DISEASE SURVEILLANCE AT DISTRICT LEVEL

A LABORATORY MANUAL

NATIONAL INSTITUTE OF COMMUNICABLE DISEASES
22 SHAM NATH MARG, DELHI - 110 054

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Government of Karnataka
Department of Health & Family Welfare
Karnataka Health Systems Development Project

Disease Surveillance at District Level

A Laboratory Manual

Office of the
Additional Director, Communicable Diseases
Karnataka Health Systems Development Project
Department of Health & Family Welfare
Bangalore.

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Role of Laboratory in Disease Surveillance

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ROLE OF LABORATORY IN DISEASE SURVEILLANCE

EPIDEMIOLOGICAL surveillance of a disease is the continuing scrutiny of all aspects of the occurrence and spread of a disease that are pertinent to effective control. It is a dynamic process involving the infectious agent, host, reservoirs, vectors and the environment as well as a complex mechanism concerned with the spread of infection and the extent to which spread has occurred. Surveillance of any particular disease includes systematic collection and evaluation of morbidity and mortality data, reports of investigation of epidemics, laboratory investigations to find out the causative agent, use and untoward effects of biologicals, insecticides and other materials used in control, assessment of immunity status of population and other relevant data for action. The introduction of laboratory techniques in epidemiological services has revolutionised the concept as well as scope of disease surveillance. Now a days laboratory support is considered an integral component of a sensitive system of surveillance.

1.1 Role of Laboratory services in surveillance:

1. Diagnosis of a syndrome

   - o Encephalitis
   - o Hepatitis
   - o Meningitis
   - o Pyrexia of unknown origin

2. Tracing the source of infection.

   - o Epidemiological markers

3. Detection of inapparent infections/carriers:

   - o Japanese Encephalitis
   - o Typhoid fever
   - o Meningococcal meningitis

4. Early detection of outbreak:

   - o Meningococcal meningitis
   - o Hospital infections.

5. Retrospective diagnosis.

- o Rheumatic heart disease
- o Subacute sclerosing panencephalitis

6. Detection of New Disease Agents

- o HIV
- o Newer Enteropathogens (*V. Cholerae* O139)

7. Monitoring of treatment

- o Antibiogram
- o Sero-Therapy.

8. Quality Control of Biologicals

- o Vaccine potency testing
- o Vaccine Safety studies

9. Prevalence studies

- o Sero-surveys
- o Immune status

10. Find out natural foci of infection

- o Plague
- o Leptospirosis

11. Controlled field trials.

- o Newer drugs/vaccines
- o Newer regimens of drugs/vaccines

12. Key to successful laboratory based surveillance lies in :

1. Right sample collection.
2. Right time to collect samples for disease surveillance.
3. Right methodology to be for transportation of sample.
4. Right laboratory to be chosen

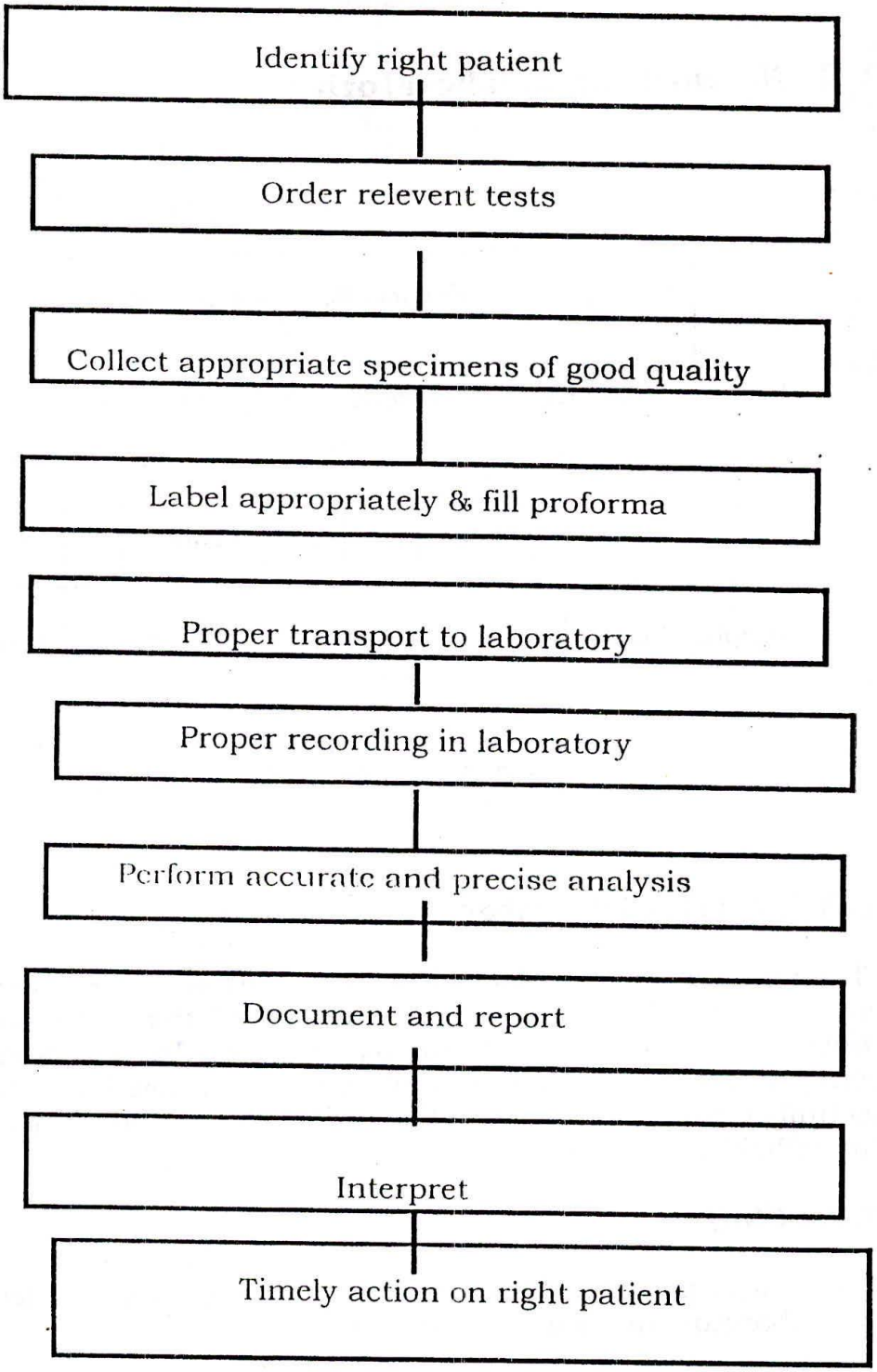

Fig 1.1:Algorithm for ensuring good laboratory results

1.2 Networking of laboratories

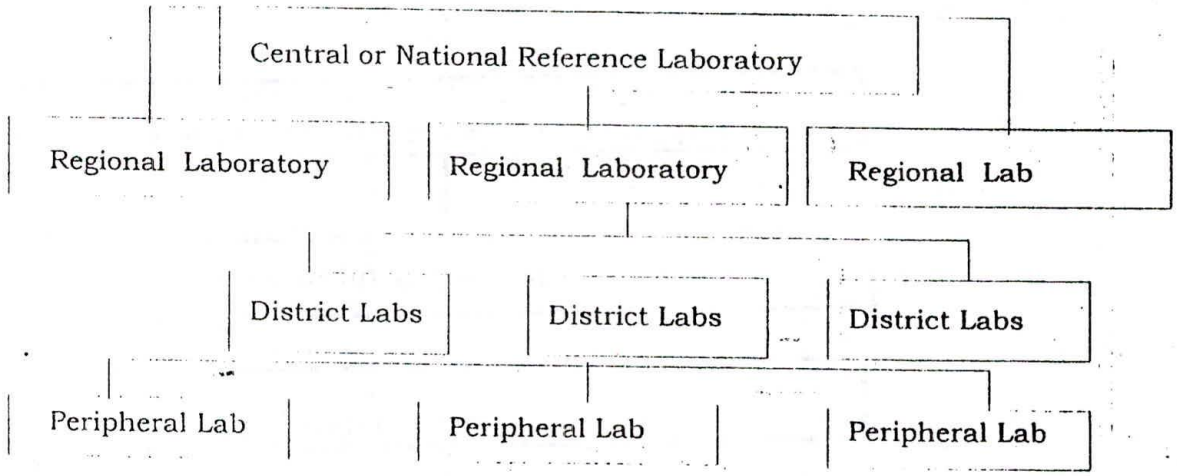

Fig 1.2: Networking of laboratories

1.3 District laboratory

These laboratories are located at the point of first contact of patients with the health care services. In most of the developing countries these are available only at primary health centre or community health centre (upgraded primary health centres). These laboratories provide technical support for preventive, curative and promotive services for the individual as well as the community.

1.3.1 Staff

The staff in peripheral laboratories should include one technician and one laboratory assistant/attendant.

1.3.2 Space

Space available in peripheral laboratories should include at least one laboratory-cum-office/record room (16 ft x10 ft) and one store-room combined with other services (16 ft x10 ft).

1.3.3 Other facilities

Other necessary facilities include

- a supply of safe water,
- a reliable source of energy (battery, electricity, solar or kerosene) and
- sterilization facilities and waste disposal

There must also be **transport** and **communication facilities** between the peripheral and intermediate laboratories for referral of samples and patients, procurement of supplies and personal discussion.

Equipment and supplies. Necessary equipment and supplies include: good microscopes, centrifuge, transport media, glassware, sterile swabs, reagents for staining (eg. Gram, Albert, Ziehl Neelsen, Romanowsky), kits and reagents for rapid diagnostic tests, sterilized syringes and needles, micropipettes and tips as well as sterile collection bottles for blood/serum and water analysis.

1.3.4 Tests to be performed

Table 1.1: Suggested microbiological tests at district laboratories

Procedure/Specimen	**Disease/Organism**
Microscopy for stained smears (Gram, Albert,Ziehl Neelsen)	
Nasopharynx and throat	Diphtheria, Vincent's angina
Sputum	Tuberculosis, pneumonia
CSF	Meningitis (pyogenic & tuberculosis)
Stool	Cholera/dysentery
Culture	Cholera
Serological tests	Enteric fever (Widal)
	RPR/VDRL
Dipstick and Particle agglutination test	HBsAg, HIV
Bacteriological analysis	Water

These laboratories are expected to undertake tests of public health as well as clinical relevance. Amongst the tests of public health relevance, diseases of greater epidemiological importance should be accorded priority.

Testing of environment samples (especially water) also falls into the priorities of public health relevance. Certain serological tests may be of use in studying epidemiological pattern of the important diseases and the same can be performed at peripheral laboratories (Table 1.1)

As far as possible, these tests should be reliable, sensitive, specific, rapid, easy to perform and cost effective.

CHAPTER- 2

Collection, Storage and Transportation of Specimens

CHAPTER- 2

COLLECTION, STORAGE AND TRANSPORTATION OF SPECIMENS

EFFECTIVE diagnostic microbiology depends upon the correct collection and timing of clinical specimens and their proper transport to the laboratory under optimal conditions. It has been observed that most important and frequent source affecting laboratory analysis is collection and transportation of the specimen. The guidelines for it must be emphasized. Specimen should be in adequate quantity.

- Specimen must be collected before the administration of antimicrobial agents.
- Contamination of specimen with externally present organisms or normal flora of body must be prevented.
- Specimen must be collected at appropriate stage of the disease.
- Specimen should not get contaminated during storage.
- Specimen handling should not be risky to individual

2.1 Precautions in handling specimens

- Apply strict aseptic techniques throughout the procedure.
- Wash hands before and after the collection.
- Collect the specimen at the optimum time.
- Make certain that the specimen is representative of infectious process (e.g. sputum is the specimen for pneumonias and not saliva) and is adequate in quantity for the desired tests to be performed.
- Collect or place the specimen aseptically in an appropriate sterile container.
- Ensure that outside of specimen container is clean and uncontaminated.
- Tightly close the container so that its contents do not leak during transportation.
- Label and date the container, complete the requisition form.
- Arrange for immediate transportation of specimen to laboratory.

