnosis of JE depends upon the proper collection of clinical material from human cases. In epidemic situations it becomes necessary to collect vector mosquitoes also for isolation of JE virus.

I. Collection of specimens from human cases

1.   Serum : Serum specimens should be collected from suspected JE cases as early in the acute phase as possible after admission to hospital or attendance at clinic. A second, convalescent specimen should be collected on discharge, or 14-21 days after onset of the disease. Collect 5 ml of venous blood under all aseptic conditions. Keep it at room temperature for about 15 minutes to enable it to clot and then at +40C in the refrigerator to allow the clot to retract. Separate the serum from the clot and transfer to a screw capped sterile container. Seal the container with adhesive tape and label the container with appropriate information viz. patient's name, identification number, date of collection etc. Place the serum in refrigerator if there is delay in transportation to laboratory.

2.   Cerebrospinal fluid (CSF) : Collect CSF specimen in sterile screw capped bottles under all aseptic precautions. Label these containers properly and store as described from serum.

3.   Brain Tissue : Obtain brain tissue within hours of patient's death during the first two weeks of illness. It is the best source for the isolation of virus. Collect small pieces of tissue from different parts of the brain-cerebral cortex, cerebellum, basal nuclei and brain stem. Immerse the brain tissue (s) thus obtained in 2ml of virus transport medium in sterile screw capped bottles. If transport medium is not available, glycerol-saline may be used. Alternatively, nutrient broth medium with antibiotics can be used.

II Storage and Transport

Place the specimens at +40C as soon as possible after collection. Do not freeze the specimens. Despatch these at the earliest possible opportunity on wet ice in a large thermos or an ice-box to the central laboratory. These can either be air-freighted or sent by rail through a special courier. The courier should drain the water and replenish ice as and when required during the journey. Every specimen must be accompanied by the pertinent information as shown in annexure-1.

III Collection of vectors

In an epidemic situation, it is desirable to collect mosquitoes from the affected areas-both indoor and outdoor, so that they may be processed for virus isolation. This may give an indication of the species acting as vector and also provide some information on the mosquito fauna of the area. Mosquitoes an be collected by standard method such as aspirator, baited traps, biting collections and light traps. The mosquitoes should be held alive in 'Barraud Cages' wrapped with moistened lint or cloth. If the collection locality is not far from the laboratory or transportation can be done within a day or two, they may be transported alive in Barraud cages. For such transportation, it is necessary to provide raisins soaked in water or a cotton pledget soaked in 10 percent solution inside the Barraud cage.

If the collection locality is far from the laboratory and immediate transportation is not possible, mosquitoes may be identified, pooled species wise and stored in liquid nitrogen, refrigerators or on dry ice for subsequent transportation to the laboratory. If facilities for liquid nitrogen or dry ice storage are not available in the field, transport medium may be used to store the mosquito pools. It is, however, necessary that such pools are constantly kept in the refrigerator or transported on wet ice.

12.9.6 Treatment

There is no specific treatment of JE. However, supportive treatment and good nursing care can significantly reduce case fatality rate. It is, therefore, important that encephalitis cases should be referred to a hospital as early as possible so that treatment is started without waiting for serological laboratory results. In the acute phase, clinical management is directed at maintaining fluid and electrolyte balance. Keeping the airway open in a comatose patient, if required oxygen may be given. Appropriate drugs for convulsions may be prescribed.

12.9.7 Prevention and control of JE

a) Surveillance

The component of JE surveillance consists of three major areas: (1) Sero-surveillance to delineate high risk population groups and to monitor JE specific antibodies in sentinel animals or birds as an indication of increasing viral activity (2) Vector surveillance in JE prone areas for monitoring vector behaviour and population build up for timely implementation of intervention methods (3) clinical surveillance through PHC system for early diagnosis and proper management of JE patients.

b) Control

(i) Interruption of Transmission

Prevention of transmission is possible through vector control. For effective control of vectors, residual insecticidal spraying has been suggested in all animal dwellings with appropriate insecticide before the onset of transmission season.

(ii) Vaccination

Three types of vaccine against JE is presently produced and used worldwide. Inactivated mouse brain (Japan, Korea, Taiwan, Thailand, Vietnam, PR China, India), Inactivated and live attenuated primary hamster kidney cells are manufactured in China. However JE vaccine produced in mouse brain is distributed commercially and available internationally.

In most areas of Asia, the mouse brain vaccine produced from the Nakayama strain is given subcutaneously in 2 doses of 0.5 ml (1.0 ml for people > 3 years) 1 to 4 weeks apart with a booster dose at 1 year and additional booster doses thereafter at 1 to 3 year intervals.

(iii) Management of cases

There is no specific curative therapy for JE patients. The cases are managed symptomatically and early diagnosis with proper and adequate management helps in reducing the associated fatality and neurological sequelae.

(iv) Health Education and Community involvement

It has been observed that there is a direct relationship between the time lag in onset of symptoms and initiation of therapy. Immediate management of cases reduces fatality to a considerable extent. Since the disease is predominantly prevalent in rural areas, generating awareness helps in early reporting. Further health education helps in encouraging personnel protection.

12.9.8 Laboratories undertaking work on JE in India

1. National Institute of Virology, Ambedkar Road, Pune
2. National Centre for Disease Control, 22-Sham Nath Marg, Delhi
3. Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raibareily road, Lucknow
4. King George's Medical College, Lucknow
5. Armed Forces Medical College, Pune
6. School of Tropical Medicine, 110 Chittaranjan Avenue, Kolkata
7. Veterinary Biological Institute, Hyderabad
8. King Institute of Preventive Medicine, Chennai

12.10   KYASANUR FOREST DISEASE

12.10.1 Introduction

In early 1957, a disease broke-out in Sagar-Sorab area and was provisionally diagnosed as "enteric fever." The zoonotic nature was apparent because it was, from the beginning, associated with the death of monkeys in the area. (Often called "monkey disease" by the villagers). Intensive investigations established the aetiology to be an arbovirus transmitted through ticks. Both the virus and the disease were named after the Kyasanur forest area in Sagar taluk of Shimoga district of Karnataka State.

12.10.2   Epidemiology

The disease is restricted to some disricts in Karnataka State, India. Districts of Shimoga, Chikkamagalore, Ukannada, Dkannada and Udupi report human and monkey deaths suspected to be due KFD. In 2004, 568 suspected cases of KFD, 4 human deaths and 86 monkey deaths have been reported from these districts. The epidemic patterns indicate irregular and unpredictable spread of the virus. When first recognized in 1957, it was active in an area of about 800 sq km. The total area now infected is perhaps more than 4,000 sq km. Serological surveys in different parts of India revealed antibodies to KFD or a closely related virus in humans and domestic animals, particularly in cattle, mainly in Kutch and Saurashtra. Enquiries have not uncovered any reports/evidence of the disease either in humans or animals in this area. These areas are arid and totally different from the KFD endemic/epizootic areas which receive heavy rains. The fauna of ticks and vertebrates are also totally different.

Spread of KFD to newer areas in recent years:
Data on the KFD incidence showed that approximately 1000 cases of KFD with a CFR of 2-10% are reported annually and all age groups are affected but incidence in working age group is high. KFD epizootics in monkeys are a regular feature as 2,442 monkey deaths were recorded from 1957 to 1975. KFDV was restricted to three taluks of Shimoga district till 1971 and subsequently reported from other districts of the state viz., Chikamagalore, Uttara Kannada, Dakshina Kannada and Udupi and later in 2012 from Chamaraja Nagar and more recently in 2016 from Belagavi district of Karnataka state. In 2013, KFDV, was detected in autopsy of dead monkeys in Nilgiris district of Tamil Nadu. Monkey deaths as well as human cases reported in Wayanad and Malappuram districts of Kerala during 2014 – 2015. KFD outbreak has been reported from North goa district of Goa state bordering Karnataka in 2015. KFD outbreak was reported from Sindhu Durg district of Maharashtra in 2016. It is evident that, the KFDV is centripetally spreading to newer areas of Karnataka as well as neighbouring states. KFD newly reported areas constitute a number of diverse biotypes such as forest, cultivated clearings and grass lands. Clearing of the forest area for cultivation causes changes in tick fauna, and is considered as the temporary risk factor.

Characteristics of New Foci: All the new foci are contiguous area in the western Ghats and ecologically they are similar to Shimoga district with full of closed forests with few open forests. The forest biotype is composed of tropical evergreen, semi evergreen, and moist deciduous forests. These areas are having cultivated and grass lands for cattle grazing but only the altitude varies. Spread of KFD to newer areas attributed to changes in environment including deforestation, new land-use practices for farming and timber harvesting, might have led to the spread of this disease to newer localities. Grazing of cattle in forest areas with infected ticks will

lead to introduction of these ticks to new areas. Presence of wild monkeys and other animal reservoirs is also conducive for the spreading of KFD to newer territories.

Seasonal incidence

Over the years, a marked and consistent pattern of seasonal incidence of KFD has been noted. The highest number of human and monkey infections occur during drier months, particularly from January to June. This period coincides with the peak nymphal activity of ticks, particularly Haemaphysalis species which is mostly incriminated in the natural cycle of KFD. The epidemic period also correlates well with the period of greatest human activity in the forest. From January onwards, the villagers frequently visit the forest for collection of firewood, timber, etc. Due to heavy monsoon rains from June through September, such visits are curtailed. From October, human activity in the forest is resumed though less because paddy harvest takes place mainly during October-December.

Age and sex incidence
The early study stressed the occurrence of a prepondrant majority of cases in young adult males. This feature, together with the seasonal pattern supported the hypothesis that the transmission of KFD takes place mainly if not exclusively in the forest.

12.10.3 Causative agent

The causative agent; Kyasanur Forest Disease (KFD) virus is an arbovirus belonging to Casal's group B. It is a member of the family Flaviviridae, genus Falvivirus. Within this group, it has a close antigenic relationship with the Russian Spring Summer Encephalitis (RSSE), Louping ill and Omsk haemorrhagic fever viruses complex.

Vectors
More than 15 species of ticks are involved in the ecology of the virus. The major vector for man is Haemaphysalis spinigera in nymphal stages. Haemaphysalis papauana kinneari and Haemaphysalis turturis are the other species found abundant in the area. Transmonsoonal persistence of the virus has also been documented in Haemaphysalis nymphs infected in nature.

Maintenance hosts
There is no evidence of person-to-person transmission of KFD virus and a number of mammalian hosts are suspected or known to be involved as maintenance hosts.

Historically, the epidemic of KFD has been associated with epizootic in monkeys, most important being black-faed langur (Presbytis entellus) the South indian macaque (Macaca radiata). However, they are effective maintenance hosts because most of them die from KFD infection.

Intensive investigations over the years have implicated several species rodents as important maintenance hosts. Special mention can be made of, porcupines, squirrels and certain species of rats, viz., Rattus raj wroughtone and Rattus blanfordi. With the possible exception of shrew and the jungle striped squirrels, the infection was not fatal to small mammals. The procupines are highly infested with mature and immature stages of ticks and also circulate high levels of the virus so as to provide effective infection threshold for the ticks.

The presence of antibodies reacting to KFD virus has been demonstrated in several species of bats collected from the epizootic-epidemic areas as well as from Pune. However, ixodid ticks are rarely found on them and the arthropod parasites investing them appear to be host - specific. Their role in the zoonoses of KFD remains enigmatic.

The high tick infestation rate and viraemia of epidemiologic significance was found in certain ground frequenting birds such as jungle fowl and red spur fowl. In several other species of birds, either the demonstration of antibodies in wild-caught birds or viraemia levels in experimental ones did not correlate with the infestation rate with the tick vectors.

The role of cattle is limited to the vector population. The adults of Haemaphysalis species including Haemaphysalis spinigera feed mainly on cattle. Therefore, they are of paramount importance in maintenance the ticks In nature. Experiments provided evidence (low or no viraermia) against the cattle as maintenance hosts of the virus.

12.10.4 Clinical features

The incubation period in man is estimated to be between 3-8 days.

Acute stage

The disease has a sudden onset with fever, headache, severe myalgia and injected conjunctive; prostration may be marked in many patients. Bradycardia, a decrease in blood pressure and signs of dehydration are commonly found. An exanthem may be seen on the palate in several patients. Gastro-intestinal disturbances and haemorrhages occur in severe cases. Bronchiolar involvement is indicated by a persistent cough and abnormal physical signs in the lungs. There is a diphasic course in a number of patients. The second phase is characterized by mild meningo-encephalitis and follows an afebrile period of 7-21 days. It is manifested by a return of fever, severe headache followed by neck stiffness, mental disturbance, coarse tremors, giddiness and abnormal reflexes.

The case fatality rate is generally estimated at about 5-10 per cent. Serious manifestations, such as haemorrhages and dehydration, resulting into death, are generally observed among the low socio-economic groups. The general state of nutrition and chronic diseases might be important contributory co-factors.

Convalescent stage

Convalescence is usually prolonged and many patients need 4 weeks or more for full recovery. During this phase, physical effort is difficult and often results in tremors due to weakness of muscles. Generally, sequelae have not been noted.

Laboratory infections

The virus is one of the most infectious ones and therefore hazardous laboratory workers; more than 120 persons have suffered from laboratory acquired infections.

The clinical features were not different from those described for infections. Mortality has not been recorded and full recovery after varying periods of convalescence, usually between 20-30 days was observed.

Differential diagnosis

In absence of any history of direct accidental exposure, different physicians attending on the laboratory acquired patients of KFD diagnosed the illness as enteric fever, influenza or

undifferentiated fever probably of viral nature. KFD aetiology could be established only after detecting the presence of the virus in the blood and/or serological evidence. In the limited areas where the virus is endemic, particularly during the seasonal, occurrence, KFD should be suspected in all patients with a sudden onset of fever.

12.10.5 Laboratory investigations

Examination of the blood shows a leucopaenia, thrombocytopaenia and decreased haematocrit during the early phase of the disease. Leucopaenia observed during the first week was followed by leucocytosis during 3rd and 5th weeks. Albuminuria appears in most cases during the acute febrile stage. Granular casts have been observed in a few patients.

In most cases, the CSF is clear with no increase in cells or alterations in proteins, chlorides or sugar. In patients developing the second phase with meningeal signs, it shows an increase in cells and proteins. The method of collection, storage and transport of the material for isolation and serological tests are the same as given for Japanese) encephalitis (JE) virus. Unlike JE virus, KFD virus can be isolated with ease from the acute phase sera of the patients.

12.10.6 Treatment and management of patients

There is no specific treatment except for supportive and symptomatic ones. Analgesics for myalgia and headache and intravenous fluids or blood should be administered for dehydration or haemorrhage, convalescence, rest and adequate diet seem to be the major requirement. No particular measures of isolation of patients seem to be indicated.

The high risk group of forest related occupations should be immunized to avoid the risk of infection of the disease. Formalin inactivated tissue culture vaccine is given to ages 6 – 65 years within 5 km radius of reported KFD activity, positive for KFD virus. Two doses of vaccine are administered to individuals at an interval of one month followed by periodic boosters after 6–9 months.

12.10.7 Prevention and control

Protection of man
Prevention of tick bites: Application of some known repelling agents (such as Dimethyl phthalate (DMP), NN-Diethyl-m-Tolumaide (DEET) and certain other proprietory preparations having these or similar chemicals, e.g. Mylol (Boots) on the exposed parts is effective from one to a few hours. Prevents tick infestation of individuals while they walk through or work in the forested areas. If the duration of stay is longer, more than one application may be necessary. Immunization with killed KFD vaccine.

Measures against ticks
Source reduction : For the control of ticks in forests, gamma isomer of benzene hexachloride (BHC) "Lindane" as wettable powder, has been found to be effective for six weeks; it is sprayed at the rate of approximately 1 kg. of gamma isomer per hectare. The spraying may be carried out in ares where monkey deaths have been reported (within a radius of 50 metres around the spot of the monkey death). It is also effective in forest tracks frequented by man for various activities.

12.10.8 Action plan for KFD control in Karnataka

Since KFD is endemic in Karnataka state for a long time the Directorate of Health Services, Karnataka with its Virus Diagnostic Laboratory, Shimoga has the expertise for the surveillance and control of the disease. They have developed a calendar of activities and coordinating KFD control measures undertaken in various other districts of the state. KFD is a seasonal disease mainly reported during early and peak summer months major preparatory aspects are being carried out during non transmission months. This is implemented by planning, processing, procuring insecticides, vaccines and drugs followed by orientation training to Medical officers and field staff during non transmission months(July – December). Conduction of district-wise workshops and action plan submission and **i**ntensive IEC about the disease and vaccination for the vulnerable groups are done during September- December**.** Strengthening of active surveillance in endemic and other forest fringed during the primary transmission period of KFD (December-June).

