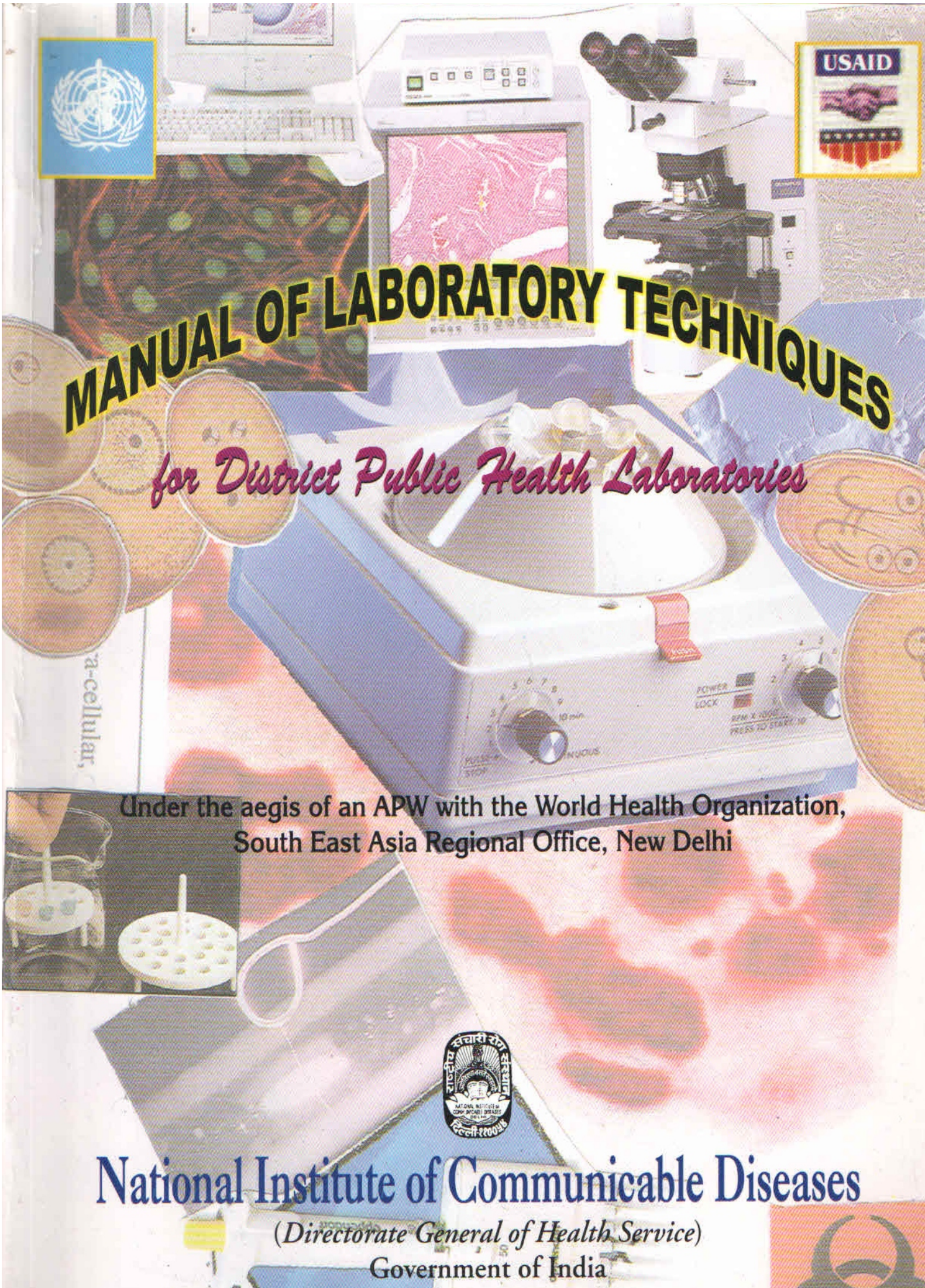

MANUAL OF LABORATORY TECHNIQUES

for District Public Health Laboratories

Under the aegis of an APW with the World Health Organization,
South East Asia Regional Office, New Delhi

National Institute of Communicable Diseases

(*Directorate General of Health Service*)

Government of India

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CONTENTS

CHAPTER -1

INTRODUCTION

The standard operating procedures (SOP) are vital documents which are essential components of quality system in any organization. These are used to ensure consistency in performing an activity. Their use is mandatory by all the staff members of the district laboratories every time they perform an activity. The accreditation and licensing procedures also demand compulsory use of SOP.

Epidemiological surveillance of a disease is the continuing scrutiny of all aspects of the occurrence and spread of a disease that is 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 intergral component of a sensitive system of surveillance.

This manual will help laboratories to play an important role in disease surveillance. This will help in

1. Diagnosis of a syndrome.
   - Encephalitis
   - Hepatitis
   - Meningitis
   - Pyrexia of unknown origin
2. Tracing the source of infection.
   - Epidemiological markers
3. Detection of inapparent infections / carriers.
   - Japanese Encephalitis
   - Typhoid fever
   - Meningococcal meningitis
4. Early detection of outbreak.
   - Meningococcal meningitis
   - Hospital infections
5. Retrospective diagnosis.
   - Rheumatic heart disease
   - Subacute sclerosing panencephalitis
6. Detection of new disease agents.
   - SARS

- Human avian influenza
- Reemerging/emerging bacterial pathogens e.g. Plague
- Drug resistant bacterial pathogens e.g.M. tuberculosis, Salmonella typhi, Methicilin resistant Staphylococcus aureus.

7. Monitoring of treatment.
   - Antibiogram
   - Sero-therapy
8. Quality control of biologicals.
   - Vaccine potency testing
   - Vaccine safety studies
9. Prevalence studies.
   - Sero-surveys
   - Immune status
10. Find out natural foci of infection.
    - Plague
    - Leptospirosis
11. Controlled field trials.
    - Newer drugs / vaccines
    - Newer regimens of drugs / vaccines

Key to successful laboratory based surveillance lies in:

- Right sample collection.
- Right time to collect samples for disease surveillance.
- Right methodology for transportation of sample.
- Right laboratory to be chosen.

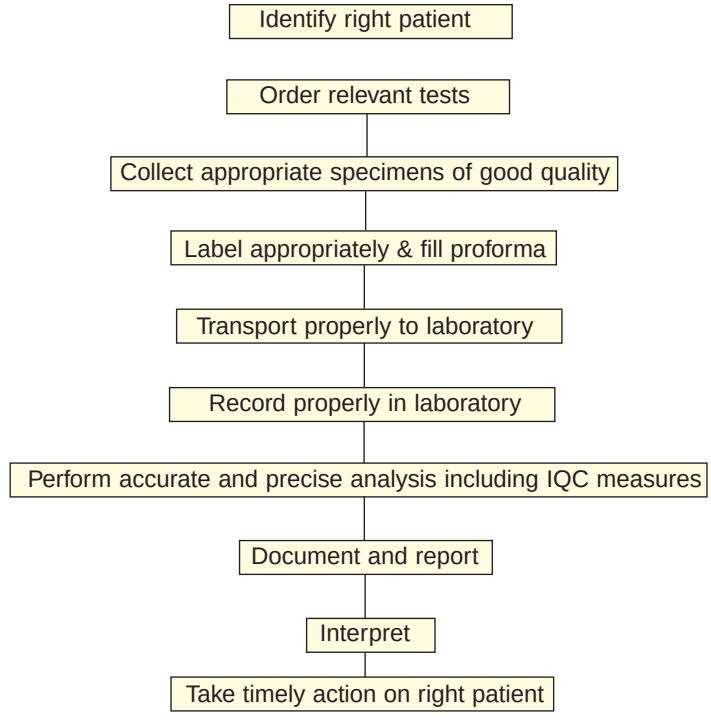


Fig: 1.1 Algorithm for ensuring good laboratory results

This manual is intended for use in the district health laboratories under the Integrated Disease Surveillance Programme. District laboratories are located at the point of first major contact of patients with the health care services. In most of the developing countries minimal laboratory services are available at primary health center or community health center (upgraded primary health centers). These laboratories provide support for preventive, curative and promotive service for the individual as well as the community. The aim of this manual is to provide guidance on the minimum basic requirement and role of district public health laboratories in disease surveillance, outbreak investigation and public health survey. The language used has been kept as simple as possible. The manual describes examination procedures that can be carried out using basic microscopy and other simple apparatus. Such procedures include the following.

- The examination of stool for intestinal parasites.
- The examination of blood for malarial parasites.
- The examination of sputum for tubercle bacilli.
- Laboratory diagnosis of pyogenic meningitis, diphtheria and cholera
- Bacteriological testing of water.
- Antimicrobial drug sensitivity testing.
- Rapid serological tests for common infectious diseases.
- Quality Assurance and biosafety in the laboratory.
- Clinical biochemistry.
- Laboratory diagnosis of zoonotic diseases.

Each laboratory procedure has been comprehensively described along with the requirement of material / equipments. Reagent requirements for each procedure, suitable tables, figures and photographs have also been given along with each procedure wherever possible.

The manual describes procedures including sample collection, transport, referral and testing, and underscores the need for biosafety networking and data analysis. The document is prepared in line with setting basic minimum lab requirements as part of the national integrated disease surveillance program. This networking of laboratory services will be as per IDSP manual guidelines in India which includes linkages and integration of surveillance data based on clinical features versus laboratory confirmation. The networking is required for

- Referral
- Supportive monitoring
- Flow of reports and data
- Teaching/Training
- Weekly/Monthly surveillance bulletin

While, in the initial phase, this is the expected function from district-level public health laboratories. States and districts are encouraged to further improve the functioning of public health laboratories, taking into account progress made and resources available

at different levels. The instructions to perform a test or activity have been described in this manual. It is suggested that the same should be rewritten by users incorporating the material and methodology to be used by them. The format described in the model SOP should be followed. Before use, SOP needs to be validated and periodically reviewed (usually after one year or whenever there is a change in methodology or material).

CHAPTER-2

OVERVIEW OF INTEGRATED DISEASE SURVEILLANCE PROJECT AND ROLE OF LABORATORY SERVICES IN SURVEILLANCE

2.1 Preamble

Disease surveillance has long been recognized as an important tool for measuring the disease burden, studying morbidity and mortality trends and early detection of outbreaks for instituting effective control measures in a timely manner. Though, the health care infrastructure in our country has grown immensely over the years, disease surveillance system did not get the desired attention resulting in frequent outbreaks. The outbreaks of plague (1994), malaria (1995), and dengue hemorrhagic fever (1996) in different parts of the country further highlighted the weaknesses in the surveillance system and brought an urgency for its strengthening so that early warning signals of outbreaks are detected and appropriate preventive and control measures are applied timely to minimize the impact of the outbreak.

Government of India launched National Surveillance Programme for Communicable Diseases (NSPCD) as a pilot project in 1997-98 with the overall goal of improving the health status of the people. The programme included capacity building at district, regional and state levels by strengthening and utilizing existing manpower, laboratory and health infra-structural facilities. The National Institute of Communicable Diseases (NICD), Directorate General of Health Services, acted as the nodal agency for monitoring and coordinating the programme at the central level. A review of the programme indicated that in the districts in which it has been made fully operational, there is a definite improvement in the capacity for early detection of outbreaks and for a structured response on a time bound basis. Keeping in view the immense utility and necessity of the scheme, it has been felt necessary to expand the programme to the entire country in a phased manner.

2.2 Integrated Disease Surveillance Project

The Government of India is initiating a decentralized, state based Integrated Disease Surveillance Project (IDSP) in the country in response to a long felt need expressed by various expert committees. The project is intended to be the backbone of public health delivery system in the country. It will be able to detect early warning signals of impending outbreaks and help initiate an effective response in a timely manner. It is also expected to provide essential data to monitor progress of on going disease control programmes and help allocate health resources more optimally. Main objectives of the project are:

- To establish a decentralized state based system of surveillance for communicable and non-communicable diseases and their risk factors so that timely and effective public health actions can be initiated in response to health challenges in the country

at the state and national levels.