2.2 Laboratory specimens required for tests for particular causative agents:

Suspected agent/ disease	*Specimen*	*Test*
Arbovirus infection	Blood or brain (-70°C) Blood or serum (+4°C)	Isolation Serology
Cholera	Rectal swabs or stool specimens in transport medium, as recommended by the laboratory.	Culture
Gastroenteritis	Stool	Culture, ELISA*
Viral Hepatitis	Serum (+4°C)	ELISA*
Legionellosis	Blood, sputum, in enrichment broth.	Culture/ FA**
Malaria	Blood smears	Staining
Meningococcal meningitis	Spinal fluid, blood	Latex agglutination
Plague	Spinal fluid, blood, Bubo fluid, blood	Staining, Culture/ FA**
Typhoid fever	Blood in enrichment broth	Culture; serology
Dysentery	Faecal specimens or rectal swabs in enrichment broth.	Culture/ microscopy
Syphilis	Blood/ serum	VDRL/ RPR

* Enzyme linked immunosorbent assay
** Fluorescent antibody test.

Because of alterations in the specimen prior to measurement, the clinical state of the patient will not be necessarily reflected by the result of the laboratory investigation despite the correct laboratory performance. Some of the important specimens and their proper collection and transportation methods are described here so as to ensure quality.

2.3 Blood for serological testing:

Blood is the most important and frequently collected clinical specimen at district level.

Blood should be drawn using sterile (preferably disposable) syringes and needles. Quantity of blood drawn should be minimum 4-5 ml. Vial in which blood is collected should be preferably sterile, dry and properly labeled. The needle and syringe used, as also the vial, should be completely DRY before collecting blood. After drawing blood, the needle should be removed from the syringe before transferring blood from syringe to the vial. Do not shake the blood that is collected in the vial. Let it stand undisturbed at room temperature for 2-4 hours. After the blood has stood at room temperature for 4-6 hours, it should be subjected to the process of serum-separation. If the facilities for separation of serum are not available, then it should be refrigerated at 4°C (NOT FROZEN).

Using a sterile Pasteur pipette, dislodge the retracted clot from the liquid portion of blood, transfer the liquid portion into a clean sterile centrifuge tube having a rubber cork.

Centrifuge at 500g for 5 minutes. Transfer the supernatant(serum) using a sterile Pasteur pipette into sterile clean, dry plastic disposable screw capped vials. Label the vials.

2.3.1 Filter paper method of collection of blood:

Transportation of liquid specimens soaked on filter paper is a simple and safe procedure. All kinds of body fluids can be absorbed onto filter paper strips (Whatman No.1). Cards with incorporated filter paper strips having marked circle (usually 3 cm diameter) are commercially available. The details of the patient are written on the card with a ball-point pen. The blood is taken from the finger prick or heal prick of an infant. The site is cleaned with 70% alcohol and wiped dry with sterile gauze. Puncture is made with a sterile disposable lancet and the first drop of blood is wiped away with sterile dry gauze.

Filter paper (marked area) is gently touched onto the second drop of blood and allow the blood to soak till the premarked area is completely filled. Punctured site should not be squeezed to prevent any haemolysis. Blood is applied only on one side of filter paper and once only.

The blood specimen is dried for 3 hours in the air in a horizontal position without letting the specimen come in direct contact with any

surface, direct heat or sunlight. These samples should not be refrigerated.

The specimen is placed in a plastic bag or an envelope along with few granules of a desiccant and sealed hermetically before transportation or mailing. If stored in a cool and dark place, such specimens give acceptable results even upto three months.

(For collection of stool, CSF , aspirates and water, see relevant chapters)

2.4 Labeling of Specimens

After collection of specimen, it should be immediately labeled and case investigation form should be filled up and should accompany each specimen. The specimen should be kept cool preferably at 2-8°C and sent to laboratory as early as possible. In case of delay the sample should be stored at 2-8°C before transporting to nearest laboratory.

Labels for specimen collection vial

Name_____ Age_____

Specimen No._____

Specimen

Date Time

2.6 Storage and Transportation:

In general, infectious materils should be kept at a low temperature during storage and transport, except the CSF samples collected from cases of pyogenic meningitis which should be maintained at room temperature. The types of refrigeration required to achieve various temperatures are as follows:

Temperature (°C)	*Type of refrigeration*
(+)2-8	**Domestic refrigerator**
(+)4	**Wet ice or frozen ice packs (cold bags)**
(-)8	**Freezer of domestic refrigerator**
(-)20	**Freezer cabinet**
(-)70	**Deep freezer or dry ice.**
(-)160	**Liquid nitrogen**

The quantity of pathogens or antibody in original clinical samples can decline during storage or transportation which seriously affects the diagnostic results. Hence special care should be taken before or during transit of materials to laboratory to protect them from heating or drying.

- **Avoid repeated thawing and freezing of specimens**
- **Freeze the specimen only if transport is assured at -20°C.**
- **Store and transport all specimens at 2-8°C (Lower compartment of refrigerator) EXCEPT CSF.**

For Transportation of specimen

- **wet ice or ice pack should be used.**
- **Specimen containers relating to single caseinvestigation should be placed in a plastic bag with an absorbent material surrounding the specimen so that even if whole specimen leaks out, it will be absorbed.**

- The laboratory report form should be sealed within a separate plastic bag and wrapped round the specimen or attached firmly to the box of specimens.
- The material should be packed in an insulated carton/carrier to transport a specimen to the laboratory.
- All specimens should be considered as potentially pathogenic and accordingly labeled with internationally accepted biohazard label.

2.7 Transportation of Virus Isolates/Specimen to Reference Laboratory:

Specimen virus isolates to be sent to other laboratories require special attention for packing of the material and strict guidelines for transportation of samples should be followed.

CHAPTER- 3
Disinfection and Sterilization

CHAPTER- 3

DISINFECTION AND STERILISATION

Definition:

Sterilisation implies complete destruction of all living micro-organisms including spores. Disinfection means destruction of vegetative forms of organisms which might cause disease or spoilage of food etc. It does not necessarily kill spores. The two terms are not synonymous.

3.1 Disinfection of used laboratory articles

Disinfection of both reusable and disposable glassware and articles contaminated with morbid or culture material is of utmost importance in the laboratory. All the specimens received in the laboratory should be considered as potentially pathogenic. The ideal method of treating such materials is to incinerate all the disposables and decontaminate the reusable articles by autoclaving. These facilities may not be available in every laboratory. For purpose of disinfection, disposal and recycling, all the articles may be divided into three categories.

- Disposables.
- Reusable articles contaminated with morbid material such as pipettes, slides, test tubes etc.
- Material containing or contaminated with bacterial cultures.

3.1.1 Disposables:

Soak the material overnight in a strong solution of disinfectant before disposing alongwith garbage; **1% sodium hypochlorite, 10% solution of formalin or 3% lysol may be used as disinfectant.**

3.1.2 Reusable articles contaminated with morbid material:

Discard the articles into a jar containing solution. Let them remain in this solution overnight. Drain off the disinfectant. Transfer the material to a metal pot or tray with cover. Pour water and boil for 15 minutes. Cool and drain off the water. Pass on the articles for washing.

3.1.3 Glassware containing culture material

Discard all the articles containing or contaminated with culture material directly in a metal box or a bucket. Place the box with material in the autoclave and decontaminate by autoclaving (see sterilisation).

Drain off culture medium and pass for washing.

3.2 Disinfection of rooms

Seal all the windows, ventilators and fire places with brown paper and adhesive tape.

Pour 500 ml of formalin and 1000 ml of water in a pan or tray and boil with the help of a spirit lamp or a bunsen burner. Spirit in the lamp should be just sufficient, to boil off the formalin and the lamp extinguishes when there is a small quantity of liquid left in the pan. Seal the door.

Open the door, next morning and spread a piece of lint soaked in ammonia on the table. This will neutralise excess of formalin present in the room.

3.3 Washing of laboratory glasswares:

3.3.1 New glassware

Usually new glassware are slightly alkaline. Before washing these have to be neutralized. The method is as follows:

- Prepare a 2% solution of hydrochloric acid in a big basin.
- Soak the new glassware in this solution for one day.
- Rinse twice with clean water and once with demineralized water and dry.

3.3.2 Dirty glassware:

- Rinse twice in lukewarm or cold water otherwise serum or blood may stick to them and may not be washed.
- Put the glassware in a bowl containing detergent solution and scrub the inside with a brush. After scrubbing soak the glassware in this solution for 2-3 hours.
- One by one, take out the articles and rinse under running tap water, then put all the glassware in a container containing tap water (no trace of detergent

should be left otherwise this may lead to false results).

- o Drain the water by putting each articles on a wall draining rack.
- o Place the articles in a wire basket and dry in a hot air oven at 60°C.
- o Plug each article with non-absorbent cotton wool or aluminium foil and store in a cupboard to avoid dust.

3.3.3 Pipettes:

- o Immediately rinse in running tap water to remove blood, urine, and serum reagent, etc.
- o If the pipettes were used for infected materials, soak them in cylinder full of disinfectant solution (2% dettol or 2% phenol) for 24 hours otherwise place in a large measuring cylinder full of water.
- o Soak in detergent and rinse as in case of dirty glassware.
- o In case the pipettes are blocked put them in dichromate solution for 24 hours. Next day clean under running tap water, check individually, rinse for a number of times or use pipette washer.

3.3.4 Syringes and needles:

- o Immediately after use remove the plunger and rinse the barrel and plunger. Syringe water through the needle forcefully. Finally remove the needle.
- o If the piston is blocked, either soak the syringe for 2 hours in hot water or pipette with the syringe standing on its end, piston down. Alternatively soak the syringe in a container of 10 vol hydrogen peroxide.
- o In case of block needles use the stylet to remove the block.

3.4 Methods of sterilisation

The common methods of sterilisation used in a microbiology laboratory can be broadly divided into three categories depending upon the materials to be sterilised.

- o Dry heat.
- o Moist heat.
- o Filtration.

3.4.1 Dry heat

The two commonly used methods of sterilisation by dry heat are:

- Red heat or flaming
- Hot air sterilisation.

Red heat or flaming

Instruments such as inoculating loops and searing irons are sterilised by this technique. For sterilisation of inoculating loop, hold the loop vertically on the blue cone of the flame for few seconds and slowly raise upwards till whole of the wire is red hot. Move the loopholder rapidly downwards through the flame so that several inches of the loop holder is also heated slightly.

Hot air sterilisation

Items to be sterilised:

This is the best method for sterilisation of dry glasswares such as test tubes, flasks, pipettes, pertidishes, assembled all glass syringes, throat swabs and other sealed materials which can withstand high temperature and where penetration by steamis not possible.

Sterilisation by hot air can be conveniently carried out in an electrically heated oven. A themostat is fitted to control the temperature. Larger units should be fitted with an air circulating fan to ensure uniform temperature in the different parts of the oven.

Autoclaving

This is sterilization by mpoist heat under pressure and is used for sterilization of bacteriological media, plastic wares etc. It has been described in details subsequently.

CHAPTER- 4
Microscopic Examination

CHAPTER- 4

MICROSCOPIC EXAMINATION

In a peripheral laboratory, microscopic examination can provide rapid and economical presumptive diagnosis which may have significant bearing upon control and prevention strategies.

4.1 Cleaning and storage of microscope slides

4.1.1 Cleaning of new slides

- Soak the slides in a vessel containing soap water solution for a few hours.
- Place the slides either in running tap water or several changes of clean water for few hours.
- The slides should be wiped dry using a dry, clean, lint-free cloth.
- Always handle the cleaned slides by the edges to avoid finger marks.