12.11 CHIKUNGUNYA FEVER

12.11.1 Introduction

Chikungunya fever is a reemerging viral disease caused by chikungunya virus (CHIKV), an RNA virus belonging to the family *Togaviridae* and genus *Alphavirus.* The disease is almost always self-limiting and is rarely fatal. Molecular characterization has demonstrated 2 distinct strain lineages that cause epidemics in Africa and Asia. These geographical genotypes exhibit differences in transmission cycles: a sylvatic cycle in Africa is maintained between monkeys and wild mosquitoes while in Asia the cycle exists between humans and *Aedes aegypti* mosquitoes. In 2005 an outbreak occurred on the French islands of La Reunion, Mayotee, Mauritius, and Seychelles. In the same year, CHIKV reappeared in India after nearly 3 decades of quiescence, with 13 lakhs suspected cases in 12 states.

Table 12.2 Clinically Supected Chikungunya Fever Cases Since 2010

Sl. No.	Affected States/UTs	2010	2011	2012	2013	2014	2015(Prov.)	2016*
1	Andhra Pradesh	116	99	2827	4827	1359	817	403
2	Arunachal Pd.	0	0	0	0	0	35	0
3	Assam	0	0	0	742	0	0	0
4	Bihar	0	91	34	0	0	3	0
5	Goa	1429	664	571	1049	1205	561	56
6	Gujarat	1709	1042	1317	2890	574	406	145
7	Haryana	26	215	9	1	3	1	0
8	Jharkhand	0	816	86	61	11	21	0
9	Karnataka	8740	1941	2382	5295	6962	20763	4574
10	Kerala	1708	183	66	273	272	175	55
11	Madhya Pd.	113	280	20	139	161	67	4
12	Meghalaya	16	168	0	0	0	78	0
13	Maharashtra	7431	5113	1544	1578	1572	391	253
14	Orissa	544	236	129	35	10	81	1

15	Punjab	1	0	1	0	2	180	0
16	Rajasthan	1326	608	172	76	50	7	0
17	Tamil Nadu	4319	4194	5018	859	543	329	30
18	Telangana	0	0	0	0	1687	2067	312
19	Tripura	0	0	0	0	34	180	11
20	Uttar Pradesh	5	3	13	0	4	0	0
21	Uttarakhand	0	18	0	0	0	0	0
22	West Bengal	20503	4482	1381	646	1032	1013	301
23	A& N Island	59	96	256	202	161	68	0
24	Chandigarh	0	1	0	1	0	1	0
25	D&N Haveli	0	0	100	2	0	0	0
26	Delhi	120	110	6	18	8	64	0
27	Lakshadweep	0	0	0	0	0	0	0
28	Puduchery	11	42	45	146	399	245	90
	Total	48176	20402	15977	18840	16049	27553	6235

* Provisional till 30th April 2016 (available at http://www.nvbdcp.gov.in/chik-cd.html cited on 19th July 2016)

12.11.2 Aetiological agent

Chikungunya virus is most commonly transmitted to humans through the bite of infected *Aedes* mosquitoes, although vertical transmission is also possible. High viremia is typical during the first 2 days of illness, declines at days 3 and 4, and usually disappears by day 5. "Silent" CHIKV infections may occur in children. Chikungunya virus infection, clinically evident or silent, is thought to confer lifelong immunity.

12.11.3 Clinical Features

Incubation following mosquito bites averages 48 hours but may be as long as 12 days. Symptom onset is abrupt and heralded by fever and severe arthralgia, followed by constitutional symptoms and rash lasting for a period of 1 to 7 days. Fever rise can be dramatic, often reaching high temperatures and accompanied by intermittent shaking chills. Arthralgias are polyarticular and migratory, and predominantly affect the small joints of hands, wrists, ankles, and feet, with lesser involvement of larger joints. Patients in the acute stage may complain bitterly of pain when asked to ambulate and they characteristically lie still. Joint swelling may occur, but fluid accumulation is uncommon. Mild articular manifestations usually resolve within a few weeks, but more severe cases may remain symptomatic for months. Generalized myalgias, back pain, and shoulder discomfort are common.

Cutaneous manifestations begin with flushing over the face and trunk and evolve to an erythematous dermatitis. The trunk and limbs are most frequently involved, but lesions may also appear over the face, palms, and soles. The rash eventually simply fades or desquamates.
Infection can infrequently result in meningoencephalitis, particularly in newborns and those with preexisting medical conditions. Chikungunya outbreaks typically result in several hundreds or thousands of cases, but deaths are rare.

12.11.4 Diagnosis

Chikungunya fever should be suspected when epidemic disease occurs with the characteristic triad of fever, rash, and rheumatic symptoms. Virus-specific IgM antibodies can be helpful with diagnosis. Chikungunya virus isolation from blood is accomplished by either in vivo (mice or mosquito) or in vitro techniques. Chikungunya virus detection is also possible in early stages using traditional polymerase chain reaction (PCR) methods. A specific and sensitive 1-step PCR assay has recently been developed as a rapid indicator of active infection by quantifying viral load in clinical samples or cell culture supernatant.

12.11.5 Treatment and Prevention

Chikungunya fever is usually self-limiting, and no specific treatment exists. Rest and supportive care are indicated during acute joint symptoms. Movement and mild exercise tend to improve stiffness and morning arthralgia, but heavy exercise may exacerbate rheumatic symptoms. Nonsteroidal anti-inflammatory drugs may be beneficial, and chloroquine has been used in severe cases. Infective persons should be protected from mosquito exposure so that they do not contribute to further transmission.

No vaccine is available, and preventive efforts are focused on vector control. Elimination of breeding sites or source reduction is important. Larvivorous fish (eg, gambusia, guppy), which eat mosquito larvae, may be introduced into local endemic areas. Protection from mosquito bites is achieved by insect repellent and use of insecticide-treated mosquito nets, especially during the daytime when the mosquitoes feed. Well-planned fogging operations are sometimes recommended in high-risk villages, where clustering of cases has been reported. Active epidemiological surveillance for CHIKV is crucial for promoting effective community education and transmission control.

12.12 YELLOW FEVER

Yellow fever (YF) is an arboviral infection caused by the yellow fever virus (Genus; Flavivirus, Family Flaviviridae). The word "yellow" in the name refers to the jaundice that occurs in some of the affected patients. The disease is highly fatal. Nearly 50 % case fatality rate has been reported in severe cases. During the ancient times yellow fever was one of the most feared death specially in the Atlantic trade route. The disease now occurs only in South America, Central America and Africa however, historically, major outbreaks have also been reported from Europe and North America too. The disease is spread by mosquito vector. Several different species of the Aedes (in Africa) and Haemogogus (in South America) can transmit the virus. The disease is a major public health concern due to the reason that the mosquitoes capable of transmitting yellow fever exist in regions where the disease does not presently occur.

The spectrum of the clinical disease can vary from mild flu like disease to classical triphasic hemorrhagic fever with hepatorenal involvement. An acute phase can last for 4 to 5 days. During the acute phase patients shows the symptoms such as fever, headache, backache, muscle pain, nausea vomiting and red eyes. Following the acute phase a temporary period of remission occurs in 5% to 20% of cases. After that a period of toxic phase can follow the period of remission and present with symptoms such as jaundice, dark urine, reduced amounts of urine production, bleeding from the gums, nose or in the stool, vomiting blood, hiccups, diarrhea, slow pulse in relation to fever.

A presumptive diagnosis of yellow fever is often based on the patient's clinical features, places and dates of travel (if the patient is from a non-endemic country or area), activities, and epidemiologic history of the location where the presumed infection occurred. Laboratory diagnosis of yellow fever is performed by testing the serum to detect virus-specific IgM and neutralizing antibodies. A direct confirmation of virus infection can be obtained during the acute phase by reverse transcription polymerase chain reaction (RT-PCR). Another direct approach is the isolation of the virus and its growth in cell culture using blood plasma or serum.

No specific treatment is available for yellow fever. In the toxic phase, supportive treatment includes therapies for treating dehydration and fever. In severe cases, death can occur between the 7th and 10th day after onset of the first symptoms.

Prevention of yellow fever includes vaccination, as well as avoidance of mosquito bites in areas where yellow fever is endemic. Institutional measures for prevention of yellow fever include vaccination programmes and measures of controlling mosquitoes. Effective vaccine against yellow fever is available and has been proven effective for reducing the occurrences of the disease worldwide. Currently, available vaccines protect against all yellow fever virus strains and are attenuated live virus vaccines derived from a virus originally isolated in 1927. This virus strain was attenuated by passage in mouse embryo tissue culture and then in chicken embryo tissue culture, resulting in the 17D strain from which all current vaccines are derived. The 17D vaccine is highly effective (approaching 100%). All individuals aged 9 months or older and living in countries or areas at risk should receive yellow fever vaccine.

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LISTERIOSIS

13.1 INTRODUCTION

Listeriosis is an emerging zoonotic illness with high mortality rate caused by bacterium *Listeria monocytogenes* belonging to genus Listeria. This bacterium has been recognized as an animal and human pathogen for more than 70 years. Listeriosis remained a relatively obscure disease, attracting limited attention until 1980's. A rise in numbers of human and animal cases in several countries and series of human food borne outbreaks in North America and Europe has generated much public concern and has drawn the attention of microbiologists to the causative organism.

13.2 ETIOLOGICAL AGENT

Of the six currently recognized species of Listeria, only *L. monocytogenes* is an important human and animal pathogen. The other species are generally believed to be non-pathogenic, although *L. seeligeri, L. welshimeri* and *L. ivanovii* have been found rarely in human infections. *L.monocytogenes* is a small (o.5x1-2mm) gram-positive non-acid fast, non-sporing rod that occurs singly or in short chains. They may show palisade and Y-shape arrangements causing confusion with corynehacterium or may be mistaken for streptococci. Listeria shows a characteristic tumbling motility at 25^0C, they are not motile at 35^0C . The organisms are facultative anaerobes, growing at optimum temperature between 30^0C and 37^0C but growth occurs at 4^0C within few days. Colonies are small (1-2 mm after 24-48 hour incubation), round, smooth and appear blue grey when examined with oblique transmitted light.

The genus and species identification can be made by the biochemical reactions.

13.3 EPIDEMIOLOGY

13.3.1 Natural habitat

Listeria species are ubiquitous in nature. They are found in soil, fresh and salt water, decaying vegetable matter, silage, sewage and animal feed. It has been isolated from faeces of both apparently healthy and diseased 42 mammalian and 22 avian species. These include common animals like cattle, buffalo, sheep, goat, pig, dog, cat, fowl and rodents. About 1-5% of humans are asymptomatic intestinal carriers as reported in the western literature.

Many foods are contamined with *L.monocytogenes*; it has been recovered from raw vegetables, milk milk products, fresh and frozen poultry and meat and various fruits and vegetables.

13.3.2 Transmission

Sporadic cases and outbreaks of listeriosis are known, in both, contaminated foods are primary vehicle of transmission. Although rare, non-food related infections could occur in veterinary

practitioners and farmers while handling infected material. Infection to fetus can be transmitted via placenta or through infected vaginal canal during delivery. Nosocomial outbreaks have been reported in nurseries. In one outbreak in maternity ward contaminated mineral oil used to clean the infants was implicated as a source of infection.

13.3.3 High Risk Groups

Neonates, the elderly, pregnant women and immunocompromised individuals are more prone to the disease. It is not a reportable disease, nevertheless Center for Disease Control and Prevention CDC) conducted active surveillance in 1980 – 82 and 1986. Data reports about 1850 cases with 425 death per year in United States. The rates of infection are highest amongst infants less than one month and elderly more than 60 years of age. Infection in antenatal patients is reported to be about 27%. Non-perinatal infection is reported from AIDS patients, organ transplant recipients and patients on immunosuppressive therapy.

13.3.4 Incubation Period

Incubation period is variable. It may range from few days to 8 weeks. Median incubation period is estimated as 3 weeks.

13.3.5 Period of Communicability

Infected individuals may shed the organismsin their stools for several months. Mothers of infected newborn infants may shed the bacteria in vaginal discharge and urine for 7 – 10 days.

13.4 CLINICAL PICTURE

Listeriosis, though a food – borne illness does not manifest as classical gastroenteritis. In fact, enteric stage of illness often goes unnotices. Clinical symptoms may range from mild influenza like illness to meningitis and meningoencephalitis.

13.4.1 Listeriosis in pregnancy

Infection can occur in any trimester but most often occurs in third trimester probably because of decline in cell-mediated immunity. Manifestations are usually mild characterized by flu-like illness, fever, headache, backache, arthralgia and myalgia sometimes accompanied by gastrointestinal symptoms. Blood culture proves diagnostic during this listerial bacterimic phase. Transplacental spread of organisms can occur leading to abortion, stillbirth, premature labor or birth of an acutely ill child. Untreated listerial infections are generally self-limiting although, if complicated by amniotis symptoms persist until spontaneous or interventional termination of pregnancy occurs. Early diagnosis and prompt treatment results in birth of healthy infant.

13.4.2 Listeriosis In Neonates

This is classified as early onset disease evident during first week of life and late onset disease manifesting thereafter. Early onset disease may be characterized by septicemia, respiratory distress and skin lesions. Disseminated abscesses may be detected in liver, spleen adrenal glands, lungs and other sites. This syndrome is called *granulomatous infantisepticum* and has a

high mortality rate. The infant acquires the disease *in utero*. Late onset disease is, in most probability, acquired during the passage from the birth canal. However, nosocomial transmission is also suggested. Meningitis is mot prominent feature and has low mortality rate.

13.4.3 Listeriosis Not Associated With Pregnancy

Immunocompromised individuals are at highest risk. *L.monocytogenes* has tropism for central nervous system; brain and meninges. CDC in 1990 reported *L.monocytogenes* as fifth most common cause of meningitis after *H.influenzae, S.Pneumoniae, N.meningitidis* and group B streptococci. Clinically listerial meningitis is not distinguishable from other bacterial meningitis. Gram stain and isolation of microorganism clinches the diagnosis. It can also cause meningoencephalitis, cerebritis and cerebral and brain stem abscess.

13.4.4 Focal Infections

Conjunctivitis, lymphadenitis, cutaneous infections, hepatic and splenic abscesses, chlecystitis, peritonitis, osteomyelitis, endocarditis, myocarditis, pericarditis, arteritis, endopthalmitis, joint infections and pleuropulmonary infections are reported.

13.5 LABORATORY DIAGNOSIS

CLINICAL SAMPLES

Blood, CSF, amniotic fluid, placenta, fetal tissue or sample from any other focal site which is normally sterile can be collected in a sterile container and transported to the laboratory as soon as possible. If delay is anticipated it can be sorted at 4^0C for 48 hours. Blood samples should preferably be inoculated in the conventional blood culture broth. Stool is preferable to rectal swabs to study the carriage rates for epidemiological purposes. Approximately one-gram stool is inoculated in a primary selective broth (US Department of agriculture broth) and transported to the laboratory. If broth is unavailable stool sample should be transported frozen. For longer period of storage freezing of specimens at -20^0C is advisable to prevent over growth of microflora.

FOOD SAMPLES

Collect at least 100-gram food sample aseptically in sterile containers. Whenever possible food should be collected in original containers e.g. ice creams, butter etc. should be collected in original packing and transported frozen. They should be thawed immediately before analysis.

CULTURE ISOLATES

Isolates of Listeria sp.can be transported to district laboratory on a non-carbohydrate containing agar slant such as tryptic soy agar.

13.5.2 Culture

Samples from sterile sites are directly plated on 5% sheep blood tryptic soy agar. Blood for culture is inoculated into conventional blood culture broth. Samples from non-sterile sites and food samples are first enriched in a primary selective enrichment broth (University of Vermont broth) at 30^0 C for 24 hours, than plated on selective differential oxford agar or PALCAM agar.

Suspect colonies are picked and plated on tryptic soy agar with 5% sheep blood for biochemical and serological identification.