- To improve the efficiency of the existing surveillance activities of disease control programs and facilitate sharing of relevant information with the health administration, community and other stakeholders so as to detect disease and risk factor trends over time and evaluate control strategies.

The Project would comprise of following four Components:

1. Integrating and decentralizing surveillance activities.
2. Strengthening Public Health Laboratories.
3. Using information technology optimally.
4. Enhancing human resource development.

The project will cover limited number of conditions based on state perceptions including 13 core and up to 5 state priority conditions for which pubic health response is available. The conditions selected initially include important communicable diseases, risk factors for non-communicable diseases, out door air pollution, drinking water contamination and road traffic accidents.

District, state & central surveillance units will be set up so that the project is able to respond in a timely manner to surveillance challenges in the country including emerging epidemics. It will ultimately integrate surveillance activities under various programmes and use existing infrastructure for its function. Besides, government sector, sentinel private practitioners, sentinel private hospitals and sentinel private laboratories will be inducted into the project. This will particularly ensure better surveillance in the urban regions of the country. The project will facilitate active participation of medical colleges in the surveillance activities.

The project will ensure uniform high quality surveillance activities at all levels by:

- Limiting number of diseases under surveillance to reduce workload at the periphery.
- Developing standard case definitions and formats for reporting.
- Developing user-friendly operational and training manuals.
- Providing training to all essential personnel and
- Setting a system of regular feed back on the quality of surveillance activity.

Laboratory infrastructure will be strengthened particularly at the district level to enhance capacity for diagnosis and investigation of epidemics and confirmation of disease conditions. Support will also be provided to network district laboratory systems with the state and regional reference laboratories in the country.

The project will effectively use the current information technology for communication, data entry, analysis, reporting, feedback and actions. Computers will be provided to facilitate integration and timeliness of surveillance. A national level surveillance network will be established up to the district level.

The project will be decentralized and the primary administrative responsibility will be at the district and state levels but will be coordinated by the centre. Administrative restructuring will include setting up surveillance committees at district, state and the central level.

2.3 Diseases under surveillance

The diseases to be included in the surveillance project are based on burden of diseases in the community, availability of public health response and special considerations and international commitments.

2.3.1 Core Diseases

(i) Regular Surveillance

Vector Borne Diseases	:	Malaria/Denguefever / DHF/Filaria
Water Borne Diseases	:	AcuteDiarrhoeal Disease (Cholera), Typhoid, Viral Hepatitis
Respiratory Diseases	:	Tuberculosis
Vaccine Preventable Diseases	:	Measles, Diphtheria
Diseases under eradication	:	Polio
Sexually transmitted diseases	:	Syphillis
Other International commitments	:	Plague
Unusual clinical syndromes (causing death/hospitalization)	:	Meningoencephalitis/ Respiratory distress/Leptospirosis/ Haemorrhagic fevers/ Other undiagnosed conditions
Antimicrobial resistance monitoring	:	for enteropathogens etc.
Other Conditions		Road traffic accidents (Linkup with police computers)

(ii) Sentinel Surveillance

Sexually transmitted diseases / Blood borne	:	HIV/HBV, HCV
Other Conditions	:	Water Quality
	:	Outdoor Air Quality (Large urban centers)

(iii) Regular periodic surveys

NCD Risk Factors	:	Anthropometry, Physical activity, Blood Pressure, Tobacco, Nutrition

2.3.2 Additional State Priorities

In addition to the core diseases, which will be under surveillance for all the states, each state will identify up to five additional conditions for which surveillance will be initiated.

2.4 Role of Laboratory Services under Integrated Disease Surveillance Project

The project envisages improving the diagnostic facilities in public health laboratories up to primary health centres. Laboratory support is essential to proper functioning of disease surveillance and hence comprehensive support will be provided for surveillance related laboratory work. Currently, the laboratory support in many areas appears weak and the available system is not integrated well with the district and state surveillance units. There is a need to define the specific roles of the various laboratories at PHCs, district and state levels and strengthen these systems. Areas that need special attention include technology and infrastructure for performing essential diagnostic tests at the district level, particularly microbiology cultures, bio-safety management and quality assurance.

The laboratory network for IDSP will be established at various levels:

- Peripheral laboratories and microscopic centers (L1 Labs)
- District public health laboratories (L2 Labs)
- State laboratories (L3 Labs)
- Regional and quality assurance laboratories (L4)
- Disease based reference laboratories (L5)

2.4.1 Peripheral laboratories (L1)

Peripheral laboratories will function so as to assist the laboratory diagnosis of:

i. Malaria
ii. Tuberculosis
iii. Typhoid
iv. Chlorination level of the drinking water source in the periphery
v. Fecal contamination of water by rapid H_2S test
vi. Proper collection and transportation of samples to the higher level of labs.

Existing peripheral laboratories at the PHC/CHC level are capable of handling microscopic examination of sputum and blood smears and are currently undertaking this activity under TB and Malaria Control Programmes. Typhoid can be diagnosed at the periphery using 'Typhi Dot' test, which can be performed easily and has established validity and reliability. Kits are available for detecting fecal contamination of water, which can be used at the periphery and these will be made available. Peripheral laboratories need minimal structural modification of the laboratory areas in the CHC to perform these functions well. Most of them are currently equipped with microscopes for performing microscopy for TB and Malaria.

In the urban regions, the microscopic centers for TB and Malaria programmes can continue to provide inputs on these diseases, but additional selected private laboratories and established and accredited laboratories will be brought into the network as sentinel institutions in the periphery.

2.4.2 District Level Laboratories (L2)

The District Level Laboratory will be the backbone of the laboratory network under IDSP. Currently, the district level laboratories are the weakest link in the programme. The district laboratories are expected to have a major role in disease surveillance and the existing infrastructure and administrative structure will be improved to undertake the following tasks:

Conditions	Tests
Tuberculosis	AFB smear examination
Malaria	Blood smear examination
Typhoid	Rapid serological diagnostic test and sample collection for blood culture
Cholera	Stool culture
Water Quality	Chlorination tests and fecal contamination by rapid H_2S test & MPN test
Diphtheria	Albert's stained smear examination
Leptospirosis	Rapid dot test
Drug resistance	In vitro drug sensitivity testing of non fastidious bacteria being cultured except Myc. tuberculosis
Dengue	Rapid strip test can be done.
Viral Hepatitis	Rapid test for HBsAg, HCV only.
HIV	Rapid test/ELISA (NACO guidelines)
Syphillis/yaws	RPR test
Meningitis	Smear examination, Rapid latex agglutination test
Non Communicable Diseases	Blood sugar, Cholestrol, Lipid Profile, KFT, LFT

*** For Plague, Measles, JE, water born Hepatitis viruses and other viral diseases: based on clinical diagnosis only sample collection to be carried out by the district laboratories and refer the samples for analysis to designated referral laboratories for the purpose.**

Most of the district laboratories are now attached to district hospitals and primarily perform the functions of a clinical laboratory. These laboratories will be staffed by a qualified person who can undertake microbiological cultures.

At the district level, integration of equipment and trained staff under Tuberculosis and Malaria programmes is crucial for effective functioning of the district level laboratory, since trained laboratory technicians are already available under these programmes.

2.5 Following is the current status and requirements of equipment at the District level

What is needed	What is available and Where	Incremental Needs
Binocular microscope with oil immersion	Binocular microscopes have been supplied by the TB and Malaria programme and also by NPSCD	Nil
Test tube rack	Available	Nil
Table top centrifuge	Available in district PH laboratory	Additional one needed
Incubator	Available at district PH laboratory	Additional one needed
Water bath	Not available	Needed
Refrigerator	Available at district PH laboratory	Nil
Autoclave	Small one available	Needed
ELISA reader and Washer	Available in some districts	Optional depending on local conditions
Hot air oven	Available at District PH laboratory	Nil
Bio-safety hood	Not available	Needed
Inoculating loops	Not available	Needed
Pasteur pipettes	Not available	Needed
Vortex mixer	Not available	Needed
- 20°C Deep freezer	Not available	Needed
Colorimeter	Not available	Needed
pH meter	Not available	Needed
Semi automated analyser	Not available	Needed
Glass distillation plant	Not available	Needed
Micro pipettes	Not available	Needed
Pipetting devices	Not available	Needed
Electronic balance	Not available	Needed
Needle destroyer (electric)	Not available	Needed

2.6 Following is the current status and requirements of some of the common reagents and supplies at the district level

What is needed	What is available and where	Incremental needs
Ziehl neelsen acid fast stain	Available with district TB laboratory	Nil
Blood culture bottles with broth	Not available	Needed
Rapid diagnostic test kit for Typhoid	Not available	Needed
Dehydrated media	Not available	Needed
HIV diagnostic kit ELISA	Available with NACO	Nil
Diagnostic kit for water quality	Not available	Needed
Screw capped bottles	Not available	Needed

The laboratories of ESI HQ and regional railway hospitals and army command hospitals in some districts can take on the role of the district public health laboratory if memorandum of understanding can be arrived at, with the state surveillance unit for

that function. The district level laboratories will be included in the IDSP computer network, so that information on identified diseases can be promptly transferred to the district surveillance unit and to the peripheral reporting units, as soon as data becomes available.

2.7 Private laboratories

Private laboratories will be brought under a licensing and accreditation system. Till accreditation is established, the identified/selected private laboratories will be included as one of the sentinel reporting units, and go for IDSP certification in a phased manner by a panel of experts to be formed.

2.8 State Level Laboratories (L3)

Most states have well developed state level laboratories capable of performing the role of L3 laboratories. Labs in medical colleges will be part of this level. The state level laboratories need not be in a single laboratory but can be a group of well-networked laboratories in the state, specific for diseases under consideration. The primary roles of the state level laboratories are to:

1. Carry out advanced laboratory tests as highlighted in Table 2.9
2. Provide quality assurance of district laboratories
3. Impart training of laboratory personnel at the district levels
4. Participate in the epidemic investigation in response to surveillance challenges
5. Link up with state and district surveillance units so that information transfer is optimized.
6. Function as the primary laboratory for NCD risk factor surveillance.
7. Referral of specimens.
8. Supportive monitoring for supplies, training, six monthly visits to the districts.

2.9 Following Tests will be performed at the State Laboratories

Conditions	Test	Confirmation	Objective
Tuberculosis	AFB culture and sensitivity	Perform in 1% of positive cultures from district level	Identify magnitude of MDR TB
Malaria	Microscopy and rapid antigen detection tests.	Confirm 1% from districts	For Q.C.
Typhoid	Sensitivity testing in S.typhi isolates	Confirm 1% of bacterial isolates at district level	Pattern of AMR for S.typhi typing
Cholera	Cholera culture and typing Cholera toxin test	1% of Cholera isolates from districts	Identify pattern of bacterial infection
Water quality	MPN method	Confirm 0.5% from district levels	For Q.C.
NCD surveillance	Blood sugar, HDL, LDL, KFT, LFT		Risk factor surveillance for NCD

Conditions	Test	Confirmation	Objective
Polio	Follow present procedures as per programme		Confirm Polio
Measles	ELISA test for Measles IgM antibodies		Confirm Measles
Leptospirosis	Rapid serological test for Leptospira and sending sample to referral lab for MAT/culture		Confirm Leptospirae
Dengue Hepatitis	IgM test for Dengue Serology for Hepatitis A, E, B, C by ELISA Kits.	Confirm 1% of samples from district	Confirm Dengue Quality control Hepatitis work
Anthrax	Microscopy/culture for presumptive Anthrax and sending sample to referral lab for confirmation.		Presumptive Anthrax
Plague	Microscopy for presumptive plague and sending sample to the referral lab for confirmation		Presumptive Plague

In each state, the state surveillance unit will select the laboratories to perform the above functions for IDSP. These laboratories will be chosen depending on the existing strength and willingness to perform the surveillance activity and can be from both the government and private sector.