4.1.2 Cleaning of used slides

- Soak the slides for at least 60 minutes in 1-2% hypo-chlorite solution.
- Wash in hot soap water scrubbing both the sides with the brush, taking particular care to wash only a few slides at a time to prevent scratching.
- Clean the slides individually with gauze or cotton wool.
- Transfer the slides to a fresh detergent solution.
- Wash in running tap water or several changes of clean water.
- Wipe dry with a clean lint free cotton cloth.

4.1.3 Storage of Slides

- Initially, after washing and cleaning, the slides should be kept in a dry place or a warm air cabinet.
- Thereafter slides should be stored in packages of 10 which should be wrapped in thick paper and secured with adhesive tape or rubber bands.

4.2 Microscopy for Pyogenic Meningitis

Pyogenic meningitis is an acute bacterial infection of the meninges, commonly caused in epidemic form by *Neisseria meningitidis, Streptococcus pneumoniae* and *Haemophilus influenzae.*

For the laboratory confirmation of the diagnosis, the following clinical specimen should be collected.

4.2.1 Cerebrospinal Fluid (CSF)

CSF following lumbar puncture should be collected in 3 separate clean sterile containers (Bijou bottles) for following investigations:

- Biochemical analysis
- Cytological examination
- Microbiological tests

General guidelines for collecting CSF

- CSF should be collected before the start of chemotherapy.
- Never refrigerate or expose it to sunlight, and transport immediately to the laboratory.
- In case of delay in transportation, keep CSF at 37°C.
- Other clinical samples which can be collected are blood and petechial fluids.

4.2.2 Examination of CSF

Macroscopic:

Look for the presence of turbidity, blood or coagulum.

Cytology

Cytologic examination to be done only when there is no coagulum in the CSF.

Material Required: Neubauer's counting chamber, WBC diluting fluid, WBC pipette, compound microscope.

The cell count should be done by the usual procedure of WBC count using a Neubauer's chamber and count the number of leukocytes per cmm of fluid.

The normal CSF should be absolutely clear, free of any coagulum and should not contain more than 0-8 lymphocytic cells/cmm.

In pyogenic meningitis, appearance of CSF is turbid and contains more than 8-10 leukocytes/cmm, the cells being predominantly polymorphs in nature.

Microbiological examination:

Microscopy

Microscopic examination is required to directly visualise the causative organism in the CSF.

REQUIREMENTS

- Clean slides
- Coverslips
- Table top Centrifuge
- Centrifuge tubes
- Pasteur pipettes
- Clean glass vials
- Reagents of Gram's staining.
- Rubber teats
- Discarding jar
- Neubauer Counting chamber
- WBC pipette.

PROCEDURE:

- Transfer about 1-2 ml of CSF in a sterile Centrifuge tube.
- Centrifuge at 3000 rpm for 5 minutes.
- Keep the supernatant fluid for Latex Agglutination test.
- From the deposit, make smears on 3 clean glass slides and air dry.
- In case of a clotted CSF, transfer 3 small pieces of clot on three different glass slides.
- Tease the clots using needles or wooden sticks or the edge of the slide and after spreading make the smears. Air dry.
- In case of scanty CSF, several drops of CSF should be placed at one particular spot on the slide, each being allowed to dry before the next is added.
- Air dry and heat fix the smear.
- Stain the smears by Gram's staining method as given in Annexure.
- Examine under microscope under oil immersion.

OBSERVATIONS:

- Presence of Gram negative bean shaped diplococci, both intracellular and extracellular suggests the presence of *Neisseria meningitidis* (Meningococcus).
- Other organisms which can be seen are *Streptococcus pneumoniae* (Pneumococcus), which appear as gram-positive diplococci, *Haemophilus influenzae* which appear as gram negative thin filaments rods.

4.3 Diagnosis of Pulmonary tuberculosis by sputum examination:

Tuberculosis is a disease of great public health importance caused by *Mycobacterium tuberculosis* and some other species of Mycobacteria.

The diagnosis of pulmonary tuberculosis can be established by demonstrating the bacillus in the sputum of the patient by microscopy.

4.3.1 Sputum collection:

- Collect the sample preferably early in the morning.
- For optimum results, 3 consecutive days samples should be tested.
- In case sputum is scanty, a 24 hour collection may be examined.
- A nebulized and heated hypertonic saline may be used to induce sputum production in patients unable to bring out the sputum.
- Sputum should be collected in a sterile wide mouthed container with a tight lid.

The sample should be delivered to the laboratory with minimum delay. Specimen that cannot be delivered or processed immediately should be refrigerated at 4-8°C for a maximum of 3-4 days.

Materials required for sputum microscopy:

- Properly collected sputum specimen
- Wooden sticks
- Clean glass slides
- Spirit lamp/Bunsen Burner
- Petri dish
- Inoculation hood
- Face masks
- Reagents for Zeihl-Neelsen staining
- Glass Rods, Plastic clay.

Procedure:

PREPARATION OF THE SMEAR

- In an inoculation hood or in an isolated room, wearing a face mask, transfer a portion of the sputum to a petri dish.
- Using a wooden stick, tease out a small portion of caseous, purulent or bloody material and transfer it to a clean slide.

- Using the same wooden stick or an inocuting wire loop, spread this material uniformly over a large area, covering at least two thirds of the slide.
- Air dry the slides and flame them immediately and stain according to the Ziehl- Neelsen staining method as given below:

4.3.2 *Ziehl Neelsen Staining (Acid fast staining)*

Requirements

- Carbol fuchsin solution
- 20% sulfuric Acid
- 25% Alcohol
- Loeffler's Methylene blue
- Distilled water

Staining Procedure:

- Put the heat fixed smears onto a platform made using two parallel glass rods over a wash basin.
- Cover the slide with carbol fuchsin and heat the slide from below until steam uses.
- Allow the stain to act for 5-8 minutes with intermittent heating, putting fresh carbol fuchsin on the slide time to time.
- Care should be taken not to allow the stain to dry on the slide.
- Wash the slide preferably using distilled water. (Do not use tap water).
- Cover the slide with 20% Sulfuric Acid. Wash the slide with water after one minute. Pour more acid and continue decolorisation till smear is just faint pink.
- Wash the slide again with water.
- Cover the slide with 25% Alcohol for 2 minutes.
- Wash with water.
- Counterstain with Loeffler's methylene blue for 30 seconds.
- Wash the smear with tap water, air dry and examine under oil immersion.

Observations:

Mycobacteria appear as bright pink, slender, slightly curved rods, whereas the background tissue, cells and other organisms are stained blue.

Note:

- Staining jars should never be used as with a positive stained slide, the bacilli may get detached from the slide and float about in staining fluid later on sticking to the negative slides

and may give false positive results.

- o After examining a positive slide, take care to wipe the lens with a clean tissue paper before examining the next slide.
- O Do not record the smear to be negative unless at least 200 microscopic fields have been thoroughly examined under oil immersion objective.

4.4 Diagnosis of Plague

Plague is an ancient scourge of mankind, which is a bacterial disease caused by *Yersinia pestis*. It is endemic in rodents and fleas. In man, plague occurs mainly in three forms, bubonic, pneumonic and septicaemic.

The presumptive diagnosis of Plague can be established by microscopic examination.

Sample collection:

- o Bubo aspirate: in bubonic plague should be collected.
- o Under all safety precautions collect bubo aspirate by puncturing the bubo with a sterile hypodermic syringe and exudate is withdrawn.
- o Sterilize the puncture site with tincture iodine.
- o 10 ml or 20 ml syringe, armed with 18/19 guage needle and a few ml of sterile saline drawn into the syringe, should be used for aspiration of bubo aspirate.
- o Bubo is then punctured and suction applied.
- o If aspiration does not produce fluid, then saline is injected into the bubo again and aspirated again.
- o Transfer the exudate into a sterile container.
- o Label the container.
- o Transport to the laboratory at 2-8°C.

4.4.1 Sputum Collection in Pneumonic Plague:

- o Collect the sputum sample in a sterile wide mouth screw capped container.
- o Label the specimen
- o Transport the specimen to laboratory at 2-8°C.

In the Laboratory:

- o Make three smears out of the same portion of exudate/sputum taking precautions not to form aerosols.
- o Air dry the smear.
- o Stain smears either by Methylene blue/Gram staining/Waysons stain.

4.4.2 Gram Stain:

This is a routine laboratory procedure used for examining specimens suspected to contain bacteriologic agents. Direct microscopic examination of specimens and cultures can provide a rapid presumptive diagnosis. Gram stain results, the shape of cell (cocci, bacilli), the type of cell arrangement (single, chained, clustered) visualized under light microscopy, can provide a quick assessment of what the etiologic agent may be.

Principle:

The Gram stain forms the cornerstone of microscopic bacteriology. It was described by Hans Christian Gram over 100 years ago. Crystal violet (gentian violet) is the primary stain that will bind to the peptidoglycan present in the cell walls of some bacterial cells. Iodine is added as a mordant to fix the dye. If the cell wall does not contain peptidoglycan then crystal violet is easily washed off with acid or alcohol (decolorizer). A secondary dye, safranin (counterstain), is added after the decolorization step. If the primary did not bind the cells will easily adsorb safranin. Thus gram-positive cells are purple, while gram-negative cells are pink/red.

Requirements:

- Crystal violet (0.5%)
- Gram's Iodine (1%)
- Acetone (100%) or Ethanol (95%)
- Safranine (0.5%)

Procedure:

- Cover the slide with crystal violet solution and allow to act for about 30 seconds.
- Pour off stain and holding the slide at an angle downwards pouron the iodine solution so that it washes away the crystal violet ; cover the slide with fresh iodine solution and allow to act for 1 minute.
- Wash off the iodine with ethanol and treat with fresh alcohol, tilt the slide from side to side until colour ceases to come out of the preparation. This is easily seen by holding the slide against a white background.

  Or

  Decolorize with 100% acetone. First, tip off the iodine and hold the slide at a steep slope. Then pour acetone over the slide from its upper end , so as to cover its whole surface.

Decolorization is very rapid and is usually complete in 2-3 second. After this period of contact, wash thoroughly with water under a running tap.

- o Apply the counterstain (0.5% safranine) for 30 seconds.
- O Wash with water and blot dry

When to use this procedure and what you expect to see

Y.pestis appears as a fat, short, gram-negative coccobacilli about 1µ by 0.5µ. Gram stains are typically done on cultures/subcultures, bubo aspirates, spleen, liver and sputum smears.

Critical value/Action to be taken:

When gram stained material reveal small coccoid gram-negative bacilli. Material should be further worked up with culture isolation and identification. No notification is needed at this time.

Interpretation:

Y. pestis appears as a fat short, gram negative coccobacilli about 1 µ by 0.5 µ.

4.4.3 Wayson stain for visualizing Yersinia pestis:

Wayson stain is a polychromatic differential stain used as a presumptive test for the presence of *Yersinia* and *Pasteurella* spp.

Principle

Basic fuschin and methylene blue in the Wayson stain bind to bacterial cells which appear under light microscopy as bipolar, closed safety pin-shaped cells. The differential polychromatic mrophology can be visualized with many differenttypes of organisms therefore Wayson stain alone is not diagnostic for *Y.pestis*.

Critical values/Action to be taken:

When stained unknown material has a characteristic "safety pin" morphology, it is Wayson stain positive. Further work-up by culture isolation and identfiication must follow. No notification is needed unless submittor specifically requests notification.

If Wayson bi-polar organisms known to have *"safety pin"* morphology cannot be visualized after staining, check reagents and check for possible technical problems. Repeat stain until characteristic morphological results are obtained with control cultures.