13.5.3 Rapid Detection Methods

Nucleic acid hybridization assay kits are available for detection of Listeria species in food. These use a synthetic DNA probe that is complementary to specific sequences of 16S RNA of Listeria species. Polymerase chain reaction can be used for rapid detection of L monocytogenes DNA in CSF and tissue. Listeriolysin gene is amplified and detected. This test is sensitive, specific and particularly useful when culture is negative due to prior administration of antimicrobials. Sandwich immunoassay using monoclonal antibodies is useful for qualitative detection of Listeria species in meats, dairy products and environmental samples.

13.5.4 Pathogenicity Tests

Both in vivo and vitro tests have been developed to check the virulence potential of Listeria species. Animal pathogenicity tests include intraperitoneal inoculation of mice, inoculation of mice, inoculation of chorioalantoic membrane of embryonated eggs and inoculation of conjunctiva of rabbits (Anton test). Cell cytotoxicity assays using human intestinal epithelial cell line, CaCo-2, have the disadvantage of not providing quantitative measurement of virulence and a few L. monocytogens show a decrease virulence to CaCo-2 cell lines as compared to animal tests.

13.5.5 Typing Methods

The considerable interest in epidemiology of Listeriosis has led to development of various typing methods using phenotypic and genotypic characters.

Phenotyping Methods

Serotyping is based on cellular (O) and flagella (H) antigen of Listeria species. Thirteen serotypes of L.monocytogenes are recognized, viz., 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4c, 4d, 4e and 7. Majority of strains causing disease in humans and animals belong to serotypes 1/2a, 1/2b, 1/2c and 4b. Thus this method has a poor discriminating power limiting its use as epidemiological marker.

Phage typing higher discrimination than serotyping. Listeria phages are isolated from lysogenic strain of Listeria species and sewage. Standard set of international phags is used to trace the sources during the outbreaks. Non-typable strains limit the usefulness of phage typing as an epidemiological marker.

In Multilocus Enzyme Electrophoresis (MEE), as the name indicates, multiple enzyme loci are examined by electrophoresis. Each mobility variant of an enzyme is designated by a numerical allele. The strain is characterized by various numerical values of the alleles examined after electrophoresis. MEE has been widely applied for epidemiological investigations of food-borne outbreaks. It has also been useful in differentiation of various Listeria species.

Genotyping Methods

DNA Microrestriction pattern, as the name suggests, the total chromosomal DNA is digested by restriction endonucleases and subjected to lectrophoresis. Large numbers of fragments are generated foming a complex pattern making It difficult to compare the patterns of several atrains. DNA Macrorestriction pattern involves pulse field gel electrophoresis with low frequency cleavage endonucleases of whole Listeria chromosomes. This method offers high discrimination for typing L monocytogenes. Random amplified polymorphic DNA (RAPD) involves amplification of homologous sequence of DNA using short arbitrarily shoosen primers. This can be used for differentiating various Listeria species. Various serotyopes of L monocytogens and various sub-types within serotypes. This method holds promise in sub-typing of L. monocytogenese in future.

13.6 ANTIMICROBIAL AGENT SUSCEPTIBILITY

In vitro, L monocytogenes is susceptible to penicillin, ampicillin, gentamycin, erythromycin, tetracycline and chloramphenicol. Penicillin or ampicillin was recommended but as b lactum antibiotics are bacteriostatic for listeria, ampicillin or penicillin plus gentamicin remains the treatment of choice for most manifestations of listeriosis. For patients intolerant to penicillin, trimethoprimsulfamethoxazol is an alternative agent. Chloramphenicol, previously regarded as alternative drug in patients allergic to penicillin is not recommended as unacceptable failures have been reported. Plasmids conferring resistance to chloramphenicol, macrolides and tetracycline have been described in the clinical isolates. Erythromycin may be used to treat listeriosis in pregnancy. Cephalosporins should never be admistered, as they are ineffective.

13.7 CONTROL

There has been a marked decline in number of invasive listeriosis cases in US since early 1990's. This was possible because in 1989, US Department of agriculture started active surveillance and enforced regulation prohibiting the sale of contaminated meat. Simple dietary practices like thorough heating of food especially from animal sources, washing raw vegetables and fruits before eating, consumption of boiled milk, reheating left over food until steaming hot before eating and keeping cooked and ready to eat food separate from raw food will go a long way in controlling leptospirosis.

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ANTHRAX

14.1 INTRODUCTION

Anthrax also called Malignant pustule, Malignant oedema, Woolsorter's disease, or Ragpicker's disease, is an acute infectious disease of animals caused by *Bacillus anthracis,* a gram-positive, spore-forming bacillus. Spores of *B.anthracis* can persist in the environment for many years in some types of soil and enter the body through skin abrasions, inhalation or ingestion and multiply to produce exotoxins.

Anthrax is primarily a disease of herbivorous animals that occasionally affects human.

14.2 HISTORICAL BACKGROUND

It is one of the oldest recorded diseases of animals, being mentioned by Moses in Exodus, and among the classical authors of Greek and Roman antiquity viz Homer, Hippocrates, Ovid, Galen, Virgil and Pliny. Devastating epidemics of the disease have been recorded by many medieval and modern writers. In the 18th and 19th centuries, it sometimes spread like plague over the southern part of Europe, taking a heavy toll of human and animal life. Anthrax was the first disease of humans and other animals in which the causative agent was definitely demonstrated as a specific microorganism by the French biologist Casimir-Joseph Davaine in 1863, and by the German bacteriologist Robert Koch in 1876 who isolated the organism in pure culture. It was also the first infectious disease against which a bacterial vaccine was found to be effective by Louis Pasteur in 1880. These discoveries led to the origin and development of the modern sciences of bacteriology and immunology.

14.3 GEOGRAPHICAL DISTRIBUTION

Anthrax is known to occur globally, though it is more often a risk in countries with less standardized and less effective public health programs. Anthrax is most common in agricultural regions where it occurs in animals. These include South and Central America, Southern and Eastern Europe, Asia, Africa, the Caribbean, and the Middle East.

14.4 CURRENT INDIAN SCENARIO

Anthrax is enzootic in southern India but is less frequent to absent in the northern Indian states. In the past years the anthrax cases have been reported from Andhra Pradesh, Jammu and Kashmir, Tamil Nadu, Orissa and Karnataka. Outbreaks of Anthrax have been reported from Mysore 1999, Orissa 2004, 2005, West Bengal 2000.

14.5 EPIDEMIOLOGY

Causative agent

The causative organism B.anthracis is a gram-positive, nonmotile, haemolytic, and spore-forming bacillus. The virulence of the organism is determined by the capsule and exotoxins; oedema toxin and lethal toxin.

Transmission

Animals usually become infected by ingestion of contaminated soil or feeds. Infected animals shed the bacilli during terminal hemorrhage, or if the blood of the dead animal is spilled accidentally. On exposure to the air, the vegetative forms sporulate. These spores are markedly resistant to many disinfectants and adverse environmental conditions and remain viable in the contaminated soil for many years. Dried or otherwise processed skins of infected animals may also harbor the spores for years. Thus, the spore forms are predominant in the environment and it is very largely through the uptake of spores that anthrax is contracted.

Cutaneous anthrax is the most common anthrax infection. Transmission occurs after exposure to infected animals and contaminated animal products such as hair, hides, wool, bones, or skin. Inhalation anthrax results from inhalation of spores in particles less than 5 μm in diameter that may reach the terminal alveoli of the lungs. Aerosols of such particles may be created by the agitation of the hair or wool in the industry settings. Intestinal and oropharyngeal anthrax results from ingestion of contaminated meat. There is no evidence that milk from infected animal transmits anthrax. The disease spreads among omnivores and carnivores through contaminated meat, bonemeal and other feeds and among wild life from feeding on anthrax carcasses. Vultures have been reported to spread the organism from one area to another.

Accidental infection may occur among laboratory workers. Direct person to person spread of anthrax is extremely rare. However, precautions should be taken with drainage and secretions of patients to prevent cutaneous anthrax. Second attacks can occur, but are rare. The disease mostly affects adults, especially males. It is due to high exposure rate among these groups. Anthrax is a seasonal disease. Climate probably acts directly or indirectly by influencing the way in which the animal comes into contact with the spores, for example, grazing closer to the soil in dry periods when grass is short and sparse.Incubation period is from a few hours to seven days. Incubation period up to 60 days is possible. Most cases occur within 48 hours of exposure.

14.6 CLINICAL MANIFESTATIONS

14.6.1 In animals

Important clinical manifestations in animals

- In ruminants, sudden death, bleeding from orifices, subcutaneous haemorrhage, without prior symptoms or following a brief period of fever and disorientation should lead to suspicion of anthrax
- In equines and some wild herbivores, some transient symptoms such as fever, restlessness, dyspnoea or agitation may be apparent

- In pigs, carnivores and primates, local oedema and swelling of face and neck or of lymph nodes, particularly mandibular and pharyngeal and/or mesenteric may be present

The incubation period in the susceptible herbivore ranges from about 36 to 72 hours. The first signs of an anthrax outbreak are one or more sudden deaths in the affected livestock. Other signs include going off feed, or producing less milk than usual. During the systemic phase, the animals become distressed, appear to have difficult breathing and cease eating and drinking. Swellings in the submandibular fossa may be apparent, and temperature may rise. If the animal fails to respond to the treatment, it lapses into coma followed by death from shock.

14.6.2 In humans

Anthrax infection occurs in three forms: cutaneous, inhalation, and gastrointestinal depending on the mode of transmission. Symptoms of disease vary depending on how the disease was contracted, but symptoms usually occur within seven days.

Cutaneous anthrax: Most anthrax infections occur when the bacterium enters a cut or abrasion on the skin, such as when handling contaminated wool, hides, leather or hair products of infected animals. The incubation period for cutaneous anthrax is 1-7 days. Skin infection begins as a painless, pruritic papule that resembles an insect bite but within 1-2 days develops into a vesicle (usually 1-3 cm in diameter) and then a painless ulcer with a characteristic black necrotic (dying) area in the center. Systemic symptoms are mild and may include malaise and low-grade fever. There may be regional lymphangitis and lymphadenopathy. Occasionally more severe form of cutaneous anthrax may occur with extensive local oedema, induration and toxemia. The infection can also spread to the bloodstream with overwhelming septicemia. About 20% of untreated cases of cutaneous anthrax will result in death. Deaths are infrequent with appropriate antimicrobial therapy.

Inhalation anthrax: Initial symptoms may resemble a common cold. After several days, the symptoms may progress to severe breathing problems and shock. Mediastinal widening is seen in the X-Ray chest. Diagnosis is difficult but inhalation anthrax should be suspected if there is a history of exposure to an aerosol that contains *B.anthracis*. Inhalation anthrax usually results in death in 1-2 days after onset of the acute symptoms.

Intestinal anthrax: The intestinal disease form of anthrax may follow the consumption of contaminated meat and is characterized by an acute inflammation of the intestinal tract. There are two clinical forms of intestinal anthrax.

- **Intestinal anthrax:** Symptoms include nausea, vomiting, fever, abdominal pain, haematemesis, bloody diarrhoea and massive ascites. Unless treatment starts early toxaemia and shock develop resulting in death.
- **Oropharyngeal anthrax:** Main clinical features are sore throat, dysphagia, fever, lymphadenopathy in the neck and toxaemia. Even with treatment mortality is high, about 50%.

Gastro-intestinal anthrax is difficult to diagnose as the symptoms are non-specific. However, history of ingesting meat of a sick animal and cases of cutaneous anthrax in the area may support diagnosis. Intestinal anthrax results in death in 25% to 60% of cases.

Meningitis may complicate any of the three primary forms. It resembles meningitis due to other causes although it is frequently haemorrhagic. Diagnosis is confirmed by demonstration of the organism in the CSF by microscopy or culture or both.

14.6.3   Case fatality rates

While Inhalation anthrax is almost always fatal, intestinal anthrax results in death in 25% to 60% of cases. The fatality rate of untreated cutaneous anthrax may be upto 20%, but is considerably reduced with early treatment.

14.7   CASE DEFINITION

14.7.1 Recommended case definition: Humans

Suspect: A case that is compatible with the clinical description and has an epidemiological link to confirmed or suspected animal cases or contaminated animal products
Probable: A suspected case that has a positive reaction to allergic skin test (in non-vaccinated individuals)
Confirmed: A suspected case that is laboratory confirmed by one or more of the following:
- Isolation of B.anthracis from a clinical specimen (e.g., blood, lesions, discharges)
- Demonstration of B.anthracis in a clinical specimen by microscopic examination of stained smears (vesicular fluid, blood, CSF, pleural fluid, stools)
- Positive serology (ELISA, Western blot, toxin detection, chromatographic assay, FAT)

Source: WHO Recommended Surveillance Standards (WHO/CDS/CSR/ISR/99.2)

14.7.2   Recommended case definition: Animals

Suspect: A case that is compatible with the clinical description. In enzootic areas all sudden death should be regarded as suspected anthrax cases.
Confirmed: A suspected case that is laboratory confirmed.

14.8   LABORATORY DIAGNOSIS

14.8.1 Collection, storage & transportation of samples from suspected anthrax cases

- Laboratory diagnosis -for anthrax should be attempted only by laboratory well trained to do so.
- High index of suspicion of the disease is important.
- Collection and transportation should be carried out under strict aseptic condition

Collection of Specimen

Human Anthrax
(a) Cutaneous Anthrax
Swabs from the lesion- Collect two swabs as follows:
- In early stage vesicular exudate from the lesions by sterile swab can be collected
- In later stage material to be taken from underneath of eschar after lifting up of eschar with sterile forcep

The swab should be put in Carry-Blair transport medium and with another swab smear on microscopic slide may be prepared and heat fixed.

(b) Intestinal Anthrax
If patient is not severely ill, a faecal specimen can be collected

If patient is severely ill ascitic fluid (peritoneal fluid) can be collected.

(c) Pulmonary Anthrax
If patient is not severely ill, sputum can be collected.

If patient is severely ill, bronchial levage can be collected.

Animal Anthrax
Anthrax should be considered as possible cause of death in herbivore's that has died suddenly with haemorrhages for nose, mouth or anal orifice.

Samples to be collected
(a) Caracass 1 to 2 days old
- Unclotted blood from nasal, buccal or anal orifice may be collected.
- If opened; body fluid, spleen and/or unclotted blood may be collected.

(b) Old putrefying carcasses
- Selection of tissues or any blood stained material may be collected.
- If opened then body fluid, spleen may be collected.

(c) If buried
- Soil or other material from burial site may be collected.

14.8.2 Storage and Transportation

(I). Storage:
All samples collected should be stored properly at room temperature, if delay in transportation takes place then specimen should be stored at 2 - 8°C.

(II).Transportation
- Specimen container should be leak proof, break-resistant plastic or glass container
- Screw cap, containers are preferable.
- After the container is closed and sealed
- Wipe with a disinfectant - a chloride solution (sodium hypochloride)
- Dry it and send it in a properly labeled triple packaging.

14.8.3 Laboratory procedures

(i)   **Materials needed for processing of clinical specimens**
- 5% Sheep blood agar plates [SBA]
- MacConkey agar plates
- Phenyl ethyl alcohol agar (PEA) plates (for stool specimens)
- Trypticase soy broth

- Clean glass microscope slides
- Sterile cotton swabs (commercially available specimen transport swabs for aerobic culture are preferred)
- Disposable bacteriologic Inoculation loops
- Clinical centrifuge with appropriate biocontainment tube holders
- Sporicidal disinfectant (0.5% sodium hypochlorite or 0.5% calcium hypochlorite)

(ii)Isolation from clinical specimens
a) Sputum specimens

Inoculate 3 routine media for sputum specimens (i .e. SBA, MacConkey agar, broth enrichment).
b) Blood specimens

1. Routine blood culture methods are sufficient.
2. There may be enough organisms in the blood to see them on direct smears by Gram stain. *B. anthracis* appears as short chains of 2-4 cells which are encapsulated as evidenced by clear zones around the bacilli. The presence of large encapsulated gram-positive rods in the blood is strongly presumptive for *B. anthracis* Identification.
3. If blood culture bottle is positive, perform a Gram stain directly and observe for encapsulated rods. These blood cultures should also be subcultured to SBA and MacConkey agar plates.

a) Swab specimens
1. Use one swab to Inoculate 3 standard media for surface wounds (e.g., SBA, MacConkey agar, or broth enrichment).
2. Prepare a smear for Gram staining with the second swab.

b) Stool specimens
1. l. Routine stool culture methods are sufficient (e.g., SBA, MacConkey agar, or PEA plates).
2. Do not use CVA or hectone agar plates.

c) CSF specimens
1. If a clinical centrifuge with appropriate bio containment tube holders is available, centrifuge the CSF specimen at 1500 X g for 15 minutes.
2. Collect the sediment and prepare a smear for Gram staining.
3. Inoculate the remainder of the sediment onto SBA and broth enrichment media (tryptic soy broth or thioglycollate).