Most state level laboratories have the infrastructure and equipment which may need to be minimally supplemented to function effectively in the roles specified as state surveillance laboratories. Consumables may need to be provided for some of the diseases for which additional load is expected. State level laboratories are expected to do AMR testing on random samples of isolates from districts and this needs additional inputs.

Most of the state level laboratories are already providing training for laboratory technicians at different levels. This may need to be tailored to suit the needs of the IDSP. Currently, the biggest lacuna in state level laboratories is their connectivity. Some laboratories have got computers but there is no infrastructure for sharing the information. Development of a network is essential. Most laboratories will be provided additional dedicated computers and software under IDSP. The success of the programme will depend on effective networking;

Peripheral ↔ District ↔ State ↔ Regional ↔ Reference

2.10 Regional, Quality Assurance and Reference laboratories (L4, L5)

IDSP will have one central and four regional reference laboratories catering to routine work and outbreak investigations. The Central laboratory at NICD will function

as the apex reference laboratory for IDSP. In addition, selected laboratories will be designated as IDSP regional reference centers in the four regions of India. Along with the above designated reference laboratories, there are a number of laboratories of high standard in the country and they could be incorporated into the IDSP as reference laboratories for various specific disease conditions. These include:

1. National Institute of Communicable Diseases, Delhi and its branches.
2. National Institute of Cholera and Enteric Diseases, Kolkatta
3. National Institute of Virology, Pune
4. PGI Chandigarh
5. CMC, Vellore
6. NIMHANS, Bangalore
7. KIPM, Chennai
8. SGPGI, Lucknow
9. TRC, Chennai

There are a number of ICMR laboratories in different parts of India, which could function as reference laboratories for IDSP apart from NICD. These could help in:

- Characterization of the strains e.g. sero-typing, phage typing, molecular finger printing
- Repository of standard strains and reagents and their supply to constituent laboratories
- Production of diagnostic anti-sera and supply to laboratories in the network
- Production of reagents for antibody detection and their supply to laboratories
- Research in production of rapid diagnostic reagents
- Standardizing of methods and procedures and provide these to constituent laboratories
- Quality assurance of laboratories

All the laboratories are requested to participate in quality assurance programme of IDSP.

CHAPTER -3

BIOSAFETY

The need for Biosafety guidelines stems from the fact that there are extreme situations in laboratory facilities in our country ranging from rooms with fans, windows, air conditioners to the more sophisticated equipment like laminar air flow system. International standards are rigid and in the developing country like India, it is not always possible though not impossible to adhere to such rigid guidelines.

Good laboratory technique is fundamental to laboratory safety. Important concepts to have lab safety are listed below.

3.1 Entry / access to laboratory area

- ⇨ Have a biohazard sign (Fig 3.1) displayed on the doors of the rooms where infectious agents are handled.
- ⇨ Entry to laboratory working area should be only for laboratory persons.
- ⇨ Doors to the laboratory should be kept closed.
- ⇨ No smoking, eating, or drinking is allowed in laboratory area.

Fig 3.1 Biohazard sign.

3.2 Personal Protection

- o While working in the laboratory always wear lab coat.
- o Have all the personnel protective equipments ready & use them as per the procedures (Fig: 3.3) strictly for highly infectious diseases outbreaks.
- o Wear gloves for all procedures that may involve direct or accidental contact with blood / infectious materials.
- o After use, gloves should be removed carefully without touching infected surface, disposed off in container containing disinfectant solution. Hands should be washed with soap & water.
- o Laboratory personnel must wash their hands after handling infectious materials / performing test procedures and before they leave the laboratory working area. See washing of hands procedure (Fig: 3.2)
- o Laboratory coat should not be worn outside the laboratory area i.e canteen, library, and toilet or staff common room.

- Eating, drinking, applying cosmetics and handling contact lens are strictly prohibited in the laboratories.
- Laboratory coat used/unused should not be placed in the same cupboard with street clothes or food articles etc.
- Lab personnel should receive suitable vaccination e.g. Hepatitis B.

3.3 General procedural precautions

- ⇨ Mouth pipetting must be strictly avoided.
- ⇨ Materials / articles must not be held in the mouth. Do not lick / wet labels for sticking.
- ⇨ All technical procedures should be such that they minimize the formation of aerosols and droplets. In the district laboratories do not perform any procedure that generates lots of aerosolization unless there is an access to biological safety cabinet.
- ⇨ Do not use hypodermic needles and syringes for pipetting devices.
- ⇨ All spills, accident or exposure to infectious materials, must be reported to laboratory in charge and a record should be maintained.
- ⇨ Display written procedures for the clean up of all spills.

3.3.1 Procedure to clean up all spills

- ⇨ Pour 1 % freshly prepared Sodium hypochlorite solution over spills in sufficient quantity.
- ⇨ Cover the spills with paper towel or absorbent materials.
- ⇨ Leave for 10 min.
- ⇨ Clean it
- ⇨ Wipe up the whole spill with fresh absorbent material using gloved hands and discard it in a contaminated waste container
- ⇨ Wipe the surface with soap and water.

3.4 Laboratory working areas

- ⇨ Keep the laboratory area neat, clean and free of materials that are not required.
- ⇨ Decontaminate the working surface after any spill and at the end of the working day using 1 % Sod. hypochlorite.
- ⇨ All contaminated materials, specimens, cultures, must be decontaminated in the laboratory premises before final disposal or cleaning for reuse.
- ⇨ If there are windows in laboratory area, they should have arthropod / mosquito & fly proof mesh.

3.5 Bio safety Management

- ⇨ Have one person responsible for bio safety activities→ Biosafety officer.
- ⇨ Health checks up of laboratory staff at regular intervals.
- ⇨ Immunization against diseases which are feasible must be given regularly, especially Hepatitis B.
- ⇨ Bio safety officer should train lower staff regularly.

3.6 Laboratory Designs and Facilities

3.6.1 Design

- ⇨ Enough space should be available
- ⇨ Smooth easily cleanable walls, ceiling and floors which should be impermeable to liquids and resistant to chemicals and disinfectants.
- ⇨ Ample illumination should be available for safe conduction of laboratory procedures.
- ⇨ Regular, continuous and dependable, quality water supply should be available which is important for laboratory techniques.
- ⇨ Wash basins with running water if possible should be provided in each laboratory room preferably near the exit door.
- ⇨ Suitably equipped first aid box should be available in the district laboratory.
- ⇨ Control programme for rodents and insects in the laboratory should be there.

3.7 Laboratory Equipment

Ensure the adequate equipment be provided and that they are used properly.

3.7.1 Essential biosafety equipment are

a) Pipetting aids → to avoid mouth pipetting

b) Screw capped tubes and bottles.

c) Autoclaves to decontaminate infectious material wastes.

d) Plastic disposable pasteur pipettes, when ever possible should be used.

e) Equipments should be validated before being taken for use and then revalidation should be done at regular intervals.

3.8 Training

Human error and poor techniques are important in non protection of laboratory workers. Thus training is important. Staff training should include safe methods adopted for commonly used laboratory procedures like :-

- Inhalation risks → using loops, streaking agarplate, pipetting, smear preparation, opening culture stocks, centrifugation, taking blood/serum samples etc.
- Ingestion risks → handling specimens, smears, cultures.
- Inoculation risks → accidental needle stick injuries.
- Handling blood and other infectious agents.
- Decontamination and disposal of infectious material.

Fig: 3.2 - Steps of good hand washing

Step 1.
Wash palms and fingers.

Step 2.
Wash back of hands.

Step 3.
Wash fingers and knuckles.

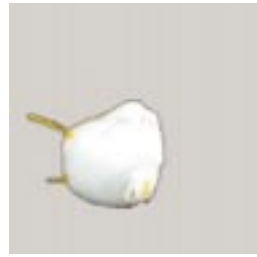
Step 4.
Wash thumbs.

Step 5.
Wash fingertips.

Step 6.
Wash wrists.

Fig: 3.3 - Protective barriers (All are not for routine testing but for handling highly infectious agents)

Goggles

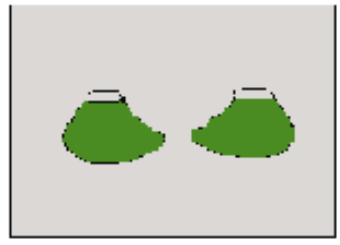


N-95 Mask

Gown (must for lab work)

Triple layer Mask

Gloves



Shoe covers

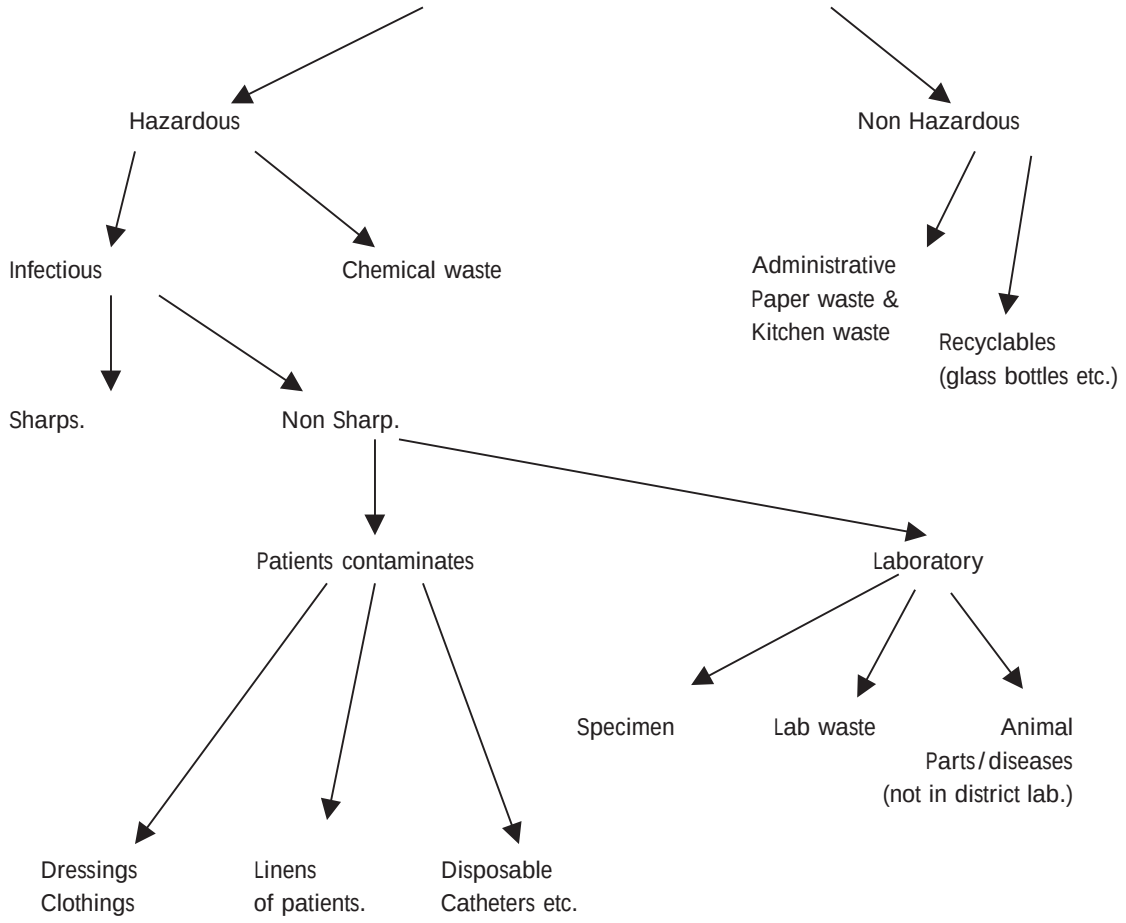
3.9 WASTE MANAGEMENT

3.9.1 What is Waste?

Any thing which has to be discarded is called waste. The laboratory organisms require appropriate handling. The most common documented transmission of infection from waste to health care worker is through contaminated metallic waste. Hospital waste is a potential reservoir of pathogenic micro-organisms. Decontamination of waste and their ultimate disposal are closely interrelated. Laboratory wastes are of different category &

can be classified as can be seen in Fig:-3.4

Fig;3.4 - Classification of Laboratory Waste / Hospital wastes.