Materials needed for this test:

Wayson stain:

- Dissolve 0.2 grams of basic fuchsin and 0.75 grams of methylene blue in 20 ml of 95% ethanol. Filter solution through Whatman #1 paper (or equivalent).
- Pour dissolved, filtered stain into 200 ml of 5% aqueous phenol. Store at room temperature. Avoid exposure to light.

Procedure:

- Prepare smear of tissue or culture on slide, air dry.
- Heat fix smear or fix in absolute methanol for 3 minutes, air dry slide. (Methanol fixation yields more contrasting staining than heat fixation).
- Flood smear with Wayson stain for 5-10 seconds.
- Wash slide in tap water, blot gently or air dry.
- Examine slide under light microscope.

Interpretation:

Consistent, striking bipolar "safety pin" morphology of small, fat bacilli are characteristic of the *Yersinia* and *Pasteurella* spp. Other bacteria may exhibit bipolar appearance as well, especially if the specimen is taken from areas with a wide variety of normal flora (nasal, pharyngeal, and fecal).

"All *Y.pestis* are Wayson positive, but all Wayson positive strains are not *Y.pestis*".

Quality control measures:

Test each lot of Wayson stain using known *Yersinia/Pasteurella* spp. (positive control) and with *Escherichia coli* or other enteric bacteria as negative controls. When examining tissue smears, controls slide prepared with plague bacilli infected and uninfected tissue smears should also be examined.

4.4.4 Methylene Blue Staining;

Material required:

- Air dried smear.
- Methylene blue stain.

Procedure:

- Fix the smear by dipping the slides in a jar containing pure Methanol for 5 minutes.
- Cover the smear with methylene blue stain.
- Leave the stain for 3 minutes.
- Wash with tap water. Air dry .
- Examine under the oil immersion of microscope.

Observation:

Characteristic bluish bacilli showing bipolar staining. Suggests presence of *Y.pestis* organisms.

4.5 Malaria

Malaria is a parasitic disease caused by *Plasmodium* species. In India, the disease is commonly caused by *P.vivax* and *P.falciparum*. The laboratory diagnosis is based on demonstration of different stages of the parasite in the peripheral blood film of the patient.

4.5.1 Collection of sample:

Peripheral blood smear:Time for taking blood:

- Collect blood either during or 2-3 hours after the peak of temperature.
- Sample should be taken before administration of antimalarial drugs.

4.5.2 Preparation of blood smear:

Both thick and thin films should be made on the same slide.

Blood sample should be collected from the tip of the ring finger of the left hand. However in small children, sample should be collected either from the heal or the tip of the big toe of the foot taking all aseptic precautions using a sterile needle or a lancet (see also page.... under "Filter paper method").

Apply gentle pressure to the finger and collect a single small drop of blood on to the mdidle of the slide. This is for the thin film. Apply further pressure to express more blood and collect 2 or 3 large drops on the slide about 1 cm from the drop intended for the thin film. Wipe the remaining blood away from the finger with cotton wool.

Thin film: Using another clean slide as a 'spreader' and with the slide with the blood drops resting on a flat firm surface, touch the small drop with the speader and allow the blood to run along its edge.

Firmly push the spreader alongwith the slide away from the largest drop keeping the spreader at an angle at 45oC. Make sure the spreader is in even contact with the surface of the slide all the time the blood is being spread.

Thick film: Always handle slides by the edges or by a corner to make the thick film as follows:

Using the corner of the spreader, quickly join the larger drops of blood and spread them to make an even thick film. The blood should not be excessively stirred but can be spread in a circular or rectangular form with 3 - 6 movements.

Allow the thick film to dry in a flat level position protected from flies, dust and extreme heat . Label the dry film with a pen or marker pencil , by writing across the thicker portion of the thin film the patient's name , or number and the date. Do not use a ball pen to label the slide.

Wrap the dry slide in clean paper and despatch with the patient's record form the laboratory as soon as possible.

The slide used for spreading the blood films must be disinfected and should then be used for the next patient, another clean slide from the pack being used as a spreader.

4.5.3 Staining of Blood smears:

GEIMSA STAIN

Materials and Reagents

:

1. Geimsa stain powder/Ready Giemsa Stain solution.
2. Alcohol
3. Methanol
4. Marking pen
5. Staining jars
6. Boric acid Borax buffer - pH 7.2.

Preparation:

- Dissolve the stain powder in alcohol as per the manufacturer's instructions.
- Prepare Borax Acid - Boric buffer as below:
  a) Dissolve 12.4 gms of Boric Acid in 1 lit. of Distilled water (I)
  b) Dissolve 19.05 gm Borax in 1 lit of Distilled water (II)

Take 50 ml of solution I and adjust the pH to 7.2 using appropriate volume of solution II. Then make up the volume to 200 ml with distilled water.

Staining Technique:

- Prepare thick and thin smear from malaria case on a glass slide.
- Dehaemoglobinize the thick smear by placing the film in a vertical position in a glass Jar containing distilled water for 5 minutes. When film becomes white, take it out and dry in upright position.
- Fix the thin smear in methanol for 15 minutes.
- Dilute the Giemsa's stain solution, one part with 9 parts of Boric buffer pH 7.2.
- Immerse the smears in this stain for 1 hour.
- Wash the smears in buffer solution.
- Blot and dry.
- Examine the slide under oil immersion of microscope.

4.5.4 J.S.B. Stain

Materials and Reagents Required:

- Eosin yellow (water soluble)
- Methylene Blue
- Potassium Dichromate
- Di-sodium hydrogen phosphate (dihydrate)
- 1% sulphuric Acid.
- Round bottom flask (2 lit.)
- Healing mantle
- Distilled water
- Staining jars.

Preparation:

J.S.B. II

Dissolve 2 gms Eosin Yellow in 1 lit. of distilled water and store in the dark for 4 weeks before use.

J.S.B.I

- Dissolve 1 gm of Methylene blue in 600 ml of distilled water and mix well.
- Add 1% sulphuric acid (6.0 ml) drop by drop and shake well.
- Add 1 gm of potassium dichromate and shake well till precipitation occurs.

- Dissolve the precipitate by adding 7 gms. of Di-sodium hydrogen phosphate dihydrate.
- Make up the volume to 1 lit.
- Boil the stain in round bottom flask over a heating mantle for one hour.
- Cool the stain and re-adjust the volume to 1 lit by adding distilled water.
- Store in dark for 4 weeks before use.

Staining technique:

- Prepare thin and thick smears from malaria cases on micro slides.
- De-haemoglobinise the thick smear.
- Fix the thin smear in methanol for few minutes.
- Take 3 staining jars for J.S.B. I, J.S.B.II and tap water.
- Dip the smears in J.S.B. II for few seconds and immediatedly wash in water.
- Drain the slides free of excess water.
- Dip the smears in J.S.B.I for 30-40 seconds.
- Wash well in water and dry.
- Examine the smears under oil immersion.

4.5.5 Observation:

Examine thin film first. If no parasite is found then only examine thick film. If parasites are seen in the thick film but the identity is not clear, the thin film should be reexamined more thoroughly so as to determine nature of infection.

Thin film examination:

o Area of the film examined should be along the upper and lower margins of tail end film as parasites are concentrated over there.

o A minimum of 100 fields should be examined in about 8-10 minutes.

c) The following stages of the parasite can be observed in a peripheral blood thin smear.

1. Ring, trophozoite, schizont and the gametocytes in case of *Plasmodium vivax.*

2.The infected erythrocytes is usually enlarged in *P.vivax* infection.

3.However, in case of P.falciparum infection, it is mainly the ring stages which are seen and occasionally schizonts and trophozoites. During the late stages of the disease even crescent shaped gametocytes can be seen in the peripheral blood.

Observation on thick smear:

- o Only elements seen are leucocytes andmalarial parasites.
- O Morphology of malarial parasites is distorted.
- o species of parasites cannot be identified.

Appearance in thick film

- o Trophozoites appear as streaks of blue cytoplasm with detached nuclear dots. The ring forms rarely seen.
- o Schizonts and gametocytes, however, retain their normal appearances (although the pigments are seen more clearly) are seen if present in smear.

4.6 Examination of blood for Microfilaria

Filariasis is a disease of the lymphatics caused mainly by the nematode *Wuchereria bancrofti* and rarely by *Brugia malayi.*

Laboratory diagnosis:

This is based on the demonstration of the larval stages of the parasite in the peripheral blood of the cases.

4.6.1 Collection of blood:

The blood should be preferably collected between 10 PM and 2 AM specially in areas where microfilaria shows nocturnal periodicity.

4.6.2 Examination of unstained preparation:

- Take 2-3 drops of blood on a clean glass slide. Put a coverslip on it.
- The rim is then smeared with vaseline to prevent drying up of the blood.
- Examine the slides under low power microscope immediately or within 24 hours of ollection of blood.
- Wriggling microfilaria present in the blood can be seen.

4.6.3 Examination of stained smear:

Thick film:

- Prepare a thick blood film as per the instruction given in the Chapter on Malaria.
- Dehemoglobinise the smear by putting the slides in a jar containing water.
- Air dry
- Fix the smear with methyl alcohol.

- Stain with Geima's stain as described earlier.
- Examine the smear under the oil immersion of the microscope.

Thin film:

o Prepare as described for malaria.
o Fix it with Methanol by dipping the smear in a jar containing methanol for 15 minutes.
o Stain it with Giemsa stain as described earlier.

Observation:

Microfilaria of *Wuchereria bancrofti* are seen.

o Size - 290 μ in length and 6-7 μ in breadth.
o It has blunt head, pointed tail and has smooth curves.
o Structureless sack called Hyaline Sheath is seen where it projects beyond the extremities of embryo.
o Somatic cells/nuclei seen as granules in central axis from head to tail end except the terminal 5 percent area. At the anterior end there is a space devoid of granules called cephalic space.
o The granules are broken at definite space serving as the landmarks for identification of the species.
  - Nerve ring, an oblique space.
  - Antereior V spot, represents the rudimentary excretory system.
  - Posterior V spot or tail spot, represents the terminal part of the alimentary canal.

Microfilaria of *Brugia malai.*

o Smaller than *Wucheraria bancrofti* (230 μ x 6 μ)
o Possess secondary kinks instead of smooth curved.
o Cephalic space is broader.
o Tail tip is not free of nuclei and nuclei are blurred.
o It lies folded with head close to tail.

CHAPTER- 5
Serological Tests

CHAPTER- 5

SEROLOGICAL TESTS:

5.1 LATEX AGGLUTINATION TEST FOR MENINGITIS

The ideal immunological test, which is also a rapid test and easy to perform in a district laboratory, is the latex agglutination (LA) test. test is done to detect the bacterial antigen (Capsular polysaccharide) in CSF samples collected from patients.

The available comercial kits are designed to provide diagnosis for meningitis caused by:-

- *N. meningitidis* serogroup A
- *N. meningitidis* serogroup C
- *Streptococcus pneumoniae*
- *Haemophilus influenzae* type b

The general procedure for performance of the test is given below, however, the laboratory personnel are advised to go through the instructions provided by the kit manufacturer, carefully, and strictly adhere to the same.

5.1.1 Equipments required: (but not supplied with the kit)

- Pasteur pipettes (sterile)
- Rubber teats
- Container with disinfectants (for discard)

5.1.2 Procedure

- Systematically heat all CSF specimens for 5 minutes at 80-100°C.
- Centrifuge the CSF samples at 2000 rpm for 10 minutes, preserve the supernatant for further use.
- Shake each latex suspension well.
- In the corresponding fields of the slide, dispense one drop of each of the latex suspension followed by one drop of the CSF supernatant.
- Mix with a stirring stick; use separate stick for each combination of CSF and latex suspension.
- Rotate the slide, and read within 2 minutes.

5.1.3 Controls: Periodically check:

a) thatnone of the four latex reagents agglutinate in presence of 0.15 mol/l NaCl solution.

b) that each of the four latex reagents do agglutinate with positive control.