(iii) Incubation and examination of cultures
a) Cultures should be incubated at 35-37° C under ambient conditions.
b) Cultures should be examined within 18-24 h of incubation. Growth of *B. anthracis* may be observed as early as 8 h after inoculation.

Differential tests for the presumptive identification of *B. anthracis*

a. Colony characteristics of B. anthracis
1. After incubation of SBA plates for 15-24 h at 35-37^0 C, well isolated colonies of B. anthracis are 2-5 mm in diameter. The flat or slightly convex colonies are irregularly round, with edges that are slightly, undulate (irregular, wavy border), and have a ground-glass appearance. There are often' comma-shaped projections from the colony edge, producing the "Medusa head" colony.
2. Colonies on SBA usually have a tenacious consistency. When teased with a loop, the growth will stand up like beaten egg white. In contrast to colonies of *B. cereus* and *B. thuringiensis,* colonies of *B. anthracis* are not ß-hemolytic. However, weak hemolysis may be observed under areas of' confluent growth in aging cultures and should not be confused with ß-hemolysls.
3. When examining primary growth media, it is important to compare the extent of growth on SBA plates with that on MacConkey agar plates. *B. anthracis* grows well on SBA, but does not grow on MacConkey agar. *B. anthracis* grows rapidly; heavily inoculated areas may show growth within 6-8 h and individual colonies may be detected within 12-15 h. This trait can be used to isolate *B. anthracis* from mixed cultures containing slower-growing organisms.

b. Gram stain morphology of B. anthraci*s*
1. Procedure
   Perform Gram stain by usual procedures.
2. Interpretation of results
B. anthracis is a large gram-positive rod (1-1.5 X 3-5 ~) that forms oval, central-to-subterminal   spores (1 X 1.5 11m) on SBA that do not cause significant swelling of the cell. Spores are not present in clinical samples unless exposed to atmospheric levels of CO_2; CO_2 levels within the body inhibit sporulation. Vegetative cells seen on Gram stain of blood and impression smears are in short chains of 2-4 cells that are encapsulated. However, cells from growth on SBA under ambient conditions, are not encapsulated and occur as long chains of bacilli. When grown on nutrient agar In the presence of 5% CO_2 or on other basal media supplemented with 0.8% sodium bicarbonate, virulent strains will yield heavily encapsulated bacilli. The capsule can be visualized microscopically using India Ink.

c. India ink staining of clinical samples (blood and CSF) for capsule
1. **Purpose**
India ink is useful for improving visualization of encapsulated B. anthracis in clinical samples such as blood, blood culture bottles, or cerebrospinal fluid (CSF).

2. **Materials**
   - Microscope slides
   - Cover glasses
   - India Ink (Bactidrop, Remel, Inc., Catalog # 21-518 or equivalent). Microscope with 100X oil immersion objective

3. Controls
a) Control strains
 (1) Positive control: *Klebsiella pneumoniae* on SBA or equivalent
 (2) Negative control: *E. coli* ATCC 25922 or equivalent

b) Method controls: Perform the test with suspensions of fresh cultures of the control strains. Control strains should be assayed on each day of testing.

c) Resolving an out-of-control result: check the purity and identity of the control strains and repeat the test.

4. Procedure

a) For the controls, transfer a small amount of growth (1 mm diameter) from each control SBA plate into 0.5 ml whole EDTA-treated blood or serum. Mix.
b) For the unknowns, take 100 µl of sample (blood, CSF)
c) Transfer 5-10 µl of unknown or control to a slide, place a cover glass on the drop, and then add 5-10 µl of India ink to the edge of the cover glass.
d) After the ink diffuses across, view the cells using 100X oil immersion objective with oil on top of the cover glass.

5. Interpretation of results

The capsule will appear as a well-defined clear zone around the cells for the positive control. No zone should be present in the negative control.

d. Motility test: Wet mount or motility medium

1 Purpose

This test determines the motility of suspect isolates. *B. anthracis* is a nonmotile species. This characteristic is unusual among *Bacillus* species and is therefore useful in the preliminary identification of *B. anthracis* isolates. Two methods are given: the wet mount and the tubed motility test.

2 Materials

 a) For wet mount procedure
 - Pre-cleaned microscope slides
 - Cover glasses
 - Sterile distilled water
 - Disposable bacteriologic inoculating loop
 - Light microscope with 40X objective and 10X eyepiece
 - Sterile glass tube
 b) For tube motility test
 - Tube motility media (Remel, Inc., Catalog # 06-1408 or equivalent), 5 ml per tube
 - Sterile disposable inoculating loop or needle

3 Controls

a) Control strains
(1) Positive control: *Pseudomonas aeuroginosa* ATCC 35032 or equivalent
(2) Negative control: *Acinetobacter* spp. ATCC 49139 or equivalent
b) Method controls: Perform the test with fresh cultures of the control strains using the same method as with unknowns. Control strains should be assayed on each day of testing.

c) Resolving an out-of-control result: Check the purity and identity of the control strains and repeat the test.

4 Procedures

Wet mount and motility

a) Wet mount procedure

1. Deliver 2 drops (approximately 0.1 ml) of sterile distilled water into the sterile glass tube.
2. Using the Inoculating loop, sample a suspicious colony from a 12-20 h culture and suspend the growth in the water. (Alternatively, a loopful of medium from a fresh broth culture can be used).
3. Transfer 1 drop of the suspension to the microscope slide and overiay with the cover glass.
4. Examine the slide under the microscope using the 40X objective (total magnification = 400X).
5. Discard slides in 0.5% hypochlorite solution.

b) Motility test-medium procedure

1. Using the sterile inoculating needle, remove some growth from an isolated, suspicious colony of an 18-24 h culture.
2. Inoculate the motility tube by carefully stabbing the needle 3-4 cm into the medium and then drawing out so that a single line of inoculum can be observed.
3. Incubate the tube aerobically at 35-37° C for 18-24 h.

c) Interpretation of results

1. For wet mount: Motile organisms can be observed moving randomly throughout the suspension. Nonmotile organisms either fail to move or move with Brownian motion.
2. For motility test medium: Nonmotile organisms, such as *B. anthracis,* will form a single line of growth that does not deviate from the original inoculum stab. Motile organisms will form a diffuse growth zone around the inoculum stab.

(iv) **Presumptive identification key for B. anthracis**

- From clinical samples, such as blood, CSF, or lesion material: encapsulated grampositive rods.
- Gram-positive, broad rod, spore-positive: *Bacillus* species.
- Spores are nonswelling and oval shaped; ground glass appearance of colonies: *Bacillus* morphology group 1 (includes *B. anthracis, B. cereus, B. thuringiensis,* and *B. cereus* var.. mycoides)
- Nonmotile: *B. anthracis* and *B. cereus* var. mycoides
- Nonhemolytic: presumptive *B. anthracis*

(v) **Confirmatory tests for *Bacillus anthracis***

A. **Animal pathogenicity**

➢ 5-10 mice injected I/P with suspension of culture material. Observe for mortality.
➢ 100% mortality within 24 hours in case of *B.anthracis*.
➢ If there is no mortality, observe for 10 days.
➢ Material (spleen) from dead mice processed for detection of *Bacillus anthracis*.

B. **Molecular methods**

➢ Direct PCR.
➢ Study of the genetic make up to detect any engineered strains.
➢ Genetic studies : There are three genes viz.
  1. 846 bp capsule gene
  2. 639 bp S-layer gene
  3. 596 bp PA gene

14.9   CASE MANAGEMENT

14.9.1 Chemotherapy

Antibiotics are effective if the disease is recognized early and the full recommended dose and course of the antibiotic is completed. If left untreated or if the antibiotic treatment is discontinued early, the disease can be fatal. General measures for treatment of shock are also necessary. Penicillin is the drug of choice. In severely affected patients or when pulmonary or gastrointestinal anthrax is suspected, Penicillin G, 2 million units per day by infusion or by slow intravenous injection should be administered until the temperature returns to normal. After that, treatment should be continued in the form of intramuscular procaine penicillin, 1 million units every 12-24 hours. Streptomycin, 1-2 grams per day intramuscularly, may act synergically with penicillin. In case of allergy to penicillin, erythromycin, tetracycline, chloramphenicol, ciprofloxacin or gentamycin can be used.

Oral antibiotics such as penicillin V (500 mg 6 hourly) or procaine penicillin (1 million units 12-24 hourly by intramuscular route) can be prescribed for mild forms of cutaneous anthrax.

Though few penicillin-resistant strains have been reported, anthrax bacillus is still by and large sensitive to penicillin all over the globe.

14.10 PREVENTION AND CONTROL

The problem of anthrax continues because of the following:

- The custom of butchering and eating roasted meat from sudden death animals and utilizing their hair, hides, bones etc.
- Lack of cooperation over reporting sudden deaths
- Long delays in diagnosis due to poor communication and inadequate local laboratory facilities
- Failure to implement policies on disposal of carcasses and subsequent disinfection and decontamination

Control measures aim at breaking the cycle of infection. It is primarily around proper disposal of anthrax carcasses, disinfection, decontamination and disposal of contaminated materials, and vaccination of exposed susceptible animals and humans in at risk occupations

14.10.1 Guidelines for an effective control programme

- *Surveillance*
All unexplained livestock deaths or suspected cases must be investigated with laboratory support

- *Reporting*
  - Mandatory reporting of sudden deaths among livestock
  - Mandatory reporting of all human cases

- *Vaccination*
All high risk persons (veterinary workers, laboratory workers, workers handling potentially contaminated industrial raw materials) should be immunized. Annual boosters are necessary if exposure continues. Immunise all animals at risk and re-immunise annually.

- *Disposal*
After confirmation as being a case of anthrax, a carcass should not be opened and should be disposed of by incineration or rendering. Deep burial after disinfection is a less favoured option. Blood from the dead animal should be collected aseptically for confirmation of diagnosis. Necropsy should not be done, as this has the risk of spread of the infection.

- *Disinfection*
Disinfectants should be available in reasonable quantities at veterinary hospitals. Veterinary assistants, surgeons and livestock owners should be trained in their use. Decontaminate soil seeded by carcasses.

- *Education*
Educate employees handling potentially contaminated articles about modes of anthrax transmission, care of skin abrasions and personal cleanliness.

- Control dust and properly ventilate all hazardous industries particularly which are handling raw animal materials.
- Do not use/sell hides of animals exposed to anthrax nor use their carcasses as food or feed supplements.
- Treat properly the effluents from hazardous industries handling animals etc.

- *Treatment*

All symptomatic animals should be treated. Immunize after cessation of treatment.

- *Intersectoral cooperation*

Good communication and cooperation including sharing laboratory facilities and knowledge between veterinary, medical and wild life services are essential to control of anthrax.

14.10.2 Measures in the event of an outbreak of anthrax

Every effort is to be made to investigate the outbreak, to confirm through laboratory diagnosis and to search for the source.

In the affected area, the following measures must be applied:

- The carcasses of infected cattle are to be either burnt at the site of death and the ashes buried deeply, or wrapped in double thickness plastic bag to prevent spilling of body fluids and removed to a more suitable site where they are burnt and the ashes buried.
- The site where the animal died is to be disinfected with 5% formaldehyde after disposal of the carcass.
- All other animals in the affected herd are to be vaccinated.
- Affected premises are to be quarantined for at least 20 days after the last case or 6 weeks after vaccination, whichever is later.
- Any milk collected from a cow, buffalo or goat showing signs of anthrax within 8 hours of milking is to be destroyed, along with any other milk that may have been mixed with the suspected milk.
- People entering infected premises are required to wear protective clothing and footwear, which are disinfected before leaving the premises.
- All cattle on neighbouring premises should also be vaccinated.
- A buffer zone, 20-30 Km wide, is to be established around the infected area within which all cattle and exposed sheep are vaccinated and quarantined.
- Persons who have handled infected animals or their carcasses should be vaccinated against anthrax, if their exposure is frequent and if the human vaccine is available.
- Such persons should avoid any contact with other persons or animals without first changing clothing, washing hands and taking appropriate disinfection measures.
- Where there is a risk of aerosolization of spores, further precautions should be considered such as damping down the material, possibly with 5% formalin, wearing facemasks etc.

Anthrax vaccination

In humans
In China and former USSR live spore vaccines are in use. However, in most other countries the same are not licensed and non-living human vaccines are used.

Vaccination of humans who are not occupationally exposed to anthrax is inappropriate

Anthrax vaccine is indicated for individuals who come in contact in the workplace with animal hides, furs, bone meal, wool, animal hair (especially goat hair), and bristles; and for individuals engaged in diagnostic or investigational activities which may bring them into contact with anthrax spores.
Anthrax vaccines intended for use in animals should not be used in humans.

Because it is not known whether the anthrax vaccine can cause foetal harm, pregnant women should not be vaccinated.
The immunization consists of three subcutaneous injections given two weeks apart followed by three additional subcutaneous injections given at 6, 12, and 18 months. Annual booster injections of the vaccine are required to maintain immunity.
Mild local reactions occur in 30% of recipients and consist of slight tenderness and redness at the injection site. A moderate local reaction can occur if the vaccine is given to anyone with a past history of anthrax infection. Severe local reactions are very infrequent and consist of extensive swelling of the forearm in addition to the local reaction. Systemic reactions occur in less than 0.2% of recipients and are characterized by flu-like symptoms.

In animals

Vaccination is the hub of anthrax control in endemic areas. Therefore, a contingency stock of a vaccine meeting acceptable standards should be available in such areas at all times. For maximum success, vaccination as a control measure must be applied together with other control measures and must be continued for a full specified period.
Anthrax vaccine is indicated for all the animals in the suspected herd, the animals on neighbouring premises and all susceptible animals if there is a reason to believe that they continue to be exposed.
The protective effect of vaccine is limited to about 1 year and therefore the animals in enzootic areas must be immunized annually. Pregnant animals should not be vaccinated.

Further Reading

1. World Health Organization. "Manual for laboratory diagnosis of anthrax." World Health Organization, Geneva, Switzerland, 2003.

2. Brazil, Melanie. "Anthrax attacked!." Nature Reviews Drug Discovery 3.1 (2004): 13-13.

3. World Health Organization, and International Office of Epizootics. Anthrax in humans and animals. World Health Organization, 2008.

STERILIZATION AND DISINFECTION PROCEDURES

15.1 STERILIZATION

Means complete destruction of all kind of living microorganisms including spores.

15.2 DISINFECTION

Destruction of vegetative forms of organisms, which might cause diseases, or spoilage of food etc.

The two terms are not synonymous.

15.3 DISINFECTION OF USED LABORATORY ARTICLES

Purpose

Laboratory garbage (reusable and disposable) is considered as potentially pathogenic both for laboratory workers as well for environment if disposed off untreated. The ideal method of treating such material (disposable) is to incinerate and decontaminate, the reusable one by autoclaving and/or chemical treatment, which ever is available/applicable. As these facilities are not available in most of the peripheral laboratories (L 1 level) the material for disinfections may be divided into the following categories as far as their safe disposal is concerned.

- ➢ Disposable (mostly plastic ware).
- ➢ Reusable, contaminated with morbid material e.g. glass pippets, slides, test tubes etc.
- ➢ Material containing or contaminated with bacterial culture.

Disinfection of disposable items

Material required
- ➢ 1 % sodium hypochlorite/3% Lysol solution.
- ➢ Glass jar.
- ➢ Biosafety bag (puncture resistant with appropriate color code).
- ➢ Gloves.

Procedure
- ➢ Freshly prepare requisite quantity of disinfectant in a jar meant for this purpose.
- ➢ Put articles to be discarded in the solution overnight.
- ➢ Drain off disinfectant.
- ➢ Collect the material in safety bags & dispose off along with other garbage at a designated place.

GLP (Good Laboratory Practices)

- Always prepare fresh solution of disinfectant before use as ready to use solution has shorter shelf life, compared to concentrated one and will be of no use if not freshly prepared.
- Care should be taken while handling & preparing the solution as it may be corrosive to skin.

Washing of Laboratory glassware

A. New glassware
Usually new glassware is slightly alkaline in nature. Before washing, this alkaline nature has to be neutralized for final use.

Material required
➢ 2% Hydrochloric acid.
➢ Big plastic basin.
➢ Demineralized water
➢ Hot air oven for drying purpose only.