3.9.2 Hospital/Laboratory Waste Management

3.9.2.1 Material required

1) Waste disposal color coded bags with biohazard symbol. blue, red, black and yellow.
2) Trolley baskets for holding the bags.
3) Autoclave for decontamination of waste on site.
4) Disinfectant solution (Sodium hypochlorite solution.).
5) Incinerator if possible (Optional).
6) Soap for hand washing and towel for drying hands.
7) Gloves.
8) Puncture proof containers plastic / metal with a biohazard symbol.

3.9.2.2 Follow management at every step from the site of generation

- Segregation.
- Collection.
- Transportation.
- Storage.
- Treatment to disinfect.
- Final disposal.

⇨ Segregate waste into the prescribed categories at the point of generation.

⇨ Color coded bags as per international norms. (Table-3.1)

3.10 Methodology

In the district lab, the lab waste handling is an essential job which needs to be under supervision of biosafety officer. Broad guidelines to be followed are :

⇨ Segregate the different category of waste at the point of generation.

⇨ Discard infectious wastes (non sharp) if possible in disinfectant solution or autoclave to render it non-infectious.

⇨ Discard sharp waste i.e. needles, blades etc in a puncture proof containers. After the container is 2/3 filled, it should be autoclaved/ shredded and land filled for disposal.

⇨ If nothing is available for disposal deep bury (as per standard guidelines) in a secure area.

3.11 Categories

All waste should be decontaminated (chemically/autoclaving) before final disposal/ reuse.

a) Non contaminated waste which can be reused or recycled, disposed off as general house hold waste.

b) Contaminated sharps disposed off in puncture proof containers fitted with cover, labeled as infectious.

c) Contaminated reusable materials for decontamination by autoclave, thereafter washing and reuse/ recycle.

d) Contaminated disposable material for autoclaving & disposal.

e) Contaminated material for direct incineration.

3.12 Quality control

- ⇨ Check that proper quality bags are purchased.
- ⇨ Autoclave monitoring & maintenance.
- ⇨ Disinfectant quality check.

3.13 Contaminated infectious materials for autoclaving and reuse

- No pre cleaning to be done.
- Transfer material to autoclave.
- Autoclave at 121°C / 15 lbs pressure for 45 minutes.
- If cleaning is required, do washing as described (Section 5.4.2).
- Re use.

3.14 Contaminated infectious waste for disposal

- Autoclave in leak proof container. i.e. autoclavable colour coded plastic bags.
- Place material in a transfer containers / trolleys with bags.
- Transport to incinerator.
- If reusable transfer containers are used they should be disinfected and cleaned before they are returned to laboratories.
- Discarding jars preferably unbreakable should be used and they should have suitable disinfectant (Sodium hypochlorite 1%) freshly prepared each day.

Table-3.1 Container and color coding for disposal of bio-medical lab wastes

Waste category	Waste class.	Type of containers	Colorcoding	Treatement of waste Disposal.
1.	Microbiology & Biochem. Lab.	Plastic holding bags with biohazard sign.	Yellow	Autoclaving/ Microwave & shredding.
2.	Waste sharps	Reusable plastic/ Metal containers	Blue	Shredding & deep buried.
3.	Discarded chemical, reagents, kits.	-------Do----	--Do--	---------Do----------
4.	Soiled wastes (Lab coats etc.)	Plastic bags with biohazard sign.	Yellow/Black.	Disinfect / Autoclave then Machine wash.
5.	Chemical wastes	Sturdy containers or Plastic holding bags.	Yellow/Black.	Incineration (not Mercury)
6.	Disposable other than sharps.	Reusable sturdy containers/Plastic bags.	Yellow/Black	Disinfect/ Autoclaving/ shredding /buried.

3.15 SODIUM HYPOCHLORITE SOLUTION PREPARATION

Dilution of sodium hypochlorite solutions
(part of stock solution: parts of water)

Required Strength	4% Stock Solution	5 % Stock Solution	10% Stock Solution	15% Stock Solution
0.1% (1g/L-1000 ppm)	1:40	1:50	1:100	1:150
0.5 % (5g/L-5000 ppm)	1:20	1:25	1:50	1:75
1% (10g/L-10,000 ppm)	1:4	1:5	1:10	1:15

Note:- Alaways prepare diluted hypochlorite solution fresh every day. If sodium hypochlorite is not available an alternative calcium hypochlorite (1%) can be used which needs to be prepared as follows

CALCIUM HYPOCHLORITE SOLUTION

Chlorine available in powder form	How to dilute to 0.1%	Chlorine available in 0.1% solution	How to dilute to 1%	Chlorine available in 1% solution
35%	2.8 gms to 1 litre in water	1000 ppm	28 gms to 1 litre in water	10000 ppm

3.16 QUESTIONNAIRE FOR BIOSAFETY

1) Name the personal protective measures to be used in district laboratory
   -
   -
   -
2) Are you doing hand washing before entering and after leaving the laboratory working area?
   YES / NO
3) How frequently sodium hypochlorite 1% disinfectant solution should be prepared in the laboratory.
   - Daily
   - Weekly
   - Monthly
4) One should always do mouth pipetting for dispensing liquid.
   YES / NO
5) How the infectious waste should be handled.
   - Just discard in garbage.
   - Put in specific color bags / Autoclave and then discard
   - Or any others describe.

CHAPTER - 4

COLLECTION, TRANSPORT & STORAGE OF CLINICAL SPECIMENS

4.1 GENERAL BIOSAFETY MEASURES

- Use disposable gloves wherever required, while collection of clinical specimen
- Wear laboratory coats while collection & handling of specimens, wherever required
- Use protective eye or face shields if procedure is likely to generate aerosols
- All laboratory waste should be handled with care to avoid injuries from sharps
- As far as possible, manual handling of waste should be avoided
- The waste should be placed in appropriate leak-proof biohazard bags (Table 3.1) and autoclaved before disposal. The clinical samples should be processed only in designated laboratory having the proper containment facility.

4.2 BLOOD SPECIMEN COLLECTION

Blood and separated serum are the most common specimens taken to investigate outbreaks of communicable diseases. Venous blood can be used for isolation and identification of the pathogen in culture and by inoculation, or separated into serum for the detection of genetic material (e.g. using the polymerase chain reaction), specific antibodies, antigens, or toxins (e.g. by ELISA). When specific antibodies are being assayed, it is often helpful to collect paired sera, i.e. an acute sample at the onset of illness and a convalescent sample one to four weeks later. Blood can also be collected by finger prick for the preparation of slides for microscopy or for absorption onto special filter paper discs for analysis. Whenever possible, blood specimens for culture should be taken before antibiotics are administered to the patient

4.2.1 Venous blood sample

4.2.1.1 Materials required

- Skin disinfectant : 70% alcohol (isopropyl alcohol, ethanol) or 10% povidone iodine, swabs, gauze pads, band aid
- Disposable latex gloves
- Tourniquet and sterile disposable syringes and needles
- Sterile screw-cap tubes, blood culture bottles (50ml for adults, 25ml for children) with appropriate media where ever required.
- Labels.

4.2.1.2 Method of collection

- Place a tourniquet above the venepuncture site
- Palpate and locate the vein.
- Disinfect the venepuncture site meticulously with 10% povidone iodine or 70% isopropyl alcohol by swabbing the skin concentrically from the centre of the venepuncture site outwards. Let the disinfectant evaporate. Do not repalpate the vein again. Perform venepuncture and collect required quantity of blood.
- Remove the tourniquet. If collection is done for blood culture, withdraw the needle and then apply swab on skin. Apply pressure to site until bleeding stops, and apply band-aid (if desired).
- Using aseptic technique, transfer the specimen to sterile sample collection bottle. For blood culture bottles, swab the top with 70% alcohol, flame it and then inoculate the blood in the bottle through the hole in the cap. Secure caps tightly.
- Blood should not be squeezed through the needle.
- Label the tube, including the unique patient identification number, using permanent marker pen.
- Do not recap used sharps. Discard directly into the sharps disposal container or destroy using a needle destroyer as per the procedure.
- Complete the case investigation and the laboratory request forms using the same identification number.
- Do not use wet tubes for collecting blood as it leads to heamolysis.

4.2.1.3 Handling of blood samples

- Keep blood culture bottles and blood sample tubes upright and secured in a screw cap container or in a rack in a transport box at room temperature.
- Cushion or suspend bottles during transport over rough terrain to prevent lysis of red cells. Place enough absorbent paper around them to soak up all the liquid in case of a spill.
- Blood sample bottle should be kept undisturbed at room temperature for 30 to 45 minutes to prevent lysis.

4.2.1.4 Separation of serum from blood

4.2.1.4.1 Additional materials required

- Sterile Pasteur pipettes and bulb, or disposable pasteur pipettes. The latter are easier to handle and can easily be disposed of in the field laboratory.
- Sterile screw-cap tubes - 2 per sample (preferably 5ml vials).

4.2.1.4.2 Method of separation

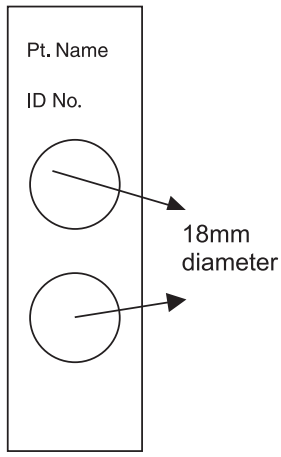
- After the blood specimen clots for 30-45 minutes at ambient temperature, keep it at 4 to 8°C for the clot to retract for a minimum of 1 to 2 hours. It can be stored at this temperature for 48-72 hours till serum is separated.
- Remove the clot aseptically using sterile Pasteur pipette and centrifuge the supernatant at low speed (1500 rpm for 5-10 minutes) to remove residual blood cells. Clear portion / serum may be aliquoted. Ensure that the centrifuge is in good condition and the tubes are properly closed and balanced to avoid breakage and spilling.
- In case a centrifuge machine is not available, and there is likely to be a delay before samples can be transported to a laboratory, allow 4-6 hours to elapse after taking the blood sample to ensure adequate clot retraction. Using the pasteur pipette, remove the clear yellow serum whilst taking care to keep the tip as far as possible from the clot, and avoid agitating the blood tube during the removal process. Transfer to plastic screw cap tubes and secure caps tightly.
- Label the tubes with the same patient details that appear on the blood sample tube.

4.2.1.4.3 Handling and transportation.

- Sera may be stored at 4-8°C for up to 10 days. If the serum needs to be stored for weeks or months before processing or sending to other lab, then keep at -20°C or in freezer compartment of refrigerator.

  Do not freeze unseparated blood samples to avoid haemolysis.

4.2.2 Capillary blood sample (finger-prick, ear lobe, and heal-prick)



4.2.2.1 Materials required.

- Disposable sterile lancets , disinfectant (70 % alcohol), swabs
- Glass slides, cover slips, slide box
- Filter paper
- Fixatives such as methanol.

4.2.2.2 Method of collection

- Disinfect finger or thumb for adults, thumb for children, or side of heel for infants by swabbing with 70% isopropyl alcohol, and prick with a sterile lancet. Wipe away the first drop of blood.
- Discard used lancets directly into the sharps disposal container.

- Collect blood directly onto labelled glass microscope slides and / or filter paper (Whatman No. 3 with circle of 18mm diameter). When collecting on a filter paper, ensure that the premarked area is completely soaked with blood on both the sides.

4.2.3 Method of preparation of blood films

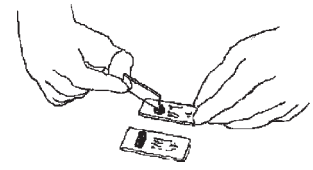
Blood films should be made by trained personnel. If this is not possible, they can be prepared from heparinized or EDTA blood specimens sent to the laboratory.