5.1.4 Reading:

- Negative reaction: The CSF latex suspension mixture remains a "milky suspension" (disregard any granules that may occur with *S.pneumonae*).
- Positive reaction: Distinct rapid agglutination occuring within 2 minutes (normally 30 seconds).

5.1.5 Interpretation:

Aglutination with one of the latex reagents indicates presence of the corresponding antigen in the CSF sample.

Advantages of LA test:

- Most sensitive method available
- Rapid
- Good field applicability
- Can diagnose the disease even in antibiotic treated patients.
- No special equipment/instrument required.

Disadvantages of LA test:

- Commercial kits not produced in India; to be imported.
- Expensive
- The test does not yield any bacterial isolate; other parameters cannot be tested.

5.2 DIAGNOSIS OF HEPATITIS B VIRAL INFECTION:

Diagnosis of Hepatitis B viral infection is very important, not only in case of chronic hepatitis and liver cirrhosis patients, but also in the screening of donor blood samples, to ensure safe blood transfusion and to control or check the spread of hepatitis B infection through unsafe blood transfusion.This is achieved by detection/demonstration of "Hepatitis-B surface Antigen" (HBsAg) or the 'Australia Antigen' in the patient/donor blood samples.

A simple latex agglutination test for rapid detection of HBsAg, which is very much feasible in the district laboratories, is described below:

LATEX AGGLUTINATION TEST FOR RAPID DETECTION OF HBsAg (AUSTRALIA ANTIGEN)

5.2.1 PRINCIPLE:

A distinct agglutination occurs, when serum sample containing HBsAg is mixed with latex particles coated with purified and highly reactive anti-HBsAg antibodies; there would be no agglutination when the serum sample does not contain HBsAg".

5.2.2 MATERIALS AND REAGENTS:

Commercial kits for this test are available in India. They contain the following reagents and accessories.

Reagent 1:	HBsAg Latex Reagent	- 1 vial
Reagent 2:	Positive control serum	- 1 vial
Reagent 3:	Negative control serum	- 1 vial
Accessories:	Disposable plastic slides	
	Disposable applicator sticks	
	Disposable plastic droppers	
	Rubber teats.	

All the reagents are stable and active, till the expiry date mentioned, provided they are stored in a refrigerator at 2-8°C. Do not freeze the reagents.

5.2.3 SPECIMEN:

- The test is performed on serum harvested from the patient's/donor's blood.
- Do not heat inactivate the test or the control sera samples.
- If delay in testing, store test sera samples in a refrigerator or deep freezer, takingcare to avoid repeated freezing and thawing of the specimens.

5.2.4 TEST PROCEDURE

- Allow the reagents to attain room temperature, and shake the vials gently to make sure that the latex reagent is completely in suspension.
- Place one drop (50 /ul) of undiluted serum in one of the circles on the slide. More circles to be filled if more than one test sera samples are to be tested. Use separate droppers for each specimen.

- o Add one drop (50 /ul) of latex reagent on to each specimen drop in circles, using a disposable dropper.
- o Mix the content of each circle, using separate disposable applicator sticks for each circle, and spread the mixture uniformly over the entire area of the circle.
- o Rock the slide gently, to and fro, for 5 minutes, and watch for agglutination.

Precautions:

1. To avoid contamination of reagents, make sure that the cap of each vial is properly and promptly applied to the same vial. Interchanging of caps and droppers lead to contamination and erroneous results.
2. Improper mixing and interchange of applicator sticks also lead to erroneous results.
3. Vigourous rocking of slides may lead to impaired agglutination.

Use of Controls: Positive and negative controls are not always required, when reagents are in continuous use. However, the performance of the kits needs checking, occasionally, using the controls.

5.2.5 Interpretation:

- o **Visible agglutination** < 5 minutes -HBsAg **Positive**
- o No agglutination -HBsAg Negative

5.2.6 LIMITATIONS:

- o Probability of FALSE POSITIVITY = 1% of all samples, due to presence of other antigens (RF).
- o FALSE NEGATIVE results may be encountered with specimens containing very high titres of HBsAg (Prozone effect). In such cases the characteristic syndrome (severe jaundice, GPT/GOT elevation) will be apparent. In that case repeat the test after diluting the specimen 1:40, with normal saline.

5.3 VDRL SLIDE FLOCCULATION TEST FOR SYPHILIS:

This is a test with high sensitivity and specificity and can be used for rapid and exact quantitative titration of the reactive sera samples.

5.3.1 PRINCIPLE:

The VDRL antigen particles, which are seen as small fusiform needles under the microscope, floculate into clumps (small, medium and large), when they comein contact with a reactive (+ve) serum.

5.3.2 *MATERIALS:*

VDRL Antigen:

It consists of a mixture of Cardiolipin, lecithin and cholesterol in definite proportions and is commercially available. Each sealed glass ampoule contains 0.5 ml (with sufficient excess for convenient withdrawal). Antigen amouples should be stored in a cool, dark place. Ampoules showing precipitate should be discarded.

Buffered Saline Solution:

10 ampoules containing 5 ml each are supplied with each package of VDRL antigen. Buffered saline is required for preparing the antigen emulsion for the test.

SLIDES:

Glass slides, 2"x3", with 12 paraffin rings of 14 mm inner diameter are used for the test. Slides of same size, with permanently fixed ceramic rings are also available commercially and may be used. The following points regarding the slides are to be noted.

- o New slides, as well as the used slides should be cleaned thoroughly.
- o Slides should be handled by the edges only, to avoid any greasy finger prints.
- o Serum within the circles will spread evenly, within the rings, only if the slides are absolurtely clean.
- o Parafin rings can be made on slides by transferring molten paraffin on to slide using a suitable mould or threaded wire rings.

5.3.3 *PROCEDURE:*

A. *Preparation of serum:*

- o Inactivate serum by heating at 56°C for 30 minutes.
- o On removal from water bath, centrifuge the serum sample if it shows particulate debris.
- o Test sera sample need to be reheated (at 56°C for 10 min.), if they are >4 hr. old since original inactivation.
- o 0.05 ml of each sample is required for testing.

B. *PREPARATION OF ANTIGEN EMULSION:*

- o Pipette out 0.4 ml of buffered saline on to the bottom of a 1 oz.reagent bottle with flat or concave inner bottom surface.

- Add 0.5 ml of VDRL antigen, drawn out from an ampoule, using a graduated pipette, directly on to saline in the reagent bottle, while rotating the bottle on a flat surface.
- The antigen should be added drop by drop, but rapidly, so that it takes approximately 6 seconds to complete the delivery of antigen.
- Blow the last drop of the antigen and continue rotation of the bottle for 10 more seconds.
- Add 4.1 ml. of buffered saline, using a graduated 5 ml. pipette.
- Stopper the bottle and shake it vigorously for about 10 seconds.
- Take care to see that the temperature of buffered saline solution and that of VDRL antigen is maintained within the range of 23-29°C, during preparation of the antigen emulsion.
- Maturation of antigen is important for increased sensitivity, maturation is complete in 15-30 minutes, after preparation.
- Store the antigen emulsion in a refrigerator, if necessary. It should be brought to room temperature and shaken gently before use.
- 5.0 ml of antigen emulsion would suffice for 250 serum tests.
- Each batch of antigen emulsion prepared must be pre-tested with known ractive and non-reactive sera samples, in order to confirm that exact pattern of distribution of antigen particles, typical of reactive and non-reactive sera samples, would result on testing.

5.3.4 ***TEST PROCEDURE***:

- *Qualitative Test:*

  * Pipette out 0.05ml of inactivated serum into one paraffin/ceramic ring on the glass slide; serum should spread.
  * Add one drop (1/60 ml) of antigen emulsion on the serum within the ring.
  * Rotate the slide for 4 minutes, by hand on a flat surface (+ or -120 times per minute covering circle of 2"dia.)

5.3.5 ***READING AND REPORTING OF RESULTS:***

- Read the test results immediately after rotation.
- Observe the slide under micrscope, using low power objective(100 x magnification)
- Antigen particles appear as small fusiform needles, they are more or less evenly spread in case of a non-reactive

serum sample, and aggregated into clumps (flocculation) in the case of a reactive serum. Grade the observations as under:

No clumps or very slight roughness	*NON-REACTIVE (N)*
Small clumps	*WEAKLY REACTIVE (W)*
Medium and large clumps	*REACTIVE (R)*

Zone reactions are possible; they are recognizable by irregular clumping. The clumps are not compact and very small and large clumps may be seen within the same microscopic field. In such cases, the results are reported on the basis of quantitative test done on the same serum.

O *Quantitative test:*

Quantitative test is performed on all positive (reactive) serum samples and on samples which show weak(W) or "rough" reaction in the qualitative tests.

* Prepare successive two-fold dilution (1:1, 1:2, 1:4, 1:8, 1:16,1:64 etc.) of serum sample to be tested, using 0.9% saline.
* Each serum dilution sample thus prepared is treated as an individual sample and tested as described under "qualitative" test.
* Results are read and graded under the microscope as before.

Reporting of results:

Results are reported in terms of the highest dilution of the serum that produces a definite positive (or Reactive, R) reaction as below. Weakly reactive is not acceptable.

Serum dilution	**1:1**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**Report**
	R	W	N	N	N	N	N	R1 dil
	R	R	W	N	N	N	N	R2 dils.
	R	R	R	W	N	N	N	R4 dils.
	W	W	R	R	W	N	N	R8 dils.
	N	W	R	R	R	N	N	R16 dils.
	W	N	N	N	N	N	N	WO dils.
	W	W	N	N	N	N	N	R1 dils.

5.4 RAPID PLASMA REAGIN (RPR) TEST FOR DIAGNOSIS OF SYPHILIS:

This test detects antibodies formed, in the blood of syphilitic patients, against Cardiolipin. These antibodies are called "Reagin". Two advantages of this test over the previously described VDRL Slide flocculation test are - (a) It does not require a microscope to read the test results; (b) The test sera/plasma sample need not be inactivated prior to testing.

5.4.1 PRINCIPLE

"Reagin formed in the blood of syphilitic patients cause flocculation of the antigen, which co-agglutinates with the charcoal particles, giving small black clumps that are readily visible without a microscope".

Commercial "Rapitest" kits, designed for carrying out 50 tests per kit, are available in India.

5.4.2 REAGENTS AND MATERIALS:

Provided in the Kit

RPR Antigen	...	1 vial
Positive Control serum	..	1 vial
Negative control serum	...	1 vial
RPR Antigen dropper	...	1
Specimen droppers (disposable)		
Rubber Teats		
Mixing sticks (disposable)		
Plastic test cards	..	9

Materials required, but not provided in the kit:

Micropipette (capable of delivering 0.05 ml of test sample)
Stop watch
Saline solution (0.9%) - Only for quantitative test.
Container with disinfectant (for discard)

Storage: The RPR antigen and control sera will remain stable and active, till the expiry date printed on the label, provided they are stored in a refrigerator between 2-8°C. They should not be frozen.

5.4.3 THE SPECIMENS:

Serum:

- Use fresh serum harvested from patient's blood sample.
- If the test cannot be conducted immediately due to some reason, store the serum sample between 2-8°C in a refrigerator, BUT NOT LONGER THAN 48 hr., after collection.

Plasma:

- Collect patient's blood into a tube/vial containing one of the anticoagulants (EDTA, Heparin, Oxalate, Sodium Flouride etc.) Avoid excess of coagulant.
- Centrifuge the blood sample, to separate the cells.
- Use the plasma sample within 18 hr. of collection.
- Inactivation of serum/plasma samples is not necessary.

PRECAUTIONS:

- Blood samples should be collected from fasting patients, since very lipaemic samples may give false +ve reactions.
- Do not use grossly haemolysed samples.
- Discard contaminated samples.