Procedure
➢ Prepare sufficient quantity of 2 % hydrochloric acid (e.g. 98 ml of water & 2.0 ml hydrochloric acid) as per the requirement in a big plastic basin.
➢ Wash the newly received glassware under running tap water-to remove the visible dust sticking inside and/or out side surface of the article.
➢ Soak the already washed articles in 2% hydrochloric acid solution.
➢ Leave them there overnight.
➢ Take the articles from 2 % hydrochloric acid and rinse in clean water twice.
➢ Finally wash using demineralized water.
➢ Allow to dry using hot air oven.
➢ Pass on for packing & sterilization for further use.

B. Non contaminated, dirty glassware
Material required

➢ 1 % detergent solution.
➢ Washing brush.
➢ Good quality water supply
➢ Wire basket
➢ Draining rack
➢ Demineralised water
➢ Hot air oven for drying.
➢ Cotton/aluminium foil for plugging

Procedure

➢ Take material, glassware etc. and rinse twice in luke warm water to remove any dirty stain sticking on them.
➢ Put the material to be washed in bowl containing I % detergent solution.
➢ Allow to boil (Electrically / by Bunsen burner flame).
➢ While in solution scrub inside & outside surface of the glassware with the help of the brush.
➢ Leave the glassware in the solution for 2 - 3 hrs.
➢ Take out each article one by one and rinse under running tap water till no trace of detergent is left, which other wise may lead to false results when used.
➢ Drain the water by putting each article on a wall draining rack or by keeping articles up side down in a wire basket.
➢ Put articles in wire basket and keep in hot air oven at 60°C for drying purpose only
➢ Take out each articles and plug using non-absorbent cotton/aluminum foil. Pass on for sterilization (dry heat/ autoclaving)
➢ In case of delay, store in dust free area.

C. Contaminated, dirty glassware
   Material required
➢ 1 % sodium hypochlorite/3% Lysol solution
➢ glass jar
➢ gloves
➢ metallic box tray
➢ Bunsen burner (or any heating device for boiling)

Procedure
➢ Freshly prepare requisite quantity of disinfectant in ajar meant for this purpose. Put articles to be disinfected in the solution overnight.
➢ Drain off disinfectant in sink fitted with tap.
➢ Transfer the material in metal box or tray with cover.
➢ Place on bunsen burner (heating device) for boiling.
➢ Wait up to 15-20 min. after the boiling starts.
➢ Put off the flame & allow cooling the material in metallic box/ tray.
➢ Drain off water.
➢ Pass on the material for washing.

Alternate Procedure for decontamination

➢ Discard all the contaminated glassware directly into metal box / puncture resistant biosafety.bags.
➢ Place box/ biosafety bags with material to be decontaminated in autoclave designated for this work only.
➢ Decontaminate the material by autoclaving.
➢ Drain off the glassware and pass on material for further washing etc.

Washing procedure
   Material required

- ➢ Material required
- ➢ I % detergent solution.
- ➢ Washing brush.
- ➢ Good quality water supply
- ➢ Wire basket
- ➢ Draining rack
- ➢ Demineralised water
- ➢ Hot air oven for drying.
- ➢ Cotton/aluminium foil for plugging

   Procedure
- ➢ Take material, glassware etc. already decontaminated (chemically/autoclaving) and rinse twice in luke warm water to remove any dirty stain sticking on them.
- ➢ Put the material to be washed in bowl containing 1 % detergent solution.
- ➢ Allow to boil (Electrically / by Bunsen burner flame).
- ➢ While in solution scrub inside & outside surface of the glassware with the help of the brush.
- ➢ Leave the glassware in the solution for 2 - 3 hrs.
- ➢ Take out each article one by one and rinse under running tap water till no trace of detergent is left, which other wise may lead to false results when used.
- ➢ Drain the water by putting each article on a wall draining rack or by keeping articles up side down in a wire basket.
- ➢ Put articles in wire basket and keep in hot air oven at 60°C for drying purpose only.
- ➢ Take out each articles and plug using non-absorbent cotton/aluminum foil. . Pass on for sterilization (dry heat/ autoclaving)
- ➢ In case of delay, store in dust free area.

15.4   METHOD OF STERILIZATION

Depending upon the nature of material to be sterilized sterilization procedures used in microbiology laboratory can be divided into the following categories.
- • Dry heat.
- • Moist heat.
- • Filtration.

   Dry heat
The commonly used methods to sterilize the material is as follows.
- • Red heat flaming.
- • Hot air sterilization.

   Red heat flaming
   Purpose
Used to sterilize material such as inoculating wire/loop, tip of the forceps, searing Iron, scalpel etc.

Material required

- Bunsen burner attached to gas supplies
- Match box

Procedure
- Light the burner with the help of match box.
- Adjust the cone of the flame to blue.
- Hold the inoculum wire/ loop/ tip of the forceps etc. vertically and heat till it gets red hot. Allow to cool before use.
- Put off the flame.

GLP

➤ Each time when heating in the Bunsen burner flame, allow to cool down the instrument. Check loop/wire etc. by touching a portion of the medium to be inoculated.

➤ Heat the loop vertically so that the entire length of the loop is heated.

➤ Dip the loop in disinfectant solution before heating to avoid splattering.

Hot air Sterilization

Purpose
The method is used for sterilizing the material like dry glass test tubes, petri dishes, flasks, glass pipettes, all glass syringes etc. and instruments like forceps, scalpels etc.

Equipment required
Sterilization by hot air should be carried out in an oven which should at least have the following features.
➤ Electrically operated (alternative gas or kerosene oil).
➤ Fitted with the thermostat to control the temperature.
➤ Device to indicate the temperature of inside chamber of the oven when in use.
➤ Should be fitted with adjustable shelves.
➤ Provided with fan to ensure uniform distribution of heat in different parts of the oven.

Procedure
- Arrange the material (pre washed & packed) to be sterilized, loosely and evenly on the racks of the oven so that air can circulate properly and heat the load evenly in the oven.
- Switch on the power supply (or alternative devise gas/ kerosene oil).
- Control the temperature of the oven by adjusting the thermostat knob.
- Note the time when desired temperature is reached (Heating time).
- Hold the load on the same temperature for the specified period as mentioned below.

Temperature	Holding Time
160° C	60 minutes.
170° C	40 minutes.
180° C	30 minutes.

- The most common temperature for hot air oven for sterilization is 160^{0}C for 60 minutes.
- On expiry of the holding time period, switch off the power supply and allow the oven to cool down slowly.
- Put down the date of sterilization on each packet and store in dust free area for future use.
- Make daily records of the equipment/ material sterilized as per the proforma given below.

Detail of all items Sterilized.	Temp. at which Sterilized	Starting time	Heating time	Holding time	Remarks
		From to	**From to**	**From to**	

GLP

- ➢ Dry up all the material before putting into sterilization in hot air oven.
- ➢ Don't place heat sensitive material inside the oven.
- ➢ As air is poor conductor of heat, do not pack the material to be sterilized in the oven too tightly.
- ➢ After holding time is over, hot air oven is switched off, wait until the temperature of the oven falls below 80°C. Only then open the door of the oven to take out the material other wise opening immediately after holding time leads to breaking of the glassware and may also cause injuries to the operator.

Moist heat

Moist heat or steam under pressure is one of the most efficient method of sterilization. Depending upon the material to be sterilized moist heat can be applied in different forms as discussed below.

Below 100^0 C

- Pasteurization 63°C - 80° C for 30minutes.
- Tyndalisation : Intermitent exposure at 75 - 80°C for 20 - 45 minutes on three successive days.
- Boiling at 100° C for 5 - 10 minutes.
- Steaming at 100°C for 1 hr.

Steaming under pressure (Autocalving)

Purpose
Saturated steam under pressure is more efficient way of sterilization as compared to dry heat because
> ➢ It provides greater lethal action.
> ➢ It is quicker in heating up the exposed articles.
> ➢ It penetrates the porous material such as cotton wool, stoppers, paper, cloth wrapper etc.

Principle
When water boils its vapour pressure is equal to surrounding atmospheric pressure. When boiling is done in a closed vessel, there is increase in the inside pressure of vessel which raises the temperature of boiling water above 100°C.

Item to be sterilized
Autoclave is mostly suitable for.
> ➢ Sterilization of culture media, aqueous solution.
> ➢ Decontamination of discarded culture and other laboratory garbage.
> ➢ Rubber guard, gloves, stoppers with rubber liner, glassware with rubber attachment, glass metal syringes, throat swabs etc.

Type of Autoclaves
In principal two type of autoclaves are used
> ➢ Pressure cooker type.
> ➢ Gravity displacement type.

Pressure cooker type

This is the most common type of autoclave used for sterilization. It has vertical chamber with a strong metal lid, which can be fastened down, and sealed with rubber gasket. An air steam discharger tap, pressure gauze and safety cum pressure adiustable valve are fitted on the lid. Water in the bottom of the autoclaves is heated electrically (or by some other device like gas burner/ kerosene oil).

Procedure
> ➢ Arrange the material (pre washed & packed) to be sterilized.
> ➢ Ensure that there is sufficient quantity of water in the chamber at the time of autoclaving by checking water level device provided with the equipment.
> ➢ Place the material to be sterilized in wire basket/ perforated container loosely.
> ➢ Place the container on perforated rack placed above the level of the water.
> ➢ Fasten the lid tightly with steam discharge valve open.
> ➢ Switch on the power and allow the water to boil.
> ➢ When water boils, steam will come out of the discharge valve so that air from chamber may be expelled.
> ➢ Wait till total air inside the chamber has been replaced by steam. This can be checked by connecting one end of the rubber tube to the discharge valve and immersing other end of tube into the bucket containing water. The discharge gas will pass through the water, steam will condense and air will bubble through water. When bubbles cease, it means that air from chamber has been expelled.

- ➢ Close the steam discharge valve.
- ➢ Adjust the valve to predetermined pressure (Normally autoclaves are adjusted at 15 Ibs/ sq inch.).
- ➢ Allow the pressure to increase to preadjusted pressure.
- ➢ Note the time when pressure gauze indicates the requisite pressure is achieved.
- ➢ Allow to continue the same for required time period as indicated below.

Temp	Pressure	Time
115°C	10 lbs/inch	20-30 min.
121 °c	15 lbs/inch	15-20 min.
132°C	27 lbs/inch	2min.

Holding time increases to 30-45min if at 121°C and 15 lbs pressure if plastic wares are sterilized
- ➢ At the end of holding time switch off the power supply.
- ➢ Allow the autoclave to cool slowly, which can be seen by gradual decrease in pressure till it shows zero reading.
- ➢ Allow the wrapping paper to be dried.
- ➢ Put date on each article and place in dust free area for future use.

GLP

- ➢ Ensure that air from chamber has been expelled completely because air steam mixture has a lower temperature than steam e.g. temperature of 50% air & 50% steam mixture will be 112° C instead pf 121^0 C provided by the pure steam.
- ➢ Air also hinders the penetration of steam in to the interior of the porous material and narrow opening container. Air being denser than steam tends to form a separate cooler layer in the bottom of the autoclave.
- ➢ As the simple autoclave lack means for drying the load after sterilization, it is therefore important to avoid placing sterilized articles in contact with unsterilized objects/ surface unless the wrapping is dried
- ➢ To check the efficacy of autoclave, each cycle should be run using chemical indicator tape.

Record Keeping
Daily recording of each run for sterilization of material should be maintained e.g.

Date	Detail of all items Sterilized.	Pressure at which SteriIized done	Starting time	Heating time	Holding time	Remarks
			From to	**From to**	**From to**	

Quality control

To check the efficacy of autoclave each run should be accompanied by placing chemical indicator which changes color if the instrument is working satisfactory. This can be achieved by placing chemical indicator tape inside the tube in the center of autoclave and check the change in color after the operation is over.

Further Reading

1. World Health Organization. Laboratory biosafety manual. World Health Organization, 2004.

2. Acosta-Gnass, Silvia I., and Valeska De Andrade Stempliuk. Sterilization manual for health centers. Pan American Health Organization, 2010.

ZIKA VIRUS DISEASE

16.1 INTRODUCTION

Zika Virus Disease (ZVD) is a mosquito-borne (Aedes) viral disease caused by Zika virus (ZIKV). It presents as mild fever, rash (mostly maculopapular), headaches, arthralgia, myalgia, asthenia, and non-purulent conjunctivitis, occurring about two to seven days after the mosquito bite. One out of five people may develop symptoms, but in those who are affected the disease is usually mild and may last between two and seven days. Its clinical manifestation is often similar to dengue, also caused by the same vector.

16.2 EPIDEMIOLOGY

The virus was first identified in a rhesus monkey in the tropical Zika Forest in Uganda in April 1947 by the scientists of the Yellow Fever Research Institute. The first human Zika Fever infection was identified in Nigeria in 1954.

Until 1981, evidence of human infection with Zika virus was reported from other African countries, such as the Central African Republic, Egypt, Gabon, Sierra Leone, Tanzania, and Uganda, as well as in parts of Asia including India, Indonesia, Malaysia, the Philippines, Thailand, and Vietnam.

Global Situation

Recent Outbreaks
Outbreak in Yap in 2007: Remote island of Yap (7,500 inhabitants), Federated States of Micronesia reported 49 confirmed and 59 probable cases of ZVD. No hospitalization, haemorrhagic manifestations, or deaths were reported. Aedes hensilli was identified as the predominant vector species. This outbreak represents transmission of Zika virus outside Africa and Asia.

Outbreak in French Polynesia in 2013-14: 5,895 patients with suspected ZIKV infections were recorded with an estimate of 28,000 ZIKV infections in early February 2014 (about 11% of the population). ZIKV was found circulating in the Pacific in 2015 in New Caledonia, Cook Islands, Eastern Island, Vanuatu, Solomon and Fiji.

May 2015: The World Health Organization reported the first local transmission of Zika virus in the region of the Americas, with autochthonous cases identified in Brazil. In December 2015, the Ministry of Health estimated that 440,000–1,300,000 suspected cases of Zika virus disease had occurred.

As of 12th February 2016, 39 countries (Table16.1) have reported locally acquired circulation of the virus since January 2007 (Source:http://who.int/emergencies/zika-virus/situation-report/who-zika-situation-report-12-02-2016.pdf). Geographical distribution of the virus has steadily expanded. World Health Organization (WHO)   declared Zika Virus Disease as a Public Health Emergency of International Concern on 1st February 2016.

Two different lineages of Zika virus (African and Asian lineage) have been reported. The African lineage is further classified into East and West African clusters. Some authors have also described three different lineages of virus; West African, East African and Asian. The Asian lineage which is the currently circulating strain is emerging as a major public health concern in the regions of the Pacific and the South America.

Table.16.1 Countries and territories with autochthonous transmission of Zika virus, 2007 – 2016

	WHO Regional Office	Country or territory
Reported autochthonous transmission (34)	AFRO	Cape Verde
	AMRO/PAHO	Barbados, Bolivia, Brazil, Colombia, Costa Rica, Curaçao, Dominican Republic, Ecuador, El Salvador, French Guiana, Guadeloupe, Guatemala, Guyana, Haiti, Honduras, Jamaica, Martinique, Mexico, Nicaragua, Panama, Paraguay, Puerto Rico, Saint Martin, Suriname, US Virgin Islands, Venezuela
	SEARO	Maldives, Thailand
	WPRO	American Samoa, Samoa, Solomon Island, Tonga, Vanuatu
Indication of viral circulation (6)	AFRO	Gabon
	SEARO	Indonesia
	WPRO	Cambodia, Fiji, Philippines, Malaysia,
Countries with outbreaks terminated (5)	AMRO/PAHO	Eastern Islands
	WPRO	Cook Islands, French Polynesia, New Caledonia, Yap
Locally acquired without vector borne transmission (1)	AMRO/PAHO	United States of America

Reservoir of infection: Unknown.

Immunity: Once a person has been infected, he or she is likely to be protected life long.

Vector: Zika virus is transmitted primarily by *Aedes aegypti* mosquito. *Aedes albopictus* mosquito also might transmit the virus. *Aedes hensilli* was the predominant vector species identified in the Yap outbreak.

About the vector: *Aedes* mosquito is common vector to transmit the viruses that cause dengue, chikungunya, West Nile fever and ZVD. *Aedes* mosquito can be recognized by white markings on its legs and a marking in the form of a lyre on the upper surface of the thorax. An infective female *Aedes* mosquito acquires the virus while feeding on the blood of an infected person and then viruses are passed on to healthy humans through its bites.