4.2.3.1 Thick films for microscopy

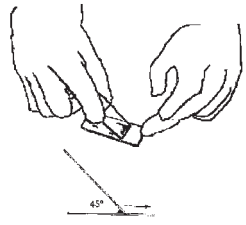
- Disinfect and prick site with a lancet as described above.
- Take one or more large drops of blood onto the centre of the slide making sure that the slide does not touch the skin.
- Spread the blood in a circle about 1 cm in diameter using the corner of another glass slide.
- Air-dry the film in a horizontal position. Do not dry the film by heating over a flame or other heat source.(SEE FIG: 4.1)
- Label the slide with patient identification number and name.

Fig: 4.1 - MAKING OF THIN & THICK SMEARS

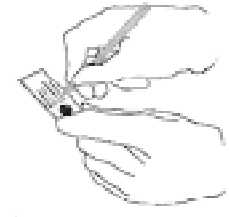
Prick finger tip with disposable sterile lancet. Touch drop of blood onto center of slide



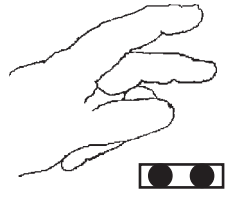
Thicksmear: spread drop of blood in circle of slide



Thinfilm: spread using another slide at angle of 45°



Lable the slide



Collection of blood on filter paper (premarked)

4.2.3.2 Thin films for microscopy

- Take another drop of blood to the glass slide about 2 cm from one end making sure that the slide does not touch the skin.
- Place the slide horizontally on a flat surface.
- Hold the side of a second clean glass slide (the spreader) on to the center of the specimen slide and move it back until it touches the drop and the blood spreads along its base.
- At an angle of about 45°, move the spreader firmly and steadily across the specimen slide and air-dry the film quickly. Do not dry over a flame or other heat source.
- Label the slide with patient identification number and name.
- Fix the dried film by dipping the glass slide in methanol for a few seconds and air dry.

4.2.3.3. Handling and transportation

- Transport air-dried and / or fixed films at ambient temperature preferably within 24 hours of specimen collection. Do not refrigerate. Thick and thin films should be kept in separate slide boxes.

4.3 CEREBROSPINAL FLUID (CSF) SPECIMEN COLLECTION

The specimen must be taken by a physician experienced in the procedure. CSF is used in the diagnosis of viral, bacterial, parasitic, and fungal meningitis.

4.3.1 Materials required

Lumbar puncture tray which includes:

- Sterile materials: gloves, cotton, towels or drapes.
- Local anaesthetic, sterilized needle, syringe.
- Skin disinfectant: 10% povidone iodine or 70% alcohol.
- Two lumbar puncture needles, small bore with stylet(sterilized).
- Six externally threaded sterile screw-cap tubes and tube rack

4.3.2 Method of collection

Only experienced clinician should be involved in the collection of CSF samples. CSF is collected directly into the separate screw-cap sterile tubes. Separate tubes should be collected for bacterial and viral processing.

- Make the patient lie on the bed in left lateral position. Ask the patient to flex the neck (so that the chin touches the chest) hip and the knee joint.

- Using the iliac crest as the reference point, palpate the joint space between the 4th and the 5th lumbar vertebrae and identify the surface anatomy.
- Disinfect the site meticulously with 10% povidone iodine or 70% isopropyl alcohol by swabbing the skin concentrically from the centre of the site outwards. Let the disinfectant evaporate. Do not repalpate the site again.
- Infiltrate the local area with the local anaestheic and wait for 4-5 minutes for the effect to appear before performing lumbar puncture
- Insert the sterile lumbar puncture needle between the 4th and 5th lumbar vertebrae to a depth of 4-5 cm, withdraw the stylet. Fluid flows freely through the needle.
- Between 1 and 2 ml of CSF is collected in each of the 3 tubes, one for culture, one for biochemical analysis and one for cytology.

Note: - Haemorrhagic CSF sample is not recommended for serological testing.

4.3.3 Handling and transportation

- In general, send the specimens to the laboratory and process as soon as possible.
- Transport CSF specimens for bacteriology at ambient temperature, generally without transport media. Never refrigerate the CSF, as many of the bacterial pathogens do not survive well at low temperatures.
- CSF specimens for virology do not need transport medium. They should be transported at 4-8°C.

4.4 STOOL SAMPLE COLLECTION

Stool specimens are most useful for microbiological diagnosis if collected soon after onset of diarrhoea (for most viruses < 48 hours and for bacteria < 4 days after the onset of illness), and preferably before the initiation of antibiotic therapy. If required, two or three specimens may be collected on consecutive / alternate days. Stool is the preferred specimen for culture of bacterial, viral, and parasitic diarrhoeal pathogens. Rectal swab samples may also be used in case of infants, debilitated patients or while carrying out direct endoscopic visualization of a lesion or any other situation where voided stool sample collection is not feasible. **In general, rectal swabs are not recommended for the isolation of viruses. As far as possible, do not collect stool samples from a bedpan.**

4.4.1 Materials required

- Clean, dry, leak-proof screw cap container (which has not been priorly washed with a disinfectant) and tape
- Appropriate bacterial transport media for transport of rectal swabs from infants

4.4.2 Method of collecting a stool specimen/ rectal swab-: Please refer to chapter 9

4.4.3 Handling and transportation. Please refer to chapter 9

- Stool specimens should be transported at 4-8°C. Bacterial / viral culture isolation rate fall significantly if specimens are not processed within 1-2 days of collection.
- Specimens to be examined for parasites should be mixed with 10% formalin as preservative (3 parts stool to 1 part preservative). Do not add formaline if there is blood and mucus in stool. Transport at ambient temperature in containers sealed in plastic bags.

4.5 RESPIRATORY TRACT SPECIMEN COLLECTION

Specimens are collected from the upper or lower respiratory tract, depending on the site of infection. Upper respiratory tract pathogens (viral and bacterial) are found in throat and nasopharyngeal specimens. Lower respiratory tract pathogens are found in sputum specimens.

4.5.1 Materials required

- Transport media - bacterial and viral.
- Throat swabs (Dacron and cotton swabs).
- Tongue depressor.
- Nasal speculum.
- 20-50 ml syringe
- Sterile screw-cap test tubes and wide-mouthed clean sterile containers (minimum volume 25ml).

4.5.2 Upper respiratory tract specimens

4.5.2.1 Method of collecting a Throat Swab

- Hold the tongue down with the tongue depressor. Use a strong light source to locate areas of inflammation and exudate in the posterior pharynx and the tonsillar region of the throat behind the uvula.
- Rub the area back and forth with a cotton or Dacron swab.
- Care must be taken to sample the posterior pharyngeal wall at the end to avoid gagging by the patient.
- Withdraw the swab without touching cheeks, teeth or gums and insert into a sterile screw-cap test tube containing appropriate transport medium required.
- Break off the top part of the stick without touching the tube and tighten the screw cap firmly.

- Label the specimen containers.
- Complete the laboratory request form.

4.5.2.2 Method of collecting per-nasal and post-nasal swabs

- Seat the patient comfortably, tilt the head back and insert the nasal speculum.
- Insert a flexible calcium alginate/Dacron swab through the speculum parallel to the floor of nose without pointing upwards. Alternately, bend the wire and insert it into the throat and move the swab upwards into the nasopharyngeal space.
- Rotate the swab on the nasopharyngeal membrane a few times, remove it carefully and insert it into a screw-cap tube containing transport medium.
- Break off the top part of the stick without touching the tube and tighten the screw cap firmly.
- Label the specimen tube.

4.5.2.3 Method of collecting nasopharyngeal wash/aspirate

- Have the patient sit with the head tilted slightly backward.
- Flush a plastic catheter or tubing with 2-3 ml of VTM/sterile normal saline.
- Instill 1-1.5 ml of VTM (viral transport medium)/sterile normal saline into one nostril.
- Insert the tubing into the nostril parallel to the palate and aspirate nasopharyngeal secretions.
- Repeat this procedure with the other nostril.
- Collect 1-2 ml in a sterile vial and transport in cold chain at 2-8°C

4.5.3 Lower respiratory tract specimens

4.5.3.1 Method of collecting sputum (Pl refer to chapter no 14)

- Instruct patient to take a deep breath and cough up sputum directly into a wide-mouth sterile container. Avoid saliva or postnasal discharge. Minimum volume should be about 1 ml.
- Label the specimen containers.
- Complete the laboratory request form.

4.5.4 Handling and transportation

- All respiratory specimens except sputum are transported in appropriate bacterial/viral media.
- Transport as quickly as possible to the laboratory to reduce overgrowth by commensal oral flora.

- For transit periods up to 24 hours, transport specimens for bacterial isolation at ambient temperature and viruses at 4-8°C in appropriate media.

4.6 POST-MORTEM SPECIMEN COLLECTION

Need to be collected during outbreak situation when causative agent in not known. Strict precautions, including respiratory protection from aerosolized particles, must be taken when carrying out post-mortem specimen collection in communicable disease outbreaks. Collect the specimens as soon as possible, preferably within 24 hour since viral titres decline while bacteria multiply rapidly after death. Experienced medical personnel may only accomplish thorough post-mortem examinations.

4.6.1 Materials required

- Barrier precautions: double gloves, sterile gown, eye goggles, mask
- For collecting blood and other fluids, refer to corresponding sections for materials
- Aseptic surgical and biopsy instruments for collecting tissue specimens
- Fixatives: saline formalin for histology
- Sterile saline, appropriate viral and bacterial transport media
- Sterile containers, sterile screw cap tubes or vials, glass slides and slide box
- Disinfectant such as household bleach diluted 1:10 in water.

4.6.2 Method of collection

- Use a separate sterile instrument for each tissue specimen from affected sites (several fragments with 1-2 grams of each is sufficient). Smaller, but adequate, specimens may be taken with a biopsy needle.
- Place different tissues in separate sterile containers containing the relevant medium (sterile saline for preparation of tissues for immunofluorescence microscopy; and microbiological transport media for the isolation of bacterial and viral pathogens, fixatives for histopathology).
- Label all containers and tighten the screw caps firmly.
- Blood may be taken from the heart cavities.
- If cerebral malaria is suspected, make several smears from the cerebral cortex on glass slides to detect Plasmodium falciparum. Label the slides and transport in a slide box.

4.6.3 Handling and transportation

- Fixed specimens can be stored and transported at ambient temperature.
- Tissue specimens for isolation of bacterial pathogens can be transported at ambient temperature in transport media for up to 24 hours.

- Transport tissue specimens for isolation of viral pathogens in viral transport medium or sterile saline at 4-8°C for 24-48 hours. For longer periods, freeze and store at -70°C.
- If rabies is suspected and brain samples are collected, freeze unfixed specimens immediately after collection. Formalin-fixed samples are also useful and may be transported at ambient temperature.

4.7 COLLECTION OF WATER SAMPLE FOR BACTERIOLOGICAL EXAMINATION

Please refer to chapter (8) on bacteriological water testing for details of materials required and procedure

4.8 LABELLING AND IDENTIFICATION OF SPECIMENS

In a diagnostic investigation the information contained in the case investigation form is collected along with the specimen. Each patient should be assigned a unique identification number. This unique identification number and the patient name should be present on all specimens, epidemiological data forms, and the laboratory transmittal forms and used as a common reference.

Preprinted labels should be used whenever possible. The label should be permanently affixed to the specimen container. It should contain the:

Patient' name	Identification No.
Specimen type	
Date of collection	Time

Glass slides for microscopy must be labeled individually,using glass marking pencil.This should not interfere with the staining process. Each slide should bear:

Patient' name
Unique Identification No.
Date of collection

4.8.1 Case Investigation Form

A case investigation form should be completed for each patient at the time of collection, and should be kept together for analysis and later reference. A laboratory request must be completed for each specimen and contain information to interpret the necessary tests. This may include:

- Patient information: age (or date of birth), sex, complete address
- Clinical information - date of onset of symptoms, clinical and immunization history, risk factors or contact history where relevant, antimicrobial drugs taken prior to specimen collection
- Laboratory information - acute or convalescent specimen, other specimens from the same patient.