5.4.4 TEST PROCEDURE

A. *Qualitative test:*

- Allow all reagents to attain room temperature.
- Place one drop of (0.05 ml) test serum or plasma, positive control and negative control sera on to separate circles on the plastic test card, using disposable specimen droppers provided.
- Shake the RPR antigen suspension gently, to resuspend the particles.
- Place one drop (0.015-0.02 ml) of the antigen suspension, on each of the circles containing test samples and the positive and negative control sera drops, using the antigen dropper provided.
- Mix the contents of each circle, using the disposable mixing sticks provided, and spreading the reagent mixture over the entire area of the circle.
- Gently rock the card, to and fro, for 6 minutes, either manually or on a mechanical shaker at 100 rpm, to ensure thorough mixing.
- Read the results at the end of 6 minutes, using a high intensity light source.

Interpretation of Results:

POSITIVE (REACTIVE)	Development of clearly visible clumps of black particles, within the test circles.
NEGATIVE	No development of clumps, the charcoal particles remain in a (NON-REACTIVE) HOMOGENEOUS GREY SUSPENSION.

A quantitative estimation is further recommended for all samples positive in the qualitative test.

B. *Quantitative test*:

- Dispense 0.05 ml (50 µl) of saline solution on to each of the circles (No.1-5) on the test card, using a micropipette.
- Dispense 0.05 ml (50µl) of the specimen (test serum/plasma) onto circle 1 and mix the two (saline and test sample) thoroughly by drawing the mixture into the micropipette, up and down several times.
- Transfer 0.05 ml (50µl) of the mixture in Circle-1, on to the drop of saline in Circle-2. Repeat the mixing action, several times, as explained above.
- Repeat transferring and mixing actions from Circle-2, through circle-5.
- Discard 0.05 ml (50 µl) from Circle-5, after mixing.
- The dilutions of specimen obtained in different circles on the test are as under:

CIRCLE	1	2	3	4	5
SALINE (ml)	0.05	0.05	0.05	0.05	0.05
SPECIMEN (Serum/Plasma ml)	0.05	-	-	-	-
MIX & TRANSFER	0.05	0.05	0.05	0.05	0.05
DILUTION	1:2	1:4	1:8	1:16	1:32

- Using the disposable mixing sticks, spread the specimen dilutions in the circles to cover the area of the circle.

Start with circle 5 and end with Circle 1. Wipe the sticks clean between circles.

- o Gently shake the RPR antigen vial to resuspend the particles, and add one drop (0.15-0.20 ml.) of antigen, on to each circle, using the antigen dropper.
- o Gently rock the card to and fro for 6 minutes (manually or on a mechanical shaker), to ensure thorough mixing.
- o Read results at the end of 6 minutes, as described above under Qualitative testing.

5.4.5 Interpretation:

The highest dilution of the sample, giving a definite positive reaction, is considered as the titre of the specimen. In case the titre exceeds 1:32, continue with double dilutions beyond that point,till the titre is obtained.

LIMITATIONS OF THE TESTS *(RPR and VDRL Slide Flocculation)*:

Both these tests are considered as "non-treponemal antibody tests", which are primarily meant as screening tests. If the tests are positive when there is no clinical evidence of syphilis, they must be repeated; if positivity persists, verifications by more specific tests (for anti-Treponemal antibody) would be necessary to confirm syphilis. In RPR and VDRL slide flocculation tests, false positive results may be obtained in diseases such as leprosy, malaria, toxoplasmosis, infectious mononucleosis and lupus erythematosUs, and also in specimens having bacterial contamination.

5.5 WIDAL TEST FOR DIAGNOSIS OF ENTERIC FEVER: (TYPHOID AND PARATYPHOID)

Widal test is an agglutination test for detection of antibodies against *Salmonella typhi* and *Salmonella paratyphi,* the common causal agents of enteric fevers.

5.5.1 PRINCIPLE:

"When serum sample containing antibodies against *S.typhi* and *S.paratyphi* AB are mixed with respective antigens, agglutination will take place".

In *S.typhi* and *S.paratyphi* AB, two types of antigens are recognised as diagnostically important:

(a) 'O' antigen or "Somatic' antigen.

(b) 'H' antigen or 'Flagellar'antigen.

'O' antigens of various species have components in common and hence only one 'O'antigen i.e. that of *S.typhi* is employed; the 'H' antigens of *Salmonella* spp. are species specific, and hence the 'H' antigens of all three, viz. *S.typhi, S.paratyphi* A and *S.paratyphi* B, are employed in the test.

Commercial test kits for WIDAL test are available in India, and using them both quantitative and quantative tests can be put up on suspected sera samples.

5.5.2 MATERIALS AND REAGENTS:

Test kit contains the following reagents and materials

Reagent 1: *S.typhi* ('H')	-	5 ml
Reagent 2: *S.typhi* ('O')	-	5 ml
Reagent 3: *S.paratyphi* A ('H')	-	5 ml
Reagent 4: *S.paratyphi* B ('H')	-	5 ml
Reagent 5: Positive control	-	1 ml
Glass slide	-	1 No.
Product Insert	-	1 No.

Materials required, but not supplied in the kit:

Small, dry and clean glass tubes (for quantitative tube test)	8/specimen
Normal saline solution	
Water bath	
Micropipette/dropper	

5.5.3 SPECIMEN:

Fresh serum (patient) free from contamination should be used. In case of delay in testing, store the sera samples at 2-8°C in a refrigerator.

Note:

- Specimen is used undiluted.
- Do not use haemolysed specimen.
- Do not heat or inactivate the specimen.

5.5.4 TEST PROCEDURE

A. Qualitative slide test for screening

o Clean the glass slide provided and wipe it dry.

- o Place a drop of undiluted serum sample to be tested in each of the first four circles.
- o Add one drop of Reagent 1, Reagent-2, Reagent-3 and Reagent 4, on to the specimen drop in Circles 1-4 respectively.
- o Mix the contents of each circle with separate mixing sticks, and spread the mixture to cover the whole circle.
- o Rock the slide gently for 1 minute.
- o Read the results at the end of one minute.

Interpretation:

A positive reaction shows agglutination, visible to naked eye, in the respective circle. Then proceed for quantitative slide test or quantitative tube test for the appropriate antigen.

B. *Quantitative slide test:*

- o Clean the glass slide supplied in the kit and proceed as follows:

Circle No.	Serum volume	Appropriate antigen	Titre
1.	0.08 ml	1 drop	1:20
2	0.04 ml	1 drop	1:40
3	0.02 ml	1 drop	1:80
4	0.01 ml	1 drop	1:160
5	0.005 ml	1 drop	1:320

- o Mix the contents of each circle, starting with circle 5 and through Circle-1, wiping the mixing stick clean between circles.
- o Rotate the slide for one minute and observe for agglutination.

Interpretation:

Titre of the serum is the highest dilution of the serum giving a positive reaction.

C. *Quantitative tube test*:

- o Take a set of 8 clean glass tubes, per specimen, per antigen.
- o Prepare dilutions of serum specimen and add appropriate antigen as below:

TUBE	1	2	3	4	5	6	7	8
Serum dilution	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Saline control
Normal saline	1.9ml	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml	1.0 ml	1.0 ml
Patient serum	0.1ml							
Transfer diluted serum		1ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 (discard ml)
Appropriate antigen	1 drop	1 drop	1 drop	1 drop	1 drop	1 drop	1 drop	1 drop

- Mix well and incubate at 37oC for 16-20 hr. and observe for agglutination.
- Repeat steps (ii) and (iii) with all antigens which showed agglutination in the screening test.
- Note the highest dilution showing clearly visible agglutination with naked eye.
  'O' antigen shows granular agglutination.
  'H' antigen shows flocular appearance.
- Saline control should remain unchanged as it is a negative control.

Interpretation:

Agglutination titre of >1:80 is suggestive of infection.

Factors affecting WIDAL Test:

Effect of antibiotic administration:

There is evidence that early treatment with antibiotics suppresses the antiboduy response by suppressing the multiplication of organisms. This may result in a low titre in WIDAL test.

Effect of past infection or typhoid vaccination:

It has been seen that th 'H' antibodies persist for a long time upto many years after typhoid vaccination. Also, many years after recovering from enteric fever, any grave negative bacterial infection can trigger a Salmonella 'H' antibody production, thereby giving a false positive result in WDAL test.

Time of collection of blood sample:

This is a very important parameter affecting the results of the WIDAL test. A single blood sample collected during the first week of the illness may give a negative WIDAL result, whereas in the same patient, a sample collected during the third week of illness may show a very high titre. Accordingly, paired samples should be collected; the first sample being taken as early as possible and the second, 10-14 days later, for optimum results.

CHAPTER- 6
Bacteriological Analysis of Water

CHAPTER- 6

BACTERIOLOGICAL ANALYSIS OF WATER

Although it is not possible to lay down fixed standards, as various types of water are examined, from a public health point of view it is generally sufficient to say that no faecal contamination has occured. Coliform bacteria present in water may not be harmful, but they indicate that water supply is contaminated with faecal matter and water is, therefore, liable to contamination with more dangerous organisms. The coliform bacilli of human origin are the most reliable indicators of faecal pollution.

The method of quantitative test for all coliform bacilli known as the 'presumptive coliform count is described below.

6.1 Collection of specimen

Collect water in presterilized bottles of 230 ml capacity with ground glass stoppers, having an over hanging rim. Sterilise the bottles by autoclaving.

Tap water:

When water is taken from tap, flame the mouth of the tap and allow the water to run for five minutes before filling the bottle.

Stream, river and lake water

Insert the bottle with its mouth closed with the stopper, a foot below the surface of water and fill with water. Bring the bottle to the surface and replace the stopper. Avoid the collection of surface water as it contains organic matter.

Precautions:

- During collection of water, avoid the contamination of the sample.
- Test the water samples as soon as possible after collection. If delay of more than 3 hours is expected, pack the water sample in ice for transport to laboratory.
- When sampling chlorinated water, add a quantity of sodium thiosulphate to the sample bottle before sterilising. This will neutralize the chlorine present in the water.

Presumptive Coliform count:

Requirements:

- Sample of water
- Sterilized test tubes.
- Quarter strength Ringer solution.
- 1 ml and 50 ml pipettes.
- Double strength MacConkey's fluid medium.
- Single strength MacConkey's fluid medium.

Method:

- o Invert the water sample 25 times to mix.
- o Flame the mouth of the bottle and discard 1/3 of the contents and mix thoroughly,
- o Using sterile graduated pipettes, the following amounts of water are added.
- - One 50 ml quantity of water to 50 ml double strength MacConkey medium in a flask.
- - Five l0 ml quantity each to l0 ml double strength MacConkey medium in test tubes.
- - Five 1 ml quantities each to 5 ml single strength MacConkey medium.
- o Incubate all tubes at 37°C for l8-24 hours.
- o All tubes showing acid and gas are regarded as presumptive positives. Reincubate negatives for further 24 hrs.
- o Using McCrady's statistical tables the probable number of coliform organisms present in 100 ml of sample can be calculated.

Interpretation:

Water samples are classified based on the presumptive count in the following way:

Class	**Preumptive coliform count l00 ml.**
1. Excellent	0
2. Satisfactory	1-3
3. Suspicious	4-10
4. Unsatisfactory	>10

Faecal Coliform Count:

From the tubes showing acid and gas in presumptive coliform count, subculture into fresh single strength MacConkey's broth or Incubate

at 44 C in a water bath. Tubes showing both acid and gas should be taken as positive for Faecal coliform. Using McCrady's tables compute the number of faecal coliform as in presumptive test. Water showing even one faecal coliform is unfit for human consumption.