The density of *Aedes* mosquito is more during monsoon and post-monsoon season. Female *Aedes* mosquito is a day biter and is most active during daylight, for approximately two hours after sunrise and several hours before sunset. Itbites many times to complete full blood meal, therefore it is known as indiscriminate feeder. The male mosquito does not bite humans or animals. *Aedes* prefers to breed in man-made containers, viz., water storage containers, coolers, discarded buckets and plastic containers, bottles, tyres and coconuts shells, etc. in which water stagnates for more than a week. It prefers to lay its eggs singly on damp surfaces just above the water line, in clean water which contains no other living species. In unfavorable conditions, the eggs can be viable for over a year in dry state, which allows the mosquito to re-emerge after winter or dry spell. The life cycle lasts for 8-10 days in favorable conditions. Aedes.aegypti rests indoor, in dark corners of the houses, on dark cloths, umbrellas, under furniture- beds, shelves, coolers, behind hanging, shoes, besides household articles, curtains etc. but rarely on walls. Outside it rests in cool and shaded places. The flight range for Aedes is generally 100 metres but it can fly up to 400 metres.

16.2.1 Mode of Transmission

ZIKV is transmitted by the bite of infected female Aedes mosquitoes. ZIKV has adapted to an enzootic cycle involving arboreal mosquitoes in Africa to a new urban cycle including humans as reservoirs and urban mosquitoes as vectors.

The virus has been isolated from several Aedes mosquito species, notably Aedes aegypti, which is widespread in the tropics and subtropics, and Aedes albopictus, which is established in many parts of Europe, especially in Mediterranean countries. In FP, Aedes polynesiensis is also suspected to contribute to ZIKV transmission.

Other modes of transmission are still under investigation. Cases of non-vector borne ZIKV transmission through sexual intercourse and perinatal transmission have been reported. Sexual transmission of Zika Virus is possible and is of particular concern during pregnancy. Sexual transmission of Zika virus from infected women to their sex partners has not been reported. However, transmission of Zika virus has been reported from men who have traveled to or reside in areas with active Zika virus transmission to their sex partners.

Recommendations for prevention of sexual transmission of Zika virus for couples in which a man has traveled to or resides in an area with active Zika virus transmission

- Couples in which a woman is pregnant should use condoms consistently and correctly or abstain from sex for the duration of the pregnancy.
- Couples in which a man had confirmed Zika virus infection or clinical illness consistent with Zika virus disease should consider using condoms or abstaining from sex for at least 6 months after onset of illness.
- Couples in which a man traveled to an area with active Zika virus transmission but did not develop symptoms of Zika virus disease should consider using condoms or abstaining from sex for at least 8 weeks after departure from the area.
- Couples in which a man resides in an area with active Zika virus transmission but has not developed symptoms of Zika virus disease might consider using condoms or abstaining from sex while active transmission persists.

Source: (http://www.cdc.gov/mmwr/volumes/65/wr/mm651 2e3er.htm?s_cid=
mm6512e3er.htm_w) Transfusion related ZIKV transmission is also a potential risk.

Transfusion-related ZIKV transmission is also a potential risk.
Incubation period: 2 to 7 days but may extend to a few weeks.

16.3 CLINICAL FEATURES

An estimated 80% of persons who are infected with Zika virus are asymptomatic. In the majority of cases, ZVD is a self-limiting disease. The symptoms are similar to other arbovirus infections such as dengue, and include fever, skin rashes, conjunctivitis, muscle and joint pain, malaise, and headache. These symptoms are usually mild and last for 2-7 days.Other reported symptoms are dizziness, oedema of the extremities, retro-orbital pain, anorexia, photophobia, gastro-intestinal disorders, sore throat, cough, aphtous ulcers, back pain, sweating and lymphadenopathies. None of these symptoms are specific and ZVD can be misdiagnosed with other bacterial and viral infections, especially with other arboviruses in endemic areas.

Complications: Birth defects like microcephaly, neurologic complications like Guillain-Barré syndrome are temporarily associated with this outbreak. Researchers are studying a potential - but unproven - link between the surge in GBS cases and Zika virus infection. There is no evidence that prior Zika virus infection poses a risk for birth defects in future pregnancies.

16.4 TREATMENT

ZVD is usually **relatively mild** and requires **no specific treatment**.

People sick with Zika virus should:
- Get plenty of rest,
- Drink plenty of fluids, and
- Receive symptomatic treatment with acetaminophen (paracetamol) for pain and fever and antihistaminic for pruritic rash.

If symptoms worsen, they should seek medical care and advice. Acetylsalicylic acid and non-steroidal anti-inflammatory drugs are **not recommended** due to the increased risk of hemorrhagic syndrome described with other arboviruses as dengue.

16.4.1 Case definitions

Suspected case - Patient with skin rash or elevation of body temperature more than or equal to 37.2 degrees with one or more of the following symptoms (not explained by other medical conditions):

- Arthralgia or myalgia
- Non purulent conjunctivitis or conjunctival hyperemia
- Headache or malaise

With history of travel to countries with indigenous transmission of ZIKV in last 2 weeks. The country details are available at www.cdc.gov/zika/geo/index.html

Confirmed case – A suspected case with laboratory positive result for the specific detection of ZIKV by RT-PCR.

16.4.2 Guidelines for sample collection

During the first week after onset of symptoms, ZVD can often be diagnosed by performing reverse transcriptase-polymerase chain reaction (RT-PCR) on serum by both conventional and real time methods. Virus-specific IgM and neutralizing antibodies typically develop toward the end of the first week of illness; cross-reaction with related flaviviruses (e.g., dengue and yellow fever viruses) is common and may be difficult to discern. Plaque-reduction neutralization testing can be performed to measure virus-specific neutralizing antibodies and discriminate between cross-reacting antibodies in primary flavivirus infections. Viral isolation is not regarded as a diagnostic tool and is recommended only for supplemental research studies in public health surveillance.

Pan American Health Organization (PAHO) has issued interim guidance for laboratory detection and diagnosis of ZVD surveillance in the Americas. An algorithm has been developed for detection of introduction of the virus into a specific area. This algorithm is addressed to reference laboratories with established capacity (molecular/antigenic and serological) to detect dengue, chikungunya), and Zika viruses. A BSL2 containment level is required to handle suspected samples. These recommendations are subject to modifications that take into account advances in knowledge of the disease and the etiologic agent.

16.5 LABORATORY DIAGNOSIS

16.5.1 Sample storage

- **Blood** (5ml in plain vial) of suspect case
- Keep refrigerated (2-8 degree C) if it is to be processed (or sent to a reference laboratory) within 48 hours
- Keep frozen (-10 to -20 degree C) if it is to be processed after the first 48 hours or within 7 days.
- Keep frozen (-70 degree C) if it is to be processed after a week. The sample can be preserved for extended periods.

16.5.2 Transportation of the sample to the reference laboratory

Always use triple layer packaging and ship within 48 hours of collection under cold chain (dry ice or at least with cooling gels). The original samples should be packed, labeled and marked. Always include the completely filled out clinical and epidemiological record (proforma on Page 7).

16.5.3 Molecular diagnosis: Blood

(5ml in plain vial) of suspect case (see previous section for case definition) should be **collected during first five days of illness** and sent to the laboratory for RT PCR with prior intimation. ZIKV RNA also has been detected in urine over an extended period in the acute phase, which means that could be an alternative sample to be considered. However, and since more studies are needed, it is recommended that the serum sample be taken during the first 5 days after the onset of symptoms for RT PCR.

16.5.4 Serological Diagnosis

As on date, this has not been standardized in India. However, the diagnosis can be undertaken by detecting ZIKV-specific IgM antibodies by ELISA or immunofluorescence assays in serum specimens from day 5 after the onset of symptoms. Since a single serum in the acute phase is presumptive, it is recommended that a second sample be taken 1–2 weeks after the first sample to demonstrate seroconversion (negative to positive) or a fourfold increase on the antibody titer (with a quantitative test). The interpretation of the serological tests is especially important for the diagnosis of ZIKV. In primary infections (first infection with a flavivirus) it has been demonstrated that antibodies cross-reaction is minimal with other genetically related viruses. However, it has been demonstrated that sera of individuals with a previous history of infection from other flaviviruses (especially dengue, yellow fever and West Nile) can cross-react in these tests. As on date no commercial kits have been approved or validated for the serological determination of ZIKV.

16.6 PREVENTION AND CONTROL

The key to control ZVD is adoption of a comprehensive approach by way of regular vector surveillance and integrated management of the Aedes mosquitoes through biological and chemical control that are safe, cost effective; and environmental management, legislations as well as action at household and community levels.

16.6.1 Integrated vector management:

1. **Vector Surveillance**
Several indices have been described and are currently used to monitor *Aedes* population**.**
Larval surveys: Water holding containers are examined for the presence of mosquito larvae and pupae in the house or premises. Indices commonly used to monitor Aedes are: House index (HI), Container Index (CI), Breteau Index (BI) and Pupae Index (PI)

Adult Surveys are done by Landing/biting collection, resting collection, Oviposition traps.

2. **Vector management**
Environmental Management
* **Environmental modification:**Long lasting physical transformation of vector habitats.Improved water supply, mosquito proofing of overhead tanks, cisterns or underground reservoirs.
* **Environmental manipulation:**Temporary changes to vector habitats as a result of planned activity to produce conditions unfavourable to vector breeding.

- **Changes in human habitations:**Efforts to reduce human-pathogen-vector interactions

3. Personal prophylactic measures:

Personal protection against mosquitoes by using mosquito coils, pyrethrum space spray and aerosols. Insecticide treated bed nets can be effectively used to protect infants and night workers while sleeping in daytime

4. Biological control

- Larvivorous fish are recommended for control of *Aedes aegypti* in large water bodies or large water containers.
- Endotoxin-producing bacteria, *Bacillus thuringiensis* serotype H-14 (Bt H-14) has been found as an effective mosquito control agent.

5. Chemical control

Chemical control measures (larvicides, adulticides) are recommended in permanent big water containerswhere water has to be conserved or stored because of scarcity of water or irregular and unreliable water supply.

Larvicide: Temephos, an organophosphate compound insecticide is being used under the public health programme. The recommended dose for application of Temephos (50 EC) is 1 ppm (1 mg per liter of water).

Adulticide: Recommended methods for the control of adult *Aedes* are:

- Pyrethrum spray used in indoor situations as space spray at a concentration of 0.1% - 0.2% @ 30-60 ml/1000 cu. ft.
  One litre of 2% pyrethrum extract is diluted with 19 litres of kerosene to make 0.1% pyrethrum formulation ('ready-to-spray' formulation). After dilution, pyrethrum extract is sprayed with Flit pump or hand operated fogging machine fitted with micro-discharge nozzle.
- Malathion fogging or Ultra Low Volume (ULV) spray by using 95% or pure technical malathion provides maximum effectiveness against target vectors.

6. Legislation:

Laws and byelaws should be enacted and implemented for avoidance of mosquitogenic conditions at construction sites, factories, etc.

7. Health education

At Household Level:

- Adult mosquitoes should be killed by using of commercially available safe aerosols (Pyrethroid-based).
- Wear protective clothing (full sleeved shirts & full pants during day time) and use mosquito nets, preferably insecticide treated ones, while sleeping, even during day time.
- Use mosquito repellents.
- Use tight-fitting screens/wire mesh on doors and windows.

- Intensify efforts to reduce actual or potential larval habitats in and around houses by:
    - Covering all water containers in the house to prevent fresh egg laying by the vector.
    - Emptying, drying water tanks, containers, coolers, bird baths, pets' water bowls, plant pots, drip trays at least once each week.
    - Regularly checking for clogged gutters and flat roofs that may have poor drainage.
    - Introducing larvivorous fishes (e.g., Gambusia / Guppy) in ornamental water tanks.

At community level:
- Identify commercial activities such as traders dealing in used tyres or small construction projects, etc, which may be creating larval habitats for the vector.
- Launch awareness campaigns on ZVD and seek cooperation for prevention of mosquito breeding and protection from mosquito bites.
- Community activities against larvae and adult mosquitoes include:
    - Cleaning and covering water storage containers.
    - Keeping the surroundings clean and improving basic sanitation measures.
    - Burning mosquito coils to kill or repel the mosquitoes/burning neem leaves, coconut shells and husk to repel mosquitoes and eliminating outdoor breeding sites.
    - Cleaning weeds and tall grass to reduce available outdoor resting places for adult mosquitoes near houses.
    - Organizing camps for insecticide treatment of community owned mosquito nets/curtains.
    - In case water containers cannot be emptied, applying Temephos (1 ppm) on weekly basis in coordination with the Health authorities.
    - Mobilizing households to cooperate during spraying / fogging.

At Institutional Level (Hospitals, Schools, Colleges, Other Institutions, Offices, etc):
- Weekly checking for Aedes larval habitats especially overhead tanks, ground water storage tanks, air coolers, planters, flower pots, etc.
- Ensuring source elimination

Source:(http://nvbdcp.gov.in/Doc/dengue_1_.%20Director_Desk%20DGHS%20meeting%20OCT%2006.pdf)

Vaccine
There is **no vaccine** against Zika fever.

India Preparedness

Travel Advisory:

The salient points include
- Non-essential travel to the affected countries to be deferred/ cancelled.

- Pregnant women or women who are trying to become pregnant should defer/ cancel their travel to the affected areas.

- All travelers to the affected countries/ areas should strictly follow individual protective

measures, especially during day time, to prevent mosquito bites (use of mosquito repellant cream, electronic mosquito repellants, use of bed nets, anddress that appropriately covers most of the body parts).

- Persons with co-morbid conditions (diabetes, hypertension, chronic respiratory illness, Immune disorders etc.) should seek advice from the nearest health facility, prior to travel to an affected country.

- Travelers having febrile illness within two weeks of return from an affected country should report to the nearest health facility.

- Pregnant women who have travelled to areas with Zika virus transmission should mention about their travel during ante-natal visits in order to be assessed and monitored appropriately.

International Airports and Ports

- All the International Airports / Ports to display billboards/ signage providing information to travelers on ZVD and to report to Immigration authorities if they are returning from affected countries and suffering from febrile illness.

- The Airport / Port Health Organization (APHO / PHO) to have quarantine/ isolation facility in identified Airports.

- Directorate General of Civil Aviation, Ministry of Civil Aviation have been requested to instruct all international airlines to follow the recommended aircraft disinfection guidelines

- The APHOs to circulate guidelines for aircraft disinsection (as per International Health Regulations) to all the international airlines and monitor appropriate vector control measures with the assistance from NVBDCP in airport premises and in the defined perimeter

Surveillance

- Provisional Case definitions for suspected and confirmed cases of ZVD have been shared with all the state surveillance units.

- Integrated Disease Surveillance Programme (IDSP) through its community and hospital based data gathering mechanism to track clustering of acute febrile illness, if any, among those who travelled to areas with ongoing transmission in the 2 weeks preceding the onset of illness.

- Rapid Response Teams (RRTs) activated at Central and State surveillance units. Each team consists of an epidemiologist / public health specialist, microbiologist and a medical / pediatricspecialist and other experts (entomologist etc.) to travel at short notice to investigate suspected outbreak.

- The Maternal and Child Health Division (under NHM) has established Sentinel Surveillance System in identified institutions representing different parts of the country to identify any unusual increase or clustering of microcephaly cases. Instructions have been issued to Sentinel

Sites for identificationand notificationof newbornswith microcephaly (Head circumference-starting above the eyebrows and ears and around the back of the head measuring less than 31 cm measured within 48 to 72 hoursof birth). The details of such identification may be notified weekly to theNational Monitoring team including "a nil" report by email. (nationalrbskunit@gmail.com).

- Sample collection and transportation guidelines shared with all the Statesurveillance units.

- ICMR Institutes, Vector Control Research Centre, Puducherry, Centre for Research in Medical Entomology, Madurai, National Institute of Malaria Research, Delhi & its Field Unit at Bengaluru, have initiated mosquito surveillance for Zika virus within the States of Tamil Nadu, Kerala, Karnataka and Puducherry. This is to gather information on circulation of virus in the community and vector population, if prevalent, in the high risk areas.

- National Blood Transfusion Council (NBTC) has advised the blood banks not to accept blood from donors till 2 weeks following complete recovery from acute viral infection and cessation of any therapy/ medications. Potential blood donors who have returned from outbreak zone should not donate blood for 4 weeks from date of return.