The receiving laboratory should record:

- Date and time when the specimen was received
- Name and initials of the person receiving specimen
- Record of specimen quality.

For a large number of patients it may be practical to submit the requests to each relevant laboratory as a 'line listing', i.e. a summary request form compiling the necessary data

An example of one such case investigation and laboratory transmittal forms is annexed (Annexure I & II).

4.9 TRANSPORT OF SPECIMENS

4.9.1 Precautions

- Before transport, notify the receiving laboratory of all shipping and specimen details in advance of specimen arrival.
- If international transport is necessary, laboratory should organize authorization to import the specimens
- While arranging for transportation, provision should also be made for information to the sender on receipt or non-receipt of the specimens.

4.9.2 Surface transport/courier service

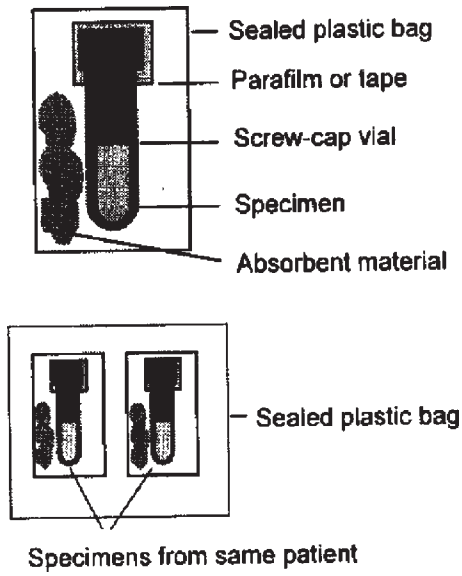
- Securely fasten transport boxes in the transport vehicle.
- In the vehicle, keep a spill kit containing absorbent materials, chlorine disinfectant, heavy-duty reusable gloves, mask, apron, goggles, and leak proof waste disposal container.
- Arrange for an adequate amount of refrigerant in case of delays in the travel schedule so that the cold chain is maintained.
- Avoid extensive vibration of samples, such as that encountered when traveling for long periods over rough roads as this can haemolyse samples, rendering them useless. If possible, separate the serum from clotted blood samples before transport.

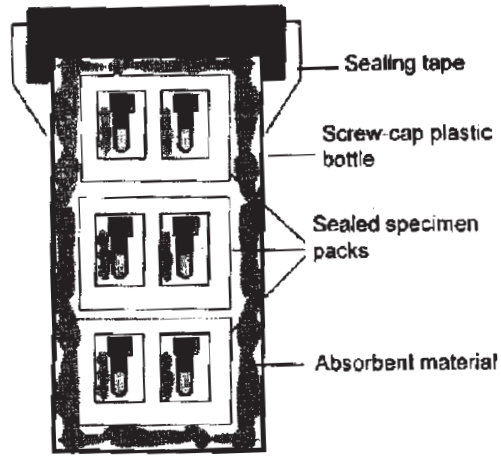
4.9.3 Air transport/postal service

- Diagnostic specimens may be sent by mail in conformance with all relevant international, national, and commercial carrier requirements.
- Contact with the postal authorities should be established prior to the collection of samples to ensure their ability to transport the materials and to verify understanding of the shipping requirements.

4.9.4 Basic triple packaging system and maintenance of transit temperature

- The specimen should be transported in a basic triple packaging system (as described below) to ensure biosafety, transient temperature and quality of the specimen.





- The specimen is in the labeled primary container which must be watertight, airtight, and wrapped in absorbent material (e.g. cotton wool) in case of leakage. After tightening the cap, apply sealing tape, for example parafilm/ water proof plastic tape, over the cap and top of the specimen container.
- Sealed specimens container must be placed in a suitably sizes plastic bags together small amount of absorbent material. The bag must be sealed, alternatively ziploc bags may be used. Two or more sealed specimens from the same patient may be placed in a larger plastic bag and sealed. **Specimens from different patients should never be sealed in the same bag.**
- Place the sealed bags containing the specimens inside secondary plastic containers with screw-capped lids. Provided the specimens have been double-bagged properly in sealed plastic bags, specimens from several patients may be packed inside the same secondary plastic container. Place additional absorbent material inside the secondary container to cushion multiple primary receptacles and absorb any leakage that may occur. Tape a biohazard label and the laboratory request form sealed in a plastic bag to the outside of this secondary container.
- The outer package or tertiary container protects the contents from physical damage and water while in transit. It should have a resistant, high-density external cover (e.g. metal, wood, or fibreboard), shock-absorbent padding

on the inside, and a tight-fitting lid. The outer package must be leak-proof and well insulated, and can contain ice, cold packs or dry ice when required. EPI vaccine carriers or other commercially made containers may be used as a tertiary container to transport. **Vaccine Carriers that have been used for specimen transport must never be reused for carrying vaccines.**

- The rigid outer package is placed within an outer carton of double-ply corrugated cardboard or plastic, and a biohazard label is applied.
- The specimen carriers and ice packs can be reused after disinfection (see Annex 11).

4.9.5 Maintenance of transit temperature

4-8 degree C

- Fit the transport box with a minimum of 4 ice packs or more if room is available, around the secondary container. This will maintain refrigeration for 2-3 days.

Note-:

- Avoid repeated thawing and freezing of specimens.
- Freeze the specimen only if transport is assured at -20^{0}C.
- Store and transport all specimens at 2-8^{0}C, except CSF obtained from suspected cases of pyogenic meningitis.

ANNEXURE-I

Case Investigation form

Date :.....

(To be filled in by the clinician/ epidemiologist)

Patient' s Name: **Patient' s I.D No.:**

Father's/ Husband's Name: **Age/ Sex:**

Address:

Date of onset of illness:

Date of hospitalization/ reporting to the district level:

Occupation:

Clinical signs & symptoms (with duration):

Treatment history:

Results of previous investigations (if any):

Any other relevant information:

Specimen details:

Nature of specimen (s)	Date of collection	Investigation required

Details of sender:

Signature:

Name of sender: **Address of sender:**

Fax: **E-mail:**

(NOTE: Please complete all the columns. Always send the sample under cold chain unless specified otherwise)

ANNEXURE-II

Laboratory Transmittal form

Date :.....

Patient' s Name:

Patient's I. D No.:

Age/ Sex:

Laboratory Reference No.:

Specimen details:

Type of Specimen	Date of Collection	Date of Receipt in lab	Type of test	Remarks (if any)	Result

Interpretation:

Details of Investigator:

Name:

Signature:

Address:

Telephone No.:

OUTBREAK INVESTIGATION KIT

- Disposable storage vials (5ml)
- Disposable sample collection vials
- Stool culture bottle
- Throat swabs
- Blood culture bottles
- Viral transport medium
- Cary Blair medium/ Stuart's transport medium
- Vacutainer (plain and EDTA)
- Syringes and needles disposable (5ml)
- Tourniquet
- Gloves
- Masks (triple layer surgical mask)
- Disposable gowns
- Puncture proof discarding bags (disposable)
- Spirit swabs/ alcohol swabs
- Band-aid
- Vaccine carrier with ice-packs
- Spirit lamp/ gas lighter
- Match-box
- Test tube rack
- Centrifuge tubes
- Lancets
- Slides and cover slips
- Rubber bands
- Ziploc plastic bags
- Absorbent material (tissue paper, cotton wool, newspaper)
- Labels
- Glass marking pen
- Adhesive tape
- Scissors
- Scalpel/ blade
- Spatula
- Forceps
- Loop holder
- Pasteur pipettes/ pipettes and pipette aids (rubber teats)
- Rapid diagnostic kits (e.g. H2S strip for water testing, latex agglutination for meningitis, card test for dengue etc.) wherever possible
- Sodium hypochlorite concentrates (4%)
- Hand disinfectant
- Laboratory request forms
- Stationery (writing pads, pens, pencils, erasers, sharpeners etc.)
- Any other, as per outbreak requirements.

CHAPTER -5

STERILIZATION AND DISINFECTION PROCEDURES

5.1 STERILIZATION

Means complete destruction of all kind of living microorganisms including spores.

5.2 DISINFECTION

Destruction of vegetative forms of organisms which might cause diseases or spoilage of food etc.

The two terms are not synonymous.

5.3 DISINFECTION OF USED LABORATORY ARTICLES

5.3.1 Purpose

Laboratory garbage (reusable and disposable) is considered as potentially pathogenic both for laboratory workers as well for environment if disposed off untreated. The ideal method of treating such material (disposable) is to incinerate and decontaminate, the reusable one by autoclaving and/or chemical treatment, which ever is available / applicable. As these facilities are not available in most of the peripheral laboratories (L1 level) the material for disinfection may be divided into the following categories as far as their safe disposal is concerned.

- ⇨ Disposable (mostly plastic ware).
- ⇨ Reusable, contaminated with morbid material e.g. glass pippets, slides, test tubes etc.
- ⇨ Material containing or contaminated with bacterial culture.

5.3.2. Disinfection of disposable items

5.3.2.1 Material required

- ⇨ 1% sodium hypochlorite / 3% Lysol solution.
- ⇨ Glass jar.
- ⇨ Biosafety bag (puncture resistant with appropriate color code).
- ⇨ Gloves.

5.3.2.2. Procedure

- ⇨ Freshly Prepare requisite quantity of disinfectant in a jar meant for this purpose.
- ⇨ Put articles to be discarded in the solution overnight.
- ⇨ Drain off disinfectant.
- ⇨ Collect the material in safety bags & dispose off along with other garbage at a designated place.

5.3.2.3

GLP (Good Laboratory Practices)

- Always prepare fresh solution of disinfectant before use as ready to use solution has shorter shelf life, compared to concentrated one and will be of no use if not freshly prepared.
- Care should be taken while handling & preparing the solution as it may be corrosive to skin.

5.3. 3 Disinfection of reusable articles contaminated with morbid material

5.3.3.1 Material required

- ⇨ Disinfectant as described above.
- ⇨ Puncture resistant biosafety bags.
- ⇨ Metallic box/ Tray.
- ⇨ Bunsen burner (Heating device for boiling).

5.3.3.2 Procedure

As described (in section 5.3.2.2) after treating the material with suitable solution of disinfectant over night, proceed further as follow.

- ⇨ Drain off disinfectant in sink fitted with tap.
- ⇨ Transfer the material in metal box or tray with cover.
- ⇨ Place on bunsen burner (heating device) for boiling.
- ⇨ Wait up to 15-20 min. after the boiling starts.
- ⇨ Put off the flame & allow cooling the material in metallic box/ tray.
- ⇨ Drain off water.
- ⇨ Pass on the material for washing.

5.3.4 Glassware containing culture material

5.3.4.1 Material required

- Biosafety puncture resistant bags.
- Autoclave.

5.3.4.2 Procedure

- Discard all the material containing/ contaminated with culture material directly into metal box / puncture resistant biosafety bags.
- Place box/ biosafety bags with material to be decontaminated in autoclave designated for this work only.
- Decontaminate the material by autoclaving (see section5.5 of this chapter).
- Drain off culture media and pass on material for further washing etc.

5.3.4.3

GLP

- Be sure that decontamination should only be done with autoclaves designated for this purpose.
- Autoclave should be checked for its efficacy using chemical indicator (Section 5.5.2.2.4.1.4).

5.4 WASHING OF LABORATORY GLASSWARE

The type of glassware i.e. new and dirty/ used is subjected to washing for further use. The method used for each type is described below.

5.4.1 New glassware

5.4.1.1 Purpose

Usually new glassware is slightly alkaline in nature. Before washing, this alkaline nature has to be neutralized for final use.

5.4.1.2 Material required

- 2% Hydrochloric acid.
- Big plastic basin.
- Demineralized water.
- Hot air oven for drying purpose only.