Most Probable Number (MPN) values/100 ml of sample, for a set of tests of one 50 ml, five 10 ml, and five 1 ml volumes. (McCrady's Statistical Table)

No. Of tubes giving positive reactions			
1x50 ml	*5x10 ml*	*5x1 ml*	*MPN/100 ml*
0	0	0	<1
0	0	1	1
0	0	2	2
0	1	0	1
0	1	1	2
0	1	2	3
0	2	0	2
0	2	1	3
0	2	2	4
0	3	0	3
0	3	1	5
0	4	0	5
1	0	0	1
1	0	1	3
1	0	2	4
1	0	3	6
1	1	0	3
1	1	1	5
1	1	2	7
1	1	3	9
1	2	0	5
1	2	1	7
1	2	2	10
1	2	3	12
1	3	0	8
1	3	1	11
1	3	2	14
1	3	3	18
1	3	4	21
1	4	0	13
1	4	1	17
1	4	2	22
1	4	3	28
1	4	4	35

1	4	5	43
1	5	0	24
1	5	1	35
1	5	2	54
1	5	3	92
1	5	4	161
1	5	5	>180

6.1 H_2S-Strip method:

In recent years a simple, reliable and easy-to-perform (by even untrained personell), 'Yes-No' test for bacteriological quality of water has been devised. This test, which is currently under field evaluation and quality standardization is expected to be adopted as the field test for water quality monitoring in the hands of peripheral health workers and community participants.

Principle:

Presence of coliform bacteria in drinking water is associated with hydrogen sulphide (H_2S)- producing organisms, and faecal pollution of water can be established by demonstration of H_2S production.

It has been claimed, by various workers, that the H2S-strip method shows 80% agreement with the conventional MPN test described above.

Description of the test device (kit):

It simply consists of a pre-calibrated 20 ml glass bottle (McCartney bottle) with a screw-cap lid, from which a strip of specially treated/coated tissue paper hangs down, internally. The whole system is sterile and needs to be opened at the time of water testing.

The paper strip inside the glass bottle (80 cm^2,folded) is pre-soaked in a concentrated medium containing peptone (20g), dipotassium hydrogen phosphate (1.5g), ferric ammonium citrate (0.75g), sodium thiosulphate (1g), Teepol (1ml) and water (50 ml); 1 ml of the concentrated medium is absorbed on to the folded tissue paper strip and dried at 50^0 C under sterile conditions. It is then introduced into the sterile bottle.

Test procedure:

- Pour the water sample to be tested for faecal pollution into the bottle, upto the precalibrated level (20 ml).

- o Incubate at 37°C or allow to stand at ambient temperature (30-37°C); no incubator is necessary under field conditions, as the bottles can be held in the pockets and body temperature can be made use of.
- o Faecal pollution is indicated if the contents of the bottle turn black.

Advantages of H_2S-Strip Test:

- o No need to measure the volume of water to be tested;
- o No need to dechlorinate the water sample, since it instantaneously dechlorinates thr sample;
- o The end point (reading) is very clear, due to development of black colour;
- o No incubator is necessary;
- o The test starts immediately on collection into the bottle, unlike other methods which start after the sample is transported to the laboratory.

CHAPTER- 7

Laboratory Diagnosis of Cholera

CHAPTER- 7

LABORATORY DIAGNOSIS OF CHOLERA:

Cholera is characterized by sudden onset of effortless and profuse watery diarrhoea. The watery stools with flakes of mucus and sweet fishy odour are characteristic of cholera. These are also popularly known as rice water stool. Cholera is caused by the organism by the name of *Vibrio cholerae* which are Gram negative usually curved by shape of coma and motile by a single polar flagellum. They are oxidase positive.

The laboratory diagnosis is based on demonstration of *Vibrio cholerae* in the stool specimen.

7.1 Collection of samples

Materials required

- Wide mouth container
- Swabs sticks (sterile)
- Carry blair transport medium
- Case investigation form.

Collection of Stool:

Voided stool

- Most preferred specimen if available.
- Should be collected before antibacterial therapy.
- Should not be collected from bed pan so as to avoid interference from outside bacteria or disinfectant used to clean bed pan.
- Patient may be instructed to void stool in wide mouth container e.g. ice cream cup and transfer 3-5 gm. stool into a sterile screw cap bottle.

Rectal swabs:

Whenever it is not possible to collect stool, a rectal swab specimen may be collected. It is a very useful and convenient sample under field condition and in cases of young babies. By this methodology 0.1-0.2 ml of liquid faeces can be collected.

- Moisten the swab in sterile normal saline, if available.
- Introduce the swab into 4 cm deep into rectum through

anal sphancter. Rotate by 90oC and withdraw the swab.

- Store the swab in stoppered container or in transport medium e.g. Carry blair so as to avoid drying.

7.2 Storage and Transportation

- Store the specimen at 2-8°C.
- Transport to the laboratory at the earliest and in case of delay use to Cary Blair transport medium and send to the nearest laboratory.

Cary Blair transport medium:

It is a semi solid transport medium usually supplied in small bijou bottles. It should be stored in air tight container so as to avoid drying.

Inoculation of Cary Blair Transport Medium:

- Insert one/two rectal swabs taken from the same patient into the medium so that the whole swab is dipped into the medium.
- Break off the extra portion of sticks and replace the screw cap.
- Label the bottles.

7.3 PROCESSING OF SAMPLES IN THE LABORATORY:

Microscopic examination and Culture:

Materials required

- Enrichment medium
  - Alkaline peptone water.
- Plating media
  - Bile Salt agar
  - Thiosulphate-citrate--Bile Salt-Sucrose (TCBS) agar
  - MacConkey Agar
- Inoculating wireloop
- Gas supply/burner
- Incubator at 37°C.
- Hand lens.

After the specimen arrives in the laboratory, it should be given laboratory code number and entered in the register before processing.

Demonstration of motility by direct microscopy:

It can be done using either the direct stool sample or using 4-6 hr. growth in alkaline peptone water.

- Prepare a hanging drop using culture growth in alkaline peptone water or direct stool suspension.
- Examine under the high power of a binocular microscope.

Observation:

A darting motility is suggestive of presence of *Vibrio cholerae.*

Culture

- Directly streak over BSA and TCBS media and also inoculate alkaline peptone water (an enrichment medium).
- Incubate overnight at 37° C in ordinary incubator.
- Examine the plates after overnight incubation while APW is to be examined after 4-6 hours.

Colony morphology on culture plates:

BSA (Bile Salt Agar): Small translucent raised flat colonies are characteristic of *Vibrio cholerae.*

TCBS: Yellow, flat smooth colonies with pale yellow periphery are suggestive of *Vibrio cholerae.*

APW: Subculture growth in alkaline peptone water after 4-6 hour onto BSA/TCBS plates. Incubate plates and APW overnight. Look for the characteristic colonies of *V.cholerae* as described earlier.

7.5 Slide agglutination test for Confirmation of *Vibrio cholerae*:

Materials required

- Glass slides (clean)
- Normal Saline
- Platinum wire loop
- Growth on BSA/TCBS
- Discarding jars with disinfectant
- Gas supply
- *V.cholerae* 01 antisera.

Procedure:

- Put a small drop of normal saline on a clean glass slide.

- Using a wire loop pick a colony from a culture plate.
- Emulsify the growth in a drop of Normal saline using wire loop.
- Add a loopful of cholera 01 non-differential antisera on to the suspension mix.
- Look for agglutination (i.e. immediate clumpingof organism within 30-60 seconds.

Observation:

A positive agglutination is indicated by immediate clumping of organism and suggests presence of *Vibrio cholerae* O1 and rest are labelled as non-agglutinating vibrios.

7.6 Stock culture and Referral to Reference Laboratory:

- Make a stab into the nutrient agar slope, the cultures resembling *Vibrio cholerae*. (Agglutinating as well as non-agglutinating strains).
- Transport the stab cultures to the reference laboratory for further testing.

7.7 Composition and source of culture media/reagents necessary for cholera laboratory:

Bile Salt Agar (BSA)

Use:

Used for isolation and enumeraton of enteric bacilli.
Readymade dehydrated media is available from Hi-Media Laboratories Pvt. Ltd., Bombay.(Product code M-739 500 gms pack)

Directions for use:

- Suspend 43 gms of media in 1000 ml of distilled water in a flask.
- Plug flask with cotton.
- Autoclave at 15 lbs pressure 121oC for 20 minutes.
- Cool to 60-70°C.
- Pour in sterilised Petridishes.
- Remove air bubbles using flame.
- Let it solidify
- Store in refrigerator.

T.C.B.S. Medium:

(Thiosulphate Citrate Bile Salt Medium)

Use: Recommended for the selective isolation and culture of *Vibrios* causing cholera and vibrios which cause food poisoning.
Readymade dehydrated medium available from Hi-Media Laboratories Pvt. Ltd. Bombay (Product Code No. M-189).

Directions for use:

- Suspend 89 gms of Media in 1000 ml of distilled water.
- Boil to dissolve completely. Do not autoclave.
- Cool at 50°C.
- Pour into sterilised petridishes.
- Allow to solidify
- Store in refrigerator.

Alkaline Peptone Water:

Use:

At pH 8.4 it is suitable for the cultivation and enrichment of *Vibrio cholerae* from infected material.
Readymade dehydrated medium available from Hi-Media Laboratories Pvt.Ltd. Bombay (Product code No: M-028 - Peptone water).

Directions for use:

- Suspend 15 gms in 1000 ml distilled water.
- Mix well.
- Adjust pH to 8.4 with NaOH.
- Dispense 2-3 in test tubes.
- Plug tubes, using cotton plugs.
- Autoclave at 15 lbs pressure at 121oC for 20 minutes.

Cary Blair Medium
(Transport Medium w/o Charcoal)

Use: Recommended for collection and shipment of clinical specimens.
Readymade dehydrated medium available from Hi-Media Laboratories Pvt.Ltd., Bombay (Product Code - M-202).

Directions for use:

- Suspend 12.6 gms of media in 99% ml of distilled water.
- Boil to dissolve completely.
- Cool to 50°C.
- Add aseptically 9 ml of 1% aqueous calcium chloride solution.

- Adjust pH 8.4.
- Distribute 5-7 ml in screw capped small bottles.
- Steam bottles for 15 minutes.
- Cool, allow to solidify.
- Bottles are kept at room temperature.

Nutrient Agar Stabs:

Use:

A general culture medium.
Readymade dehydrated media supplied by Hi-Media Laboratories Pvt.Ltd., Bombay (Product Code M-012 Nutrient Agar W/1% peptone).

Directions for use:

- Suspend 35 gms of media in 1000 ml of distileld water.
- Boil to dissolve medium completely.
- Pour 2-3 ml in sugar tubes.
- Plug tubes with cotton plugs.
- Autoclave at 15 lbs pressure at 121°C.
- Cool to solidify.

CHAPTER- 8

Safety Precautions in Laboratory

CHAPTER- 8

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 equipment.**
- **Selection and use of essential biosafety equipment.**
- **Safe labooratory procedures.**

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

- **Avoid mouth pipetting**
- **Avoid eating, drinking, smoking and storing eatables inthe laboratory.**
- **Decontaminate the working area at least once a day and more frequently after the spillage of potentially infective material.**
- **Wash your hands 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, direct contact with blood or infectious materials.**
- **Decontaminate all liquid or solid waste before disposal.**
- **Perform all technical procedures in a way that minimises 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 and illumination for safe conduction of laboratory rpocedures.**
- **Design smooth easily cleanable walls, ceilings anfloors which should be impermeable to liquids andresistant to chemicals and disinfectants.**
- **Ensure a dependable and good quality water supply.**
- **Make suitably equipped 'first aid' rooms 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 thehighly susceptible individuals.**

- Provide safety systems covering fire and electrical emergencies.
- Control rodents and insects in the laboratory.
- Don't permit the entry of the experimental animals which are not to be used in the laboratory.
- Immunize your staff handling blood and blood products against Hepatitis B.