Laboratories strengthened for ZVD:

National Institute of Virology (NIV), Pune and National Centre for Disease Control (NCDC), Delhi, have the capacity to undertake diagnosis of ZVD by RT-PCR test.

Further, ten Viral Research & Diagnostic Laboratories of Department of Health Research and ICMR and ICMR institutes are strengthened:
1. King Institute of Preventive Medicine, Chennai
2. NIV Field Unit, Allapuzzha
3. Manipal Centre for Virus Research, KMC, Manipal
4. BJ Medical College, Ahmedabad
5. Regional Medical Research Centre, Bhubaneshwar
6. National Institute for Cholera & Enteric Diseases, Kolkata
7. King George Medical University, Lucknow
8. Regional Medical Research Centre, Dibrugarh
9. Regional Medical Research Centre, Jabalpur
10. Jawaharlal Institute of Post-Graduate Medical Education & Research, Puducherry

Risk Communication:
- The States/ UT Administrations to create increased awareness among clinicians including obstetricians, pediatricians and neurologists about ZVD and its possible link with adverse pregnancy outcome (fetal loss, microcephaly etc.). There should be enhanced vigilance to take note of travel history to the affected countries in the preceding two weeks.

- The Central/ State Government to take all necessary steps to work closely with technical institutions, professionals and global health partners. The public needs to be reassured that there is no cause for undue concern.

Further Reading

1. Petersen, Lyle R., et al. "Zika virus." New England Journal of Medicine 374.16 (2016): 1552-1563.

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6. National Guidelines on Zika Virus Disease. Ministry of Health and Family Welfare. (Available at: http://www.ncdc.gov.in/index2.asp?slid=566&sublinkid=224)

CRIMEAN-CONGO HAEMORRHAGIC FEVER (CCHF)

17.1 INTRODUCTION

Crimean-Congo haemorrhagic fever (CCHF) is a viral haemorrhagic fever caused by tick-borne virus of the nairovirus group The disease was first described in the Crimea (former USSR) in 1944 and given the name Crimean haemorrhagic fever. In 1969, it was recognized that the pathogen causing Crimean haemorrhagic fever was the same as that responsible for an illness identified in 1956 in the Congo, and linkage of the two place names resulted in the current name for the disease and the virus.

Global Scenario

The geographic range of CCHF virus is the most extensive among the tick borne viruses that affect human health, and the second most widespread among all medically important arboviruses, after Dengue viruses. The disease is endemic in many countries in Africa, Europe Middle East and Central Asia with sporadic outbreaks recorded in Kosovo, Albania, Iran, and Turkey. CCHF outbreaks constitute a threat to public health services because of its epidemic potential, its high case fatality ratio (10-40%), its potential for nosocomial (hospital acquired infection) outbreaks and the difficulties in its treatment and prevention (**Source WHO**).

Indian Scenario

In India, the first laboratory confirmed outbreak was reported in January, 2011 in Gujarat where 7 cases and 2 deaths were reported from Ahmedabad. This had nososcomial transmission. Subsequently outbreaks of CCHF have been reported from different parts of Gujarat Amreli, Anand, Aravalli, Jamnagar, Kutch, Mehsana, Morbi, Patan, Sabarkantha, Surendranagar, Rajkot, Kheda). Three outbreaks have been reported from Rajasthan (Chittorgarh, Jodhpur, Sirohi) in 2014 and 2015. An outbreak was also reported from Muradabad, UP in 2015. (Table 17.1)

Table 17.1: Cases (C) & Deaths (D) of CCHF reported under IDSP 2011-16 (Data till 9/3/16)

	State	2011		2012		2013		2014		2015		2016	
		C	D	C	D	C	D	C	D	C	D	C	D
1.	Gujarat	9	4	2	1	18	8	5	4	16	8	4	2
2.	Rajasthan	-	-	-	-	-	-	1	0	5	3		
3.	Uttar Pradesh	-	-	-	-	-	-	-	-	1	1		
	Total	9	4	2	1	18	8	6	4	22	12	4	2

17.2 EPIDEMIOLOGY

Causative Agent: CCHF virus belongs to family Bunyaviridae, genus Nairovirus. It is an enveloped, single stranded negative sense RNA virus with tripartite genome. Since it is an enveloped virus, it can be readily inactivated. Suitable disinfectant solutions include 1 % sodium hypochlorite, 10% aqueous solution of household bleach, solution of glutaraldehyde (2% or as recommended by the manufacturer) and phenolic disinfectants (0.5%-3%). Soaps and detergents can also inactivate the virus and should be used liberally for washing hands. The virus gets inactivated by heating at 56^0 C for 30 minutes. CCHF virus is stable for upto 10 days in blood kept at 40^0C.

Vector: Ticks are arthropods which suck blood from animals and humans. A number of tick genera are capable of becoming infected with CCHF virus, but the most efficient and common vector of CCHF are the member of Hyalomma genus, the family Ixodidae.
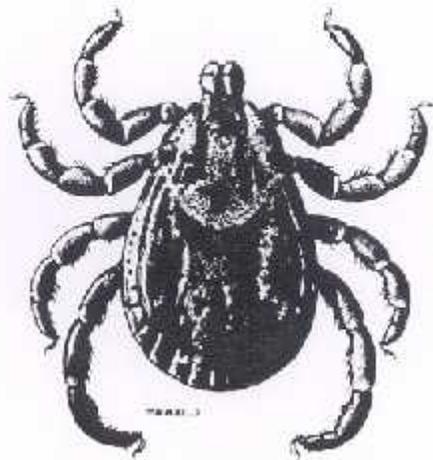

Fig 17.1. Adult hard tick, *Dermacentor andersoni*, family *Ixodidae* (from Lane and Crosskey 1993)

The ticks are also natural reservoir of CCHF virus. The Hyalomma ticks are hard ticks, can be easily distinguished by four pairs of legs in adults and lack of clear segmentation of the body. The adult ticks are flat and oval in shape. The ticks have four life stages viz. eggs, larvae, nymph and adults. Larva and nymph remain on the same host. The engorged nymph drops to the soil where it moults to adult from which seeks new host. Larvae, nymph and adults need blood meal for their maturation. Both larvae and nymph largely feed on lower vertebrates (such as rodents, rabbits, hare etc.) while the adults feed on higher vertebrates (such as cattle, goat, sheep etc.). Male and female ticks suck blood. Both male and female can act as a vector for disease transmission. Transovarial transmission (transmission of the virus from infected female ticks to offspring via eggs) and trans-stadial (ie, from larvae to nymph to adult) transmissions have been demonstrated amongst some vector species, indicating a mechanism which may contribute to maintaining the circulation of the virus in nature.

Reservoir: Hard ticks are the reservoir and the vector for CCHF virus. In addition, infected animals may also act as reservoir during the period of viraemia. The CCHF virus may infect a wide range of wild animals and domestic ruminant animals such as hares, rats, camel, cattle,

sheep and goats. Many birds are resistant to infection, but ostriches are susceptible and may show a high prevalence of infection in endemic areas. Animals become infected with CCHF from the bite of infected ticks. The most important source for acquisition of the virus by ticks is believed to be infected small vertebrates on which immature Hyalomma ticks feed. Domestic ruminant animals, such as cattle, sheep and goats, are viraemic for around one week after becoming infected.

Environmental factors: Ecological changes, poverty, social instability, poor health services, and absence of standard infection control practices have contributed to increased transmission of the CCHF virus.

Mode of transmission

- **Animal to Human Transmission:** Human beings may acquire the CCHF virus by direct contact with blood or other tissues of infected livestock or they may become infected through a tick bite or crushing of infected tick. Meat itself is not a risk because the virus is inactivated by post-slaughter acidification of the tissues and would not survive cooking.
- **Human to Human Transmission:** Humans can become infected if blood, body fluids and wastes from patients with the disease comes into contact with broken skin or mucous membranes, as occurs when medical care personnel sustain accidental needle stick injury. In advanced stages of the disease, aerosol contact of blood of the patient can also lead to transmission of the virus.
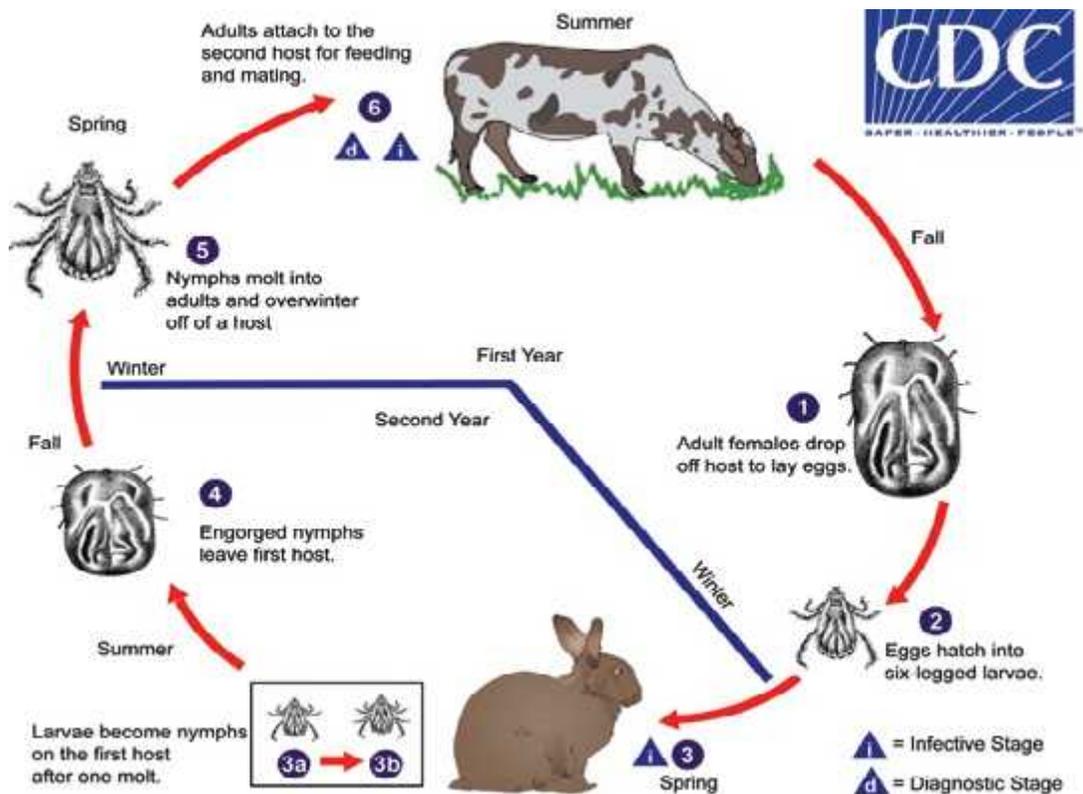
Fig 17.2 . Life cycle of the two-host ixodid (hard) ticks

Population at Risk: In endemic countries, majority of cases have occurred in those involved with the livestock industry, such as agricultural workers, slaughterhouse workers and veterinarians. Health care workers attending on suspect/ probable/confirmed CCHF cases and not following contact precautions are at high risk of getting infection. Hospital acquired infection outbreaks (Nosocomial spread) have been reported in many countries.

Transmission cycle: CCHF virus circulates in an enzootic tick–vertebrate–tick cycle, and there is no evidence that the virus causes disease in animals.

Incubation period: The incubation period for the illness depends upon the mode of acquisition of the virus. Following infection via tick bite, the incubation period is usually one to three days, with a maximum of nine days. The incubation period following contact with infected blood or tissues is usually five to six days, with a documented maximum of 13 days.

Communicability: Highly infectious in the hospital settings. Nosocomial infections are common after exposure to blood and secretions.

Susceptibility: Immunity after infection is probably lifelong.

17.3 CLINICAL FEATURES

Onset of symptoms is sudden, with fever, myalgia, dizziness, neck pain, stiffness, backache, headache, sore eyes and photophobia. There may be nausea, vomiting and sore throat early on, which may be accompanied by diarrhoea and generalized abdominal pain. Over the next few

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days, the patient may experience sharp mood swings, and may become confused and aggressive. After two to four days, the agitation may be replaced by sleepiness, depression and lassitude, and the abdominal pain may localize to the right upper quadrant, with detectable hepatomegaly (liver enlargement). Other clinical features include tachycardia, lymphadenopathy and a petechial rash both on internal mucosal surfaces, such as in the mouth and throat, and on the skin. The petechiae may give way to ecchymoses (like a petechial rash, but covering larger areas) and other haemorrhagic phenomena such as melaena (bleeding from the upper bowel, passed as altered blood in the faeces), haematuria (blood in the urine), epistaxis (nosebleeds) and bleeding from the gums. There is usually evidence of hepatitis. The severely ill may develop hepatorenal and pulmonary failure after the fifth day of illness.

The mortality rate from CCHF is approximately 9-50% with death occurring in the second week of illness. In those patients who recover, improvement generally begins on the ninth or tenth day after the onset of illness.The convalescence period begins in survivors about 10–20 days after the onset of illness. In the convalescent period, labile pulse, tachycardia, temporary or complete loss of hair, polyneuritis, difficulty in breathing, xerostomia, poor vision, loss of hearing and loss of memory have been reported. Malaria, Leptospirosis, Rickettesial diseases, Meningococcemia, Dengue Haemorrhagic Fever, Haemolytic Uremic Syndrome, and Thrombocytopenic Purpura form the differential diagnosis of CCHF.

17.4 LABORATORY DIAGNOSIS

As CCHF virus is classified as risk group 4 virus and hence the clinical samples should be handled in specially-equipped, high biosafety level laboratories (BSL 3 plus or 4).

Sample Collection

- **Ante-mortem:** Blood sample: Serum/Plasma
- **Postmortem:** Tissue sample (liver, spleen, bone marrow, kidney, Lung and brain)

In the first few days of illness diagnosis is achieved by virus/genome detection in blood or tissue samples.

Collection: Samples should be collected with all biosafety precautions including PPE and should be accompanied with detailed history of patient on the performa which can be obtained from the testing laboratory.

Transportation of sample: Sample should be safely packed in Triple container packing and should be transported under cold chain to the reference laboratory with prior intimation. However, in the areas where obtaining such container is difficult the samples can be sent as follows:

- The case sheets with complete information about the samples should be completely filled in Case report Form (separate sheet) and provided along with the samples.
- The blood sample [Serum or plasma or blood in EDTA] should be kept in screw cap plastic vials, with proper label.

- The sample containing vials should be kept in good quality plastic bags which should either be sealed by heat or tied with rubber bands so that inside material, if leaks, should not come out of the bag.
- This plastic bag should be placed in another plastic container which should be sealed with adhesive tape. This carrier should then be placed in another plastic bag sealed with rubber bands and be placed in a thermocol or vaccine carrier containing ice.
- If plastic container is not available then good quality of double plastic bags can be used.
- The case sheets with complete information should be placed in a plastic bag or envelop and be pasted outside of the thermocol or vaccine container.
- Person handling the sample should wear gloves and a gown to avoid direct contact with the infectious material. After completing the packing of samples, person should thoroughly wash hand with soap and water.
- Before dispatching the container, Bleach can be used for disinfection. A 1:100 dilution of bleach or 5% Lysol solution should be used to clean the outer surfaces of the container.

Virus Isolation: It should always be carried out in maximum bio-containment laboratory i.e. BSL -4. The virus may be isolated from blood or tissue specimens in the first five days of illness, and grown in cell lines.

Molecular Technique: The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is the test of choice for laboratory diagnosis of CCHF virus infection for detecting virus specific genome. It is a sensitive and specific method. Specificity and sensitivity can be further enhanced by using automated real time PCR. Viral antigens may often be detected in tissue samples using immune-fluorescence or "ELISA".

Serology: IgG and IgM antibodies may be detected in serum by enzyme-linked immunoassay (the "ELISA") from about day six of illness. IgM remains detectable for up to four months, and IgG levels decline but remain detectable for up to five years.
Recent or current infection is confirmed by demonstrating seroconversion or a fourfold or greater increase in antibody titre in paired serum samples or detection of IgM antibodies by IgM capture ELISA (MAC ELISA) in a single sample.