5.4.1.3 Procedure

- Prepare sufficient quantity of 2 % hydrochloric acid (e.g. 98 ml of water & 2.0 ml hydrochloric acid) as per the requirement in a big plastic basin.
- Wash the newly received glassware under running tap water to remove the visible dust sticking inside and/or out side surface of the article.
- Soak the already washed articles in 2% hydrochloric acid solution.
- Leave them there overnight.
- Take the articles from 2 % hydrochloric acid and rinse in clean water twice.
- Finally wash using demineralized water.
- Allow to dry using hot air oven.
- Pass on for packing & sterilization for further use.

GLP

- ❖ Care should be taken while using HCl.
- ❖ Add acid to water drop by drop by constant stirring (and not vice versa)

5.4.2 Dirty glassware

5.4.2.1 Material required

- 1 % detergent solution.
- Washing brush.
- Hot air oven for drying.
- Draining rack.
- Cotton / aluminum foil for plugging.
- Good quality water supply.
- Wire basket.
- Demineralized water.

5.4.2.2 Procedure

- Take material, glassware etc. already decontaminated (chemically / autoclaving) and rinse twice in luke warm water to remove any dirty stain sticking on them.
- Put the material to be washed in bowl containing 1% detergent solution.
- Allow to boil (Electrically / by Bunsen burner flame).
- While in solution scrub inside & outside surface of the glassware with the help of the brush.
- Leave the glassware in the solution for 2 - 3 hrs.
- Take out each article one by one and rinse under running tap water till no trace of detergent is left, which other wise may lead to false results when used.
- Drain the water by putting each article on a wall draining rack or by keeping articles up side down in a wire basket.
- Put articles in wire basket and keep in hot air oven at 60°C for drying purpose only.
- Take out each articles and plug using non-absorbent cotton / aluminum foil.
- Pass on for sterilization (dry heat / autoclaving)
- In case of delay, store in dust free area.

5.5 METHOD OF STERILIZATION

Depending upon the nature of material to be sterilized, sterilization procedures used in microbiology laboratory can be divided into the following categories.

- Dry heat.
- Moist heat.
- Filtration.

5.5.1 Dry heat

The commonly used methods to sterilize the material is as follows.

- Red heat flaming.
- Hot air sterilization.

5.5.1.1 Red heat flaming

5.5.1.1.1 Purpose

Used to sterlize material such as inoculating wire / loop, tip of the forceps, searing iron, scalpel etc.

5.5.1.1.2 Material required

- Bunsen burner attached to gas supplies.
- Match box.

5.5.1.1.3 Procedure

- Light the burner with the help of match box.
- Adjust the cone of the flame to blue.
- Hold the inoculum wire/ loop/ tip of the forceps etc. vertically and heat till it gets red hot. Allow to cool before use.
- Put off the flame.

5.5.1.1.4

GLP

- Each time when heating in the bunsen burner flame, allow to cool down the instrument. Check loop/ wire etc. by touching a portion of the medium to be inoculated.
- Heat the loop vertically so that the entire length of the loop is heated.
- Dip the loop in disinfectant solution before heating to avoid splattering.

5.5.1.2 Hot air Sterilization

5.5.1.2.1 Purpose

The method is used for sterilizing the material like dry glass test tubes, petri dishes, flasks, glass pipettes, all glass syringes etc. and instruments like forceps, scalpels etc.

5.5.1.2.2 Equipment required

Sterilization by hot air should be carried out in an oven which should at least have the following features.

- Electrically operated (alternative gas or kerosene oil).
- Fitted with the thermostat to control the temperature.
- Device to indicate the temperature of inside chamber of the oven when in use.
- Should be fitted with adjustable shelves.
- Provided with fan to ensure uniform distribution of heat in different parts of the oven.

5.5.1.2.3 Procedure

- Arrange the material (pre washed & packed) to be sterilized, loosely and evenly on the racks of the oven so that air can circulate properly and heat

the load evenly in the oven.

- Switch on the power supply (or alternative devise gas/ kerosene oil).
- Control the temperature of the oven by adjusting the thermostat knob.
- Note the time when desired temperature is reached (Heating time).
- Hold the load on the same temperature for the specified period as mentioned below.

Temperature	Holding Time
160 °C	60 minutes.
170 °C	40 minutes.
180 °C	30 minutes.

- The most common temperature for hot air oven for sterilization is 160 °C for 60 min.
- On expiry of the holding time period, switch off the power supply and allow the oven to cool down slowly.
- Put down the date of sterilization on each packet and store in dust free area for future use.
- Make daily records of the equipment/ material sterilized as per the proforma given below.

Date	**Detail of all items Sterilized.**	Temp. at which Sterilized	Starting time	Heating time	Holding time	Remarks
			From/ to	**From/ to**	**From/to**	

5.5.1.2.4

GLP

- ⇨ Dry up all the material before putting into sterilization in hot air oven.
- ⇨ Don't place heat sensitive material inside the oven.
- ⇨ As air is poor conductor of heat, do not pack the material to be sterilized in the oven too tightly.
- ⇨ After holding time is over, hot air oven is switched off, wait until the temperature of the oven falls below 80°C. Only then open the door of the oven to take out the material other wise opening immediately after holding time leads to breaking of the glassware and may also cause injuries to the operator.

5.5.2 Moist heat

Moist heat or steam under pressure is one of the most efficient method of sterilization. Depending upon the material to be sterilized moist heat can be applied in different forms as discussed below.

5.5.2.1 Below 100^{O}C

- Pasteurization 63°C - 80°C for 30minutes.
- Tyndalisation : Intermitent exposure at 75 - 80°C for 20 - 45 minutes on three successive days.
- Boiling at 100°C for 5 - 10 minutes.
- Steaming at 100°C for 1 hr.

5.5.2.2. Steaming under pressure (Autocalving)

5.5.2.2.1 Purpose

Saturated steam under pressure is more efficient way of sterilization as compared to dry heat because

- ⇨ It provides greater lethal action.
- ⇨ It is quicker in heating up the exposed articles.
- ⇨ It penetrates the porous material such as cotton wool, stoppers, paper, cloth wrapper etc.

5.5.2.2.2 Principle

When water boils its vapour pressure is equal to surrounding atmospheric pressure. When boiling is done in a closed vessel, there is increase in the inside pressure of vessel which raises the temperature of boiling water above 100°C.

5.5.2.2.3 Item to be sterilized

Autoclave is mostly suitable for.

- ⇨ Sterilization of culture media, aqueous solution.
- ⇨ Decontamination of discarded culture and other laboratory garbage.
- ⇨ Rubber guard, gloves, stoppers with rubber liner, glassware with rubber attachment, glass metal syringes,throat swabs etc.

5.5.2.2.4 Type of Autoclaves

In principal two type of autoclaves are used

- ⇨ Pressure cooker type.
- ⇨ Gravity displacement type.

5.5.2.2.4.1 Pressure cooker type

This is the most common type of autoclave used for sterilization. It has vertical chamber with a strong metal lid, which can be fastened down, and sealed with rubber

gasket. An air steam discharger tap, pressure gauze and safety cum pressure adjustable valve are fitted on the lid. Water in the bottom of the autoclaves is heated electrically (or by some other device like gas burner / kerosene oil).

5.5.2.2.4.1.1 Procedure

- ⇨ Arrange the material (pre washed & packed) to be sterilized.
- ⇨ Ensure that there is sufficient quantity of water in the chamber at the time of autoclaving by checking water level device provided with the equipment.
- ⇨ Place the material to be sterilized in wire basket / perforated container loosely.
- ⇨ Place the container on perforated rack placed above the level of the water.
- ⇨ Fasten the lid tightly with steam discharge valve open.
- ⇨ Switch on the power and allow the water to boil.
- ⇨ When water boils, steam will come out of the discharge valve so that air from chamber may be expelled.
- ⇨ Wait till total air inside the chamber has been replaced by steam. This can be checked by connecting one end of the rubber tube to the discharge valve and immersing other end of tube into the bucket containing water. The discharge gas will pass through the water, steam will condense and air will bubble through water. When bubbles cease, it means that air from chamber has been expelled.
- ⇨ Close the steam discharge valve.
- ⇨ Adjust the valve to predetermined pressure (Normally autoclaves are adjusted at 15 lbs / sq inch.).
- ⇨ Allow the pressure to increase to preadjusted pressure.
- ⇨ Note the time when pressure gauze indicates the requisite pressure is achieved.
- ⇨ Allow to continue the same for required time period as indicated below.

Temp	Pressure	Time
115°C	10 lbs / inch.	20 - 30 min.
121°C	15 lbs / inch	15- 20 min.
132°C	27 lbs / inch	2 min.

Holding time increases to 30-45min if at 121°C and 15 lbs pressure if plastic wares are sterilized

- ⇨ At the end of holding time switch off the power supply.
- ⇨ Allow the autoclave to cool slowly which can be seen by gradual decrease in pressure till it shows zero reading.

- ⇨ Allow the wrapping paper to be dried.
- ⇨ Put date on each article and place in dust free area for future use.

5.5.2.2.4.1.2

GLP

- ⇨ Ensure that air from chamber has been expelled completely because air steam mixture has a lower temperature than steam e.g. temperature of 50% air & 50% steam mixture will be112°C instead of 121°C provided by the pure steam.
- ⇨ Air also hinders the penetration of steam in to the interior of the porous material and narrow opening container. Air being denser than steam tends to form a separate cooler layer in the bottom of the autoclave.
- ⇨ As the simple autoclave lack means for drying the load after sterilization, it is therefore important to avoid placing sterilized articles in contact with unsterilized objects/ surface unless the wrapping is dried
- ⇨ To check the efficacy of autoclave, each cycle should be run using chemical indicator tape.

5.5.2.2.4.1.3 Record Keeping

Daily recording of each run for sterilization of material should be maintained e.g.

Date	Detail of items to be sterilized	Pressure at which sterilization done	Starting time		Heating time		Holding time	Chemical indicator tape (colour changed)
			From	**To**	**From**	**To**		

5.5.2.2.4.1.4 Quality control

To check the efficacy of auotclave each run should be accompanied by placing chemical indicator which changes color if the instruments is working satisfactory. This can be achieved by placing chemical indicator tape inside the tube in the center of autoclave and check the change in color after the operation is over.

Indicator tape Before Autoclaving

Indicator tape After Autoclaving

CHAPTER - 6

COMMON STAINING TECHNIQUES IN A DISTRICT LABORATORY

6.1 GRAM'S STAINING

This is one of the most common staining procedure used for examining specimens suspected to contain bacteriologic agents. Direct microscopic examination of specimens and cultures can provide a rapid presumptive diagnosis. Gram staining can give information regarding the shape of cell,the type of cell arrangement (single chained, clustered) the gram reaction that can provide a quick assessment of what the etiologic agent may be.

6.1.1 Principle

Certain bacteria when treated with one of the basic para rosaniline dyes such as Methyl Violet, Crystal Violet or Gentian Violet (which is a mixture of two preceding dyes) and then with Iodine, fix the stain so that subsequent treatment with a decolourizing agent- e.g. alcohol or acetone does not remove the colour. Other organisms however, are decolourized by this process. Thus, if a mixture of various organisms are stained and subjected to decolourizing agents, it is found that some retain the dye, and these are termed **Gram-Positive** whereas others are completely decolourized and are termed as **Gram-Negative**.

In order to render the decolourized organism visible, and to distinguish them from those retaining the colour, a counter stain is then applied. This counter stain is usually red so that the gram-negative organism which appear reddish pink in colour may easily be differentiated from gram-positive organism, which retain the original violet stain.

6.1.2 Specimens

Smear prepared from any of the following e.g. throat swab, nasal swab, ear discharge, pleural fluid, C.S.F, uretheral discharge, sputum, centrifuged deposit of urine, bacterial culture, vaginal discharge etc.can be stained by this method.

6.1.3 Materials

1) Crystal violet (0.5%)
2) Iodine crystals.
3) Potassium iodide
4) Acetone (100%) or Ethanol (95%)
5) Safranine (0.5%)
6) Distilled water
7) Ethyl alcohol.