CHAPTER- 9
Common Laboratory Equipment

CHAPTER 9

COMMON LABORATORY EQUIPMENT

9.1 Incubator

Incubator is an apparatus having a desired temperature. The heating device used can be gas, oil or electricity. Maintenance of uniform temperature within the incubator is essential and is achieved by fan, blower or a waterjacket containing heated water.

Ideal temperature for most of the medically important bacteria is 35°+2°C. However for some organisms different temperatures are necessary e.g. atypical mycobacteria (22°C-45°C), fungi (22°C) etc. Some organisms may need extra gaseous element e.g. 5-10% CO2 incubator for Brucella. The size of the incubator may vary from a small table top to a large walkin type rooms. A proper temperature recording thermometer and a small tray of water inside the incubator to prevent excessive drying of air are the two other essential requirements.

9.2 Hot Air Oven

It is used for sterilisation of the following materials:

a Dry glass materials like test tubes, Petridishes, flasks, pipettes, syringes.

b Instruments like forceps, scalpels, throat swabs, etc.

c Sealed materials which can stand heat and when penetration of steam is not possible.

The instrument is electrically operated and should be equipped with a fan to have unfirom temperature inside, the required temperature for sterilisation is generally 160°C for 1 hour.

Operation of Hot air Oven

- Arrange the material to be sterilised loosely and evenly on the racks of the ovenallowing free circulation of air and thereby even heating of the load.
- Air is poor conductor of heat so do not pack the load tightly.
- Switch on the power supply and control the temperature of the oven by adjusting thermostat. When the desired

temperature is reached, note the time. Time taken for the oven to reach the desired temperature is called 'heating up period'.

- Hold the load in the oven at this temperature for a definite period of time. This period known as 'holding up period' is dependent upon the temperature employed. At 160°C the holding up period is 60 minutes, at 170°C for 18 minutes, at 180°C 7.5 minutes and at 190°C it is 90 seconds.
- The most common temperature for hot air sterilisation is 160°C for one hour. When the temperature is raised further, cotton plugs and paper wrappings get charred.
- On the expiry of holding up period, switch off the power supply and allow the load to cool.
- Open the oven door only when the temperature fall below 80°C, otherwise it may result in breaking up of glassware and also cause injuries to the operator.
- Dry up the instruments before placing them in the hot air oven.
- Do not place the heat sensitive materials inside.

9.3 Water Bath:

Water bath is a water container having an electrically operated heating device to provide a fixed and uniform temperature. A thermometer is inserted inside the water bath for recording temperature. A mixer immersed inside water is also desired to maintain uniform temperature throughout the water bath.

A few applications of water bath are:

37°C Water bath -	required during performance of WIDAL test;
44°C Water bath -	required in faecal coliform count (water bacteriology) test.
56°C Water bath -	for inactivating complement in the serum.

9.4 Centrifuge

For an average laboratory a small table top centrifuge with a maximum revolutions per minute of 6000 and capable of accommodating 10-12 tubes of 15 ml capacity is sufficient. The tubes should be placed exactly opposite to each other, should be of the same weight and should contain same amount of fluid. The speed is adjusted by a rheostat and should be allowed to rise slowly. A timer for fixed duration of centrifugation is preferred.

A few common uses are:

- o Sediment examination of urine - 1500 rpm for 5 minutes.
- o Separation of serum from clotted blood - 1500 rpm for 15 minutes.
- o Concentration of microfilaria from blood - 2000 rpm for 2-5 minutes.

9.5 pH METER

A pH meter consists of an electrode pair which is sensitive to hydrogen ion concentration due to the development of an electrical gradient which is directly proportional to the hydrogen ion concentration. The electrodes commonly used are one of glass for the unknown and other of colomel to be used as a standard precautions while using pH meter are:

- o The electrodes specially the glass ones should be handled carefully to prevent breakage due to contact with hard surface.
- o Sufficient time should be given to warm up the instrument before use.
- o Frequent standardizations of the pH meter should be made using standard buffer solution.
- o Electrodes are to be washed with a stream of distilled water between measurements.
- o The electrodes should never be removed from the solution when the measuring circuit is closed.
- o When not in use, the electrodes must be kept immersed in water or electrode solution.

9.6 Refrigerator:

Refrigferators are essential for storage of degradable laboratory substances like media, reagents, antisera, antibiotic discs etc. Refrigerators can vary in their capacity ranging from table top to a large walk-in-type. The usual temperature needed is 4o+2°C which is maintained comfortably by household use refrigerators. Substances to be kept at frozen state like sera may be kept in the freezer units of the same. Proper recording of the temperature is very important to avoid deterioration of biological materials.

9.7 Microscope

- o Place a slide on the stage, specimen side up and the centre of the section to be examined as accurately as possible over the hole in the centre of the stage.
- o Adjust the mirror until it reflects the maximum amount of the light through the specimen with the low objective in position, lower the body tube by means of the coarse adjustment until

the objective is about 1/4" from the slide.

- Look through the eye piece and slowly raise the objective with the coarse adjustment until the specimen is in approximate focus. Never focus downward while looking through the eye piece. Bring the specimen to sharp focus with the fine adjustment. Adjust the iris diaphgram and substage. Condense until the light intensity is optimum.
- After examining the specimen with the low power objective shift to the high dry objective by rotating the nose piece until the objective clicks into place.
- Look through the eye piece and slowly raise the body tube with the coarse adjustment until the specimen comes into approximate focus. Then bring the image into final accurate focus by using the fine adjustment. Once the specimen is in focus adjust the mirror and the iris diaphgram to give the clearest possible image.
- Focussing of the oil immersion objective:- First use the low power objective to locate the portion of the specimen to be examined. Raise carefully the body tube, and then rotate the nose piece until the oil immersion objective clicks into the position. Now place a drop of immersion oil on the portion of the slide directly under the objective watching the object from the side carefully lower it into the oil. Do not allowthe objective to touch the slide. Look through the occular and slowly focus upward with the fine adjustment until the age appears. Once it appears do the fine adjustment and adjust the mirror and iris diaphragm to obtain optimum illumination.

Maintenance

- Never touch the lenses if they become dirty, wipe them gently with lens paper.
- Always remove oil from the oil immersion objective after its use. If by accident, oil should get on either of the low power, wipe of the objective immediately with the lens paper. If oil becomes dry or hardened on a lens, remove it with lens paper lightly moistened with xylol.
- Keep the stage of the microscope clean and dry.
- Do not tilt the microscope when working with the oil immersion system.
- When the microscope is not in use, keep it covered in a microscope compartment. Never apply force to the microscope. Never allow the objective lenses to touch the cover glass or the slide. Never lower the body tube with the coarse adjustment while looking through the microscope. Never exchange the objective or occulars of different microscopes.
- Store the microscope in its cabinet when not in use.

9.8 Autoclave

Principle

Water boils when its vapour pressure equals the pressure of surrounding atmosphere. The temperature at sea level is 100°C. When water is boiled within a closed vessel at increased pressure, the boiling point of water is increased and so is the temperature of steam produced. This principle is employed in sterilising material by steam at temperature higher than 100°C and the process is called autoclaving.

For autoclaving in the laboratory, the most agreeable and commonly used method is to use steam at 121°C for 15 to 30 minutes depending upon the particular material to be sterilised.
Items to be sterilised:

Autoclaving is most suitable for culture media, aqueous solutions, decontamination of discarded cultures and specimens, rubber items such as gloves, stoppers with rubber liner, glass ware with attached rubber tubings such as transfusion sets, glass metal syringes, etc. Autoclaves designed for laboratory work and capable of handling mixed loads should be used.

9.8.1 Types of autoclave

Only autoclaves designed for laboratory work and capable of dealing with a 'mixed load' should be used. 'Porous load' and 'bottled fluid sterilizers' are rarely satisfactory for laboratory work. There are two varieties of laboratory autoclave

- Pressure cooker types; and
- Gravity displacement models with automatic air and condensate discharge.

Pressure Cooker type laboratory autoclaves:

The most common type is a device for boiling water under pressure. It has a vertical metal chamber with a strong metal lid which can be fastened down and sealed with a rubber gasket. An air and steam discharge tap, pressure gauge and safety valve are fitted in the lid. Water in the bottom of the autoclave is heated by external gas burners, an electric immersion heater or a steam coil.

Operating Instructions:

There must be sufficient water inside the chamber. The autoclave is loaded and the lid is fastened down with the discharge tap open. The

safety valve is then adjusted to the required temperature and the heat is turned on.

When the water boils, the steam will issue from the discharge tap and carry the air from the chamber with it. The steam and air should be allowed to escape freely until all of the air has been removed. This may be tested by attaching one end of a length or rubber tubing to the discharge tap and inserting the other end into a bucket or similar large container of water. Steam condenses in the water and the air rises as bubbles to the surface; when all of the air has been removed from the chamber, bubbling in the bucket will cease. When this stage has been reached, the air-steam discharge tap is closed and the rubber tubing removed. The steam pressure then rises in the chamber until the desired rpessure, usually 15 lb/in2, is reached and steam issues from the safety valve.

When the load has reached the required temperature the pressure is held for 15 min.

At the end of the sterilizing period, the heater is turned off and the autoclave allowed to cool.

The air and steam discahrge tap is opened very slowly after the pressure gauge has reached zero (atmospheric pressure). If the tap is opened too soon, while the autoclave is still under pressure, any fluid inside (liquid media, etc.) will boil explosively and bottles containing liquids may even burst. The contents are allowed to cool. Depending on the nature of the materials being sterilized, the cooling (or 'run-down') period needed may be several hours for large bottles of agar to cool to 80°C, when they are safe to handle.

Autoclaves with air discharge by gravity displacement

These autoclaves are usually arranged horizontally and are rectangular in shape, thus making the chamber more convenient for loading. A palette and trolley system can be used.

The jacket surrounding the Gravity displacement autoclave consists of an outer wall enclosing a narrow space around the chamber, which is filled with steam under pressure to keep the chamber wall warm. The steam enters the jacket from the mains supply, which is at high pressure, through a valve that reduces this pressure to the working level. The working pressure is measured on a separate pressure gauge fitted to the jacket. This jacket also has a separate drain for air and condensate to pass through.

The steam enters the chamber from the same source which supplies steam to the jacket. It is introduced in such a way that it is deflected

upwards and fills the chamber from the top downwards, thus forcing the air and condensate to flow out of the drain at the base of the chamber by gravity displacement. The drain is fitted with strainers to prevent blockage by debris. The drain is usually fitted with a thermometer for registering the temperature of the issuing steam. The temperature recorded by the drain thermometer is often lower than that in the chamber. The difference should be found with thermocouple tests. A 'near to steam' trap is also fitted.

The autmatic steam trap or 'near-to-stream' trap is designed to ensure that only saturated steam is retained inside the chamber, and that air and condensate, which are at a lower temperature than saturated steam, are automatically discharged. It is called a 'near-to-steam' trap because it opens if the temperature falls to about 2oC below that of saturated steam and closes within 2oC or near to the saturated steam temperature. The trap operates by the expansion and contraction of a metal bellows , which open and close a valve. The drain discharges into a tundish in such a way that there is a complete airbreak between the drain and the dish. This ensures that no contaminated water can flow back from the waste-pipe into the chamber.

Operation of a gravity displacement autoclave:

If the autoclave is jacketed, the jacket must first be brought to the operating temperature. The chamber is loaded, the door is closed and the steam-valve is opened, allowing steam to enter the top of the chamber. Air and condensate flow out through the drain at the bottom. When the drain thermometer reaches the required temperature a further period must be allowed for the load to reach that temperature. This should be determined initially and periodically for each autoclave. Unless this is done the load is unlikely to be sterilized. The autoclave cycle is then continued for the holding time. When it is completed the steam valves are closed and the autoclave allowed to cool until the temperature dial reads less than 80oC. Not until then is the autoclave safe to open. It should first be 'cracked' or opened very slightly and left in that position for several minutes to allow steam to escape and the load to cool further.