17.5 MANAGEMENT AND TREATMENT

Supportive therapy: General supportive therapy is the mainstay of patient management in CCHF. Intensive monitoring to guide volume and blood component replacement is required.
- In case of hypotension and hemodynamic instability patient should be managed on standard guidelines for the treatment of shock which includes resuscitation, fluid supplements (crystalloids/ colloids) and ionotropic support.
- In suspected secondary bacterial infection patient should be treated on standard guidelines / practice for community acquired/ nosocomial infections. Proton pump inhibitors can be considered on case to case basis.
- There is no definite role of steroids for managing this illness per se. Correction of coagulation abnormalities (only if present) with the use of PRP/SDP; FFP, cryoprecipitate, as per indications may be considered.
- Platelet transfusion may be considered if there is significant bleeding with thrombocytopenia.

- Use Paracetamol for fever, avoid other NSAID.
- Ventilator/ renal support may be provided as per standard guidelines.

There is currently no specific antiviral therapy for CCHF. However, ribavirin has been shown to inhibit in-vitro viral replication in Vero cells and reduced the mean time to death in a suckling mouse model of CCHF. Additionally, several case reports have been published that suggest oral or intravenous ribavirin is effective for treating CCHF infections.

Ribavirin is a member of the nucleoside anti metabolite drugs that interfere with duplication of viral genetic material. This is the only antiviral known to have some affect on the viruses causing VHF.

Ribavarin treatment protocol:
For adults (IV/Oral)

- 2 gm loading dose
- 4 gm/ day in 4 divided doses(6 hourly) for 4 days
- 2gm/day in 4 divided doses for 6 days

For Children

Intraenous
- 17 mg/kg loading dose
- 17 mg/kg 6 hourly, day 1-4
- 8 mg/kg 8 hourly, day 5-10

Oral
- 30 mg/kg loading dose
- 15 mg/kg 6 hourly, day 1-4
- 7 mg/kg 6 hourly, day 5-10

For management patients may be categorized as follows

Category-A: Those that have relatively mild disease (fever <38.50C, No systemic bleeding, Alanine Transaminase (SGPT) levels < 150 IU, Platelet count > 50,000). These patients improve spontaneously in about day 10 of illness. Patient can be managed with supporting therapy and regular monitoring for worsening of symptoms. These patients do not require Ribavirin.

Category-B: Those who are in the first 5 days of illness and are severely ill with high grade fever (> 38.50C), local and systemic bleeding manifestations, having Alanine Transaminase (SGPT) levels of 150 IU or more, Aspartate Aminotransferase (SGOT) of 200 IU or more, platelets (< 50,000) or Activated

Partial Thromboplastin Time (APTT) of 60 seconds or more. Even if the patients still look comparatively well at this stage these clinical path values are markers of poor prognosis if recorded during the first 5 days of illness and persons in this group should be treated as soon as possible with ribavirin. Those who are recognized and treated early enough respond remarkably well to ribavirin.

Category C: Patients first seen/recognized as CCHF after day 5 and are in comatose/terminal state with DIC and multi-organ failure. Treatment with ribavirin is indicated but the prognosis is very poor.

Category B&C patients, even if they subsequently test negative, should receive the full course of ribavirin.

Adverse effects: The most common side effect of Ribavirin is mild to moderate haemolytic anaemia which is reversible. Anaemia associated with ribavirin therapy is often asymptomatic and can be managed by monitoring blood count and serum biochemistry. Ribavirin administered as an intravenous bolus has been reported to induce rigors; consequently, it is recommended that the drug be administered as an infusion over 10-15 minutes. There have been reports of pancytopenia and pancreatitis associated with use of intravenous ribavirin.

Contra-indications and precautions: Ribavirin is contraindicated for treatment in pregnant women. Ribavirin has demonstrated significant teratogenic and embryocidal potential in all animal species in which adequate studies have been conducted. It can be given to pregnant women only if the benefit of ribavirin therapy appears to outweigh any fetal risk. Given the high risk of CCHF-related mortality both for pregnant women and foetuses, ribavirin still may be recommended.

Ribavirin is contraindicated in patients with chronic anaemia and haemoglobin levels below 8 g/dl, and in patients with severe renal impairment (creatinine clearance <30 ml/min). The drug may accumulate in patients with impaired renal function. These patients should be carefully monitored during therapy with ribavirin for signs and symptoms of toxicity, such as anaemia.

Chemoprophylaxis: Prophylactic administration of oral Ribavirin to contacts of CCHF patients is not recommended. Symptomatic contacts can be given therapeutic dose as mentioned above. Consider full therapeutic dose of Ribavirin for Health Care Workers with severe exposure (Needle stick injury, direct contact with blood /body fluids). For person with mild exposure observe and closely monitor HCW for any symptoms.

17.6 PREVENTION AND CONTROL

There is no vaccine available for either people or animals.

In disease affected areas:
- Educate public about the mode of transmission and the means for personal protection.
- Tick control in the affected areas in cattle can be undertaken in consultation with Animal husbandry department.
- To minimize exposure, wear light clothing that covers legs and arms, tuck pants into socks, regularly examine clothing and skin for ticks, and apply tick repellent such as DEET (N,N-diethyl toluamide) to the skin or permethrin (a repellent and contact acaricide) to the clothings.
- Persons working with livestock or other animals in the endemic areas should take practical measures to protect themselves. They include the use of repellents on the skin (e.g. DEET) and clothing (e.g. permethrin) and wearing gloves or other protective clothing to prevent skin contact with infected tissues or blood.

- Safe handling of dead bodies using personal protection measures.

Infection Prevention and Control in Health Facilities:

- Universal Infection Prevention and Control practices should be strictly adhered to in all healthcare facilities dealing with suspected, probable and confirmed cases.
- At the point of entry into the healthcare facility, patients with signs and symptoms consistent with CCHF should be identified as quickly as possible, separated from other people.
- Patients with suspected or confirmed CCHF should be managed in an isolation room for the period of communicability and barrier-nursing techniques should be followed to avoid nosocomial infection.
- Only designated medical / para-medical staff and attendants should attend the patient. Non-essential staff and attendants should not be allowed to enter the room.
- All staff entering the room should wear personal protective equipments.
- Hand washing / Hand sanitization before and after clinical examination/ conducting procedures on the patient.
- Persons coming within 3 feet of the patient should wear face shields or surgical masks with eye protection (including side shields). Use HEPA filter masks if patients have prominent respiratory, GI, or hemorrhagic symptoms.
- Specimens of blood or tissues taken for diagnostic purposes should be collected and handled using universal precautions. Sharps (needles and other penetrating surgical instruments) and body wastes should be safely disposed of using appropriate decontamination procedures.
- If large amounts of blood or other body fluids are present in the environment, use leg and shoe coverings.
- Before exiting the room, discard all used protective barriers and clean shoes with a hospital disinfectant or solution of household bleach. If possible, use an anteroom for putting on and removing protective barriers and for storing supplies.
- Hospital clothing, bed sheets and other linen used in patient care should be treated as infectious and autoclaved and incinerated.
- All used materials such as syringes, gloves, canulla, tubing etc used for patient care should be collected in autoclavable bag, autoclaved and incinerated.
- All instruments, equipments etc should be decontaminated/ autoclaved before re use.
- Surfaces should be decontaminated with liquid bleach.
- CCHFV can be inactivated by disinfectant including 1% hypochlorite and 2% gluteraldehyde.
- Avoid spills, needle pricks, injury and accidents during case management.
- Healthcare workers who have had contact with tissue or blood from patients with suspected, probable or confirmed CCHF should be followed up with daily temperature and symptom monitoring for at least 14 days after the putative exposure.
- Hospital waste management practices should be as per standard guidelines.
- The patient and attendants need to be examined for ticks using universal precautions. Application of acaricidal agents is recommended if there is evidence of tick infestation.

Monitoring and Laboratory Testing for Contacts of CCHF Cases

Contacts include family, neighborhood and health care facility contacts

All contacts should be advised to self-monitor twice daily for any clinical symptoms (such as fever, muscular pain or bleeding) for 14 days (maximum) from the day of last contact with the patient or other source of infection.

In case of onset of any symptom, he/she should immediately report to the nearest health facility. Appropriate laboratory testing is blood recommended in cases meeting the case for CCHF definition.

Dead body disposal

- Only trained personnel should handle remains during the outbreak.
- Personnel handling remains should wear personal protective equipment (gloves, gowns, apron, surgical masks and eye protection) and closed shoes.
- Spray dead body with 1:10 liquid bleach. Wrap with a winding sheet. Spray the winding sheet with bleach solution.
- Place the wrapped and bleached body in leak-proof bag plastic bag. Seal with adhesive tape and transport
- Disinfect ambulance / transport vehicle.
- Protective equipment is not required for individuals driving or riding a vehicle to collect human remains.
- Remains should not be washed or embalmed.
- Protective equipment should be put on at the site of collection of human remains and worn during the process of collection and placement in a body bag.
- Protective equipment should be removed immediately after remains have been placed in a body bag and then placed inside a coffin.

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Further Reading

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EBOLA VIRUS

18.1 INTRODUCTION

The emergence of Ebola Virus Disease (EVD) in West Africa is unprecedented in many ways. It is certainly one of the largest and deadliest outbreaks in recent times. It began in Guinea in late 2013 and spread to neighboring countries of Liberia and Sierre Leone. The outbreak has mounted exceptional concern, preparedness and response worldwide as it is dreaded as one of the most virulent disease causing high fatality in humans. It has no specific treatment or vaccine despite it being known since 1976 when it first appeared in Democratic Republic of the Congo (DRC) in two simultaneous outbreaks in Nzara, Sudan, and Yambuku, DRC.

Formerly known as Ebola hemorrhagic fever, in the current outbreak EVD involved the health care workers and further weakened the already compromised health care system in the affected countries.

18.2 CAUSATIVE AGENT

Ebolavirus is one of 3 members of the Filoviridae family (filovirus), along with genus Marburgvirus and Cuevavirus. There are 5 distinct species of Ebolavirus viz.Bundibugyo ebolavirus (BDBV), Zaire ebolavirus (EBOV), Reston ebolavirus (RESTV), Sudan ebolavirus (SUDV) and Taï Forest ebolavirus (TAFV). While BDBV, EBOV, and SUDV have been associated with large EVD outbreaks in Africa, RESTV and TAFV have not yet been implicated in a human outbreak. The RESTV species, found in Philippines and the People's Republic of China, can infect humans, but no illness or death in humans has been reported.

18.3 EPIDEMIOLOGY

The natural reservoir of Ebola viruses has not yet been proven conclusively. However, fruit bats Hypsignathusmonstrosus, Epomopsfranqueti and Myonycteristorquata, may be the natural hosts in Africa. Human beings can get infected and initiate human to human transmission on contact with infected animals or their carcasses. In Africa, infection has been documented through the handling of infected chimpanzees, gorillas, fruit bats, monkeys, forest antelope and porcupines found ill or dead or in the rainforest.

18.4 TRANSMISSION

The most common mode of human-to-human transmission is direct contact through broken skin or unprotected mucous membranes e.g. the eyes, nose, or mouth, with the blood or body fluids (urine, feces, saliva, semen, and other secretions) of a person who is sick or has died of EVD. However, infection cannot be transmitted before the appearance of symptoms. Transmission may also occur with contaminated needles or infected animals. Ebola does not spread through the air or by water.

People are infectious as long as their blood and secretions contain the virus. Ebola virus has been isolated from semen for more than 5 months after onset of illness. High Risk Group includes those who are in close contact viz. relatives or Health Care Workers (HCWs) of an EVD patient, handlers of dead body of an EVD patient.

18.5 CLINICAL FEATURES AND LAB DIAGNOSIS

After an incubation period of 2-21 days (average 8-10 days) patient develops fever and non specific symptoms such as severe headache, fatigue, muscle pain, vomiting, diarrhea, abdominal (stomach) pain, or unexplained hemorrhage (bleeding or bruising). Patient may rapidly progress to multi-organ involvement, hemorrhages and shock. Ebola virus is a group 4 agent and requires high containment facility. EVD can be diagnosed by RT PCR, antigen detection or IgM antibody detection. Virus isolation is technically demanding and is carried out in BSL 4 facility. National Centre for Disease Control (NCDC), Delhi and National Institute of Virology (NIV), Pune were designated to undertake diagnosis of EVD in high containment (BSL3 / 4) facility.

18.6 TREATMENT

There is no specific anti-viral drug or vaccine available for EVD. Symptomatic treatment is given and complications are managed as they appear. Maintenance of hydration, electrolyte balance and managing infections remains the mainstay of the treatment. ZMapp (Mapp Biopharmaceutical Inc.), cocktail of three different monoclonal antibodies that bind to protein of Ebola virus and some investigational vaccines and drugs are being developed/supported by NIH and Department of Defense (DoD), USA.

18.7 PREVENTION AND CONTROL

Undoubtedly, EVD with its high mortality and morbidity, rapid and high rate of human-to-human transmission and no approved vaccine or therapeutics, has heightened anxiety across the world. However, the strategies to control EVD are simple and time-tested epidemiological principles of early detection, prompt response and prevention of further spread. The importance of communicating clear and accurate information to all stakeholders including the general public is the need of the hour. Central and state governments have demonstrated high-level commitment and preparatory activities are being undertaken.

All the health care workers who come in contact with the suspect/confirmed case of EVD should wear complete PPE which includes double gloves, boot covers that are waterproof and go to at least mid-calf, single-use impermeable gown, respirators, including either N95 respirators or powered air purifying respirator (PAPR), face shield, surgical hoods to ensure complete coverage of the head and neck. Waterproof apronshould be used if Ebola patient has vomiting or diarrhea. The enhanced guidance is centered on the principle of "no skin exposure" when PPE is worn.

Government of India has taken a proactive stand to control EVD. India is among the top five financial contributors to the United Nations' ebola response. WHO, on August 8th, 2014 has

declared it as Public Health Emergency of International Concern. No one in the world is safe unless the epidemic is controlled in West Africa and that there are close to 45,000 Indians in the affected countries. Early recognition, immediate institution of infection control practices and contact tracing is therefore the most critical aspect.

The Joint Monitoring Group (JMG) of various stakeholders of different ministries under the chairpersonship of Director General of Health Services met frequently to discuss the evolving situation of EVD, review and revise the preparedness activities. Guidelines for surveillance and contact tracing, health care providers, hospital infection control, environmental control, clinical case management, collection storage and transportation of samples were also prepared and available on the NCDC website.

Further Reading

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ABBREVIATIONS

ARS	Anti rabies serum
APHO	Airport Health Organization
ATN	Acute tubular necrosis
CSF	Cerebrospinal fluid
CIE	Counter immunoelectrophoresis
CCHF	Crimean-Congo haemorrhagic fever
CAT	Computed axial tomography
CDC	Centre for Disease Control
DFA	Direct fluorescent antibody
ERIG	Equine rabies immunoglobulins
ELISA	Enzyme linked immunosorbent
FAT	Fluorescent antibody test
FITC	Fluorescein isothiocyanate
HA	Hemagglutination assay
HIA	Hemagglutination inhibition assay
HRIG	Human rabies immunoglobulins
HDCV	Human diploid cell vaccine
IDSP	Integrated Disease Surveillance Programme
IIF	Indirect Immunofluorescence Test
IFAT	Indirect fluorescent antibody test
IHA	Indirect haemagglutination
JE	Japanese encephalitis
KFD	Kyasanur forest disease
MHA	Muller Hinton Agar
MAT	Microscopic agglutination test
NIV	National Institute of Virology
NHM	National Health Mission

NVBDCP	National Vector Borne Disease Control Programme
NTV	Neural tissue vaccine
PCEC	Purified chick embryo cell vaccine
PVRV	Purified vero cell rabies vaccine
PKDL	Post kala-azar dermal leishmaniasis
PCR	Polymerase chain reaction
PHA	Passive haemagglutination assay
RRT	Rapid Response Teams
RFFIT	Rapid fluorescent focus inhibition test
SBA	Sheep blood agar
SRBC	Sheep red blood Cells
TE Buffer	Tris-EDTA buffer
TCV	Tissue culture vaccine
VL	Visceral leishmaniasis
WHO	World Health Organization
ZVD	Zika virus Disease

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Zoonotic diseases of public health importance is a manual on endemic and remerging zoonotic diseases in India such as Arbovirus, Rickettsia, Plague, Anthrax, Rabies and Leptospirosis etc. It also gives insight into various newer emerging diseases like Zika virus, Crimean Congo Haemmorhagic fever and Ebola Virus. This manual will provide an overview to the public health experts, laboratory professionals, State directorates, veterinary experts, airport health officials, medical and paramedical professionals about various epidemiological and laboratory aspects and measures for prevention and control of zoonotic diseases as "One Health" approach.