6.1.4 Preparation of Reagents / Stains

6.1.4.1 Solution 'A (Crystal violet-0.5%)

Crystal violet -------- 0.5 gm.

Distilled water -------- 100 ml

6.1.4.2 Solution 'B' (Gram's Iodine-1%)

Iodine ---------- 1gms.

Potassium Iodide --- 2.0 gm

Distilled water ------- 100 ml

Grind potassium iodide and iodine and dissolve in not more than 20 ml of distilled water. Make up the volume to 100 ml with distilled water.

6.1.4.3 Solution 'C' (Safranine-0.5%)

Dissolve 0.5 gm safranine in 10 ml alcohol and make up the volume to 100 ml with distilled water.

6.1.5 Procedure

- Appropriate smear is made on a clean glass slide.
- The smear is fixed by passing the slide over flame 2-3 times quickly.
- 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 pour on the iodine solution on the slide 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 seconds. After this period of contact, wash thoroughly with water under a running tap
- Apply the counterstain (0.5% safranine) for 30 seconds.
- Wash with water and blot dry.
- Examine the smear under oil immersion microscopy.

6.1.6 Result

Violet stained bacteria ---------- Gram-Positive

Reddish pink bacteria ---------- Gram- Negative

6.1.7 Quality Control

To obviate errors from over decolorizing, a control smear of a known gram positive organism (.e.g. a pure culture of Staphylococcus aureus) may be prepared on one end of slide and a smear of gram negative organism e.g. E.coli be prepared on the other end of the slide. The smears thus prepared should be stained by the grams stain as described. The smear prepared from Staphylococcus aureus should show violet coloured bacteria whereas the one made from E. coli should show pink coloured bacteria.

6.2 ALBERT'S STAINING

Albert's staining is most common staining technique used for examining the specimens, suspected to have Corynebacterium diphtheriae; which are gram positive rods of varying length and often containing volutin (polyphosphate) granules which appears purple black against the light green cytoplasm of the bacillus.

6.2.1 Specimens

Throat swab/nasal swab smear or bacterial culture from suspected cases of C. diphtheriae.

6.2.2 Materials

- ⇨ Toluidine blue
- ⇨ Malachite green
- ⇨ Glacial acetic acid
- ⇨ Alcohol (25% ethanol)
- ⇨ Iodine
- ⇨ Potassium iodide
- ⇨ D. Water.

6.2.3 Preparation of Albert's Stain

6.2.3.1 Albert's -I

- ⇨ Toluidine blue ---------- 1.5gm
- ⇨ Malachite green ---------- 2.0 gm
- ⇨ Glacial acetic acid ---------- 10ml
- ⇨ Alcohol (25% ethanol) ----- 90ml
- ⇨ D. water ---------- 1000 ml.

Dissolve the dyes in alcohol and add to the water and acetic acid. Allow to stand for one day and then filter.

6.2.3.2 Albert's -II

- ⇨ Iodine---------- 6 gms
- ⇨ Potassium Iodide---- 9 gms
- ⇨ D.water---------- 900 ml

6.2.4 Procedure for staining

- ⇨ Make a smear, dry in air, and fix by heat.
- ⇨ Cover slide with Albert's stain -I and leave for 4-6 minutes.
- ⇨ Wash in water and blot dry.
- ⇨ Cover the slide with Albert's stain -II and allow to act for 1-2 mins.
- ⇨ Wash, blot dry and examine under oil immersion lens of microscope.

6.2.5 Result

C.diphtheriae appear as bacilli with dark green protoplasm and purple black granules, other bacteria will stain light green.

6.2.6 Quality Control

In order to check the quality of the reagents and the staining procedure, prepare smear of a standard Corynebacterium diphtheriae culture **(positive control)** and another smear from standard E.coli culture **(negative control).** Stain both smear using the standard Albert staining procedure as described C.diphtheriae appear as bacilli with dark green protoplasm and black granules, whereas smear of E.coli will stain light green. These control cultures can be supplied by NICD, Delhi on request

6.3 STAINING FOR MALARIAL PARASITE

6.3.1 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.

6.3.2 Collection of sample

6.3.2.1 Peripheral blood smear: Time for taking blood

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

6.3.3 Preparation of blood smear

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

- Apply gentle pressure to the finger and collect a single small drop of blood on the middle of the slide. This is for the thin film. Apply further pressure to express moreblood 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.

6.3.3.1 Thin film

Thin film and thick film prepration. Refer to section 4.2.3 of chapter-4

6.3.2.2 Thick film

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 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 dispatch with the patient's records form to 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.

6.3.4 Staining of blood smears

6.3.4.1 Giemsa Stain

6.3.4.1.1. Material and Reagents

1) Giemsa stain powder/ ready Geimsa stain solution.
2) Alcohol.
3) Methanol.
4) Marking pens.
5) Staining jars.
6) Boric acid buffer -pH 7.2.

6.3.4.1.2 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 ----Solution-I
  b) Dissolve 19.05 gm borax in 1 lit of distilled water -Solution-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.

6.3.4.1.3 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 geimsa'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 smear in buffer solution.
- Blot dry.
- Examine the slide under oil immersion of microscope.

6.4 J.S.B.STAIN (JASWANT SINGH AND BHATTACHARJEE STAIN) DEVELOPED AT NICD, DELHI

6.4.1 Material and reagents required

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

6.4.2 Preparation

6.4.2.1 J.S.B.- II

Dissolve 2 gm eosin yellow in 1 lit. of distilled water and store in the dark for 4 weeks before use.

6.4.2.2 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 gm. 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.

6.4.3 Staining technique

- Prepare thin and thick smear from malaria cases on micro slides.
- De- haemoglobinise the thick smear (ref. section 6.3.4.1.3)
- 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 smear in J.S.B-II for few seconds and immediately wash in water.
- Drain the slides free of excess water.
- Dip the smear in J.S.B-I for 30-40 seconds.
- Wash well in water and dry.
- Examine the smears under oil immersion.

6.4.4 Observation

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

6.4.5 Thin film examination

- Area of the film examined should be along the upper and lower margins of tail end film as parasites are concentrated over there.
- A minimum of 100 fields should be examined in about 8-10 minutes.

6.4.5.1 The following stages of the parasite can be observed in a peripheral blood thin smear

- Ring, trophozoite, schizont and the gametocytes in case of Plasmodium vivax.
- The infected erythrocytes are usually enlarged in P. vivax.
- 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.

6.4.6. Observation on thick smear

- Only elements seen are leucocytes and malarial parasites.
- Morphology of malarial parasites is distorted.
- Species of parasites cannot be identified.

6.4.6.1 Appearance in thick film

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

6.5 NEGATIVE (INDIA INK) STAINING

Negative staining is sometimes a very useful technique for demonstrating the capsulated organisms like Meningococcus/Pneumococcus/Cryptococcus, the causative agents of meningitis

6.5.1 Requirements

- Glass sides.
- Coverslips
- India Ink (Available commercially).
- Pasteur pipettes

6.5.2 Procedure

1) Using a Pasteur pipette, put a drop of CSF sediment (obtained after Centrifuging the CSF at 2000-3000 rpm for10-15 mts) or any other appropriate specimen on a clean glass slide.
2) Add another smaller drop of Indian Ink (about 1/3rd the size of CSF drop) on the slide. Mix the two drops thoroughly using a match stick.
3) Put a coverslip on the resultant mixture.
4) Examine the preparation under the microcope first using 40x objective and then 100x objective to look for the characteristic encapsulated cells

CHAPTER - 7

PREPARATION OF COMMON CULTURE MEDIA

7.1 DE- HYDRATED CULTURE MEDIA

7.1.1 Some points to be remembered

- Always receive media prior to the expiry date label marked on the container and for media preparation follow the manufacturer's instructions.
- Most dehydrated culture media are hygroscopic; hence always keep container bottle tightly closed.
- Store media bottle in dark place at room temperature away from moisture.
- Since there could be batch-to-batch variation of media, it is advisable to check every new receipt (new batch) of media with growth characteristics of control strains (ATCC, NCTC, MTCC strains) and or locally available strains growth of which is desirable.
- It is advisable to use freshly prepared culture plates as far as possible & culture plate should be used within 2-5 days of preparation.
- Prepared media is kept in fridge in sealed plastic bags to prevent media getting dried up.
- For media preparation distilled water / de-ionized water should be used and all glassware's for preparation should be thoroughly washed and autoclaved as per the laid down procedure.
- Avoid formation of air bubbles during media pouring as far as possible.
- For sterility checking of the media, incubate at least 1-2 uninoculated plate from each new batch of prepared media at 37°C incubator and check for growth of any contaminating bacteria after over-night incubation.
- Media pouring room should be a clean, enclosed space without any outside air current and with UV light facility preferably.
- Following autoclaving of media there is usually a fall in pH of about 0.2 to 0.4, hence adjust the pH to a bit higher side (0.2 - 0.4 or so) than the pH indicated on the media bottle prior to autoclaving.
- All pH measurement are to be done at room temperature i.e. at 25°C
- pH adjustments are done with either 1N-NaOH for alkalinity or with 1N-HCl for acidity.
- While boiling dehydrated culture media, gently heat with mixing in between so as to prevent overheating of the media.
- Heat sensitive ingredients that need to be added in the media may be pre-sterilized prior to addition in the media.

- Non- absorbent type of cotton wool is used for plugging test tubes / flasks etc. of media always.
- For preparing 1 Litre of Media, bigger glass flask either 2 or 3 litre is chosen to avoid boiled media touching the cotton plug by way of bumping of the media.
- Arrange all reagents / materials / glassware like bijou bottles, flask, test tubes, distilled water, pH paper, 1N-NaOH, 1N-HcL, etc. before starting media preparation.
- Most culture media available these days are of dehydrated media - which need to be just mixed by heating and then distributed on to plates / tubes.
- Though there is no need to check pH of dehydrated media (as the media comes pre checked for pH) still it is useful to check pH once after the media gets dissolved in water so as to ensure correctness of pH as per the bottle label, and in case of any change of pH observed following dissolution the same may be corrected with addition of 1N-NaOH or 1N-HCl.

7.2 MACCONKEY'S BROTH (DOUBLE STRENGTH) WITH NEUTRAL RED

7.2.1 Water Testing Media

7.2.1.1 Ingredients — gms / litre.

Peptic digest of animal tissue	40.00 gm.
Lactose	20.00 gm.
Bile Salts	10.00 gm.
Sodium Chloride	10.00 gm.
Neutral Red	0.15 gm.

PH 7.4 + 0.2

7.2.1.2 For double strength media

Weigh exactly 80.15 gm of dehydrated media. Dissolve it in one litre distilled water using (2 to 3 litre glass flask). Heat till media dissolves. Now distribute this media in about 10 ml amounts to test tubes of size 15 x 150 mm. with inverted DURHAM'S tube (one in each tube). Plug the tube with cotton. Now autoclave at 15 lbs pressure for 15 minutes.

7.2.1.3 For single strength media

40 gm of dehydrated media is dissolved in similar manner in one litre distilled water. 5 ml of this media is distributed in 15 x 125 mm test tubes with durham's tube. Tubes are plugged and autoclaved as mentioned above

7.2.1.4 For flask media

50 ml of double strength Media (Section 7.2.1.2) is poured in conical flask (250 ml capacity) and a durham's tube is put in each flask. The Flask is plugged later & autoclaved at 15 lbs pressure for 15 minutes.

7.2.1.5 Use: - It is a liquid media used for detection of coliform bacteria and determination of MPN counts from water samples

7. 3 MACCONKEY'S AGAR (MAC)

7.3.1 Purpose

For the isolation and differentiation of enteric organism. It differentiates lactose fermenting from non-lactose fermenting gram-negative enteric bacilli

7.3.2 Ingredients

Ingredients	gm / Litre
Bacto peptone	17 gm
Bacto proteosepeptone	3 gm
Bacto lactose	10 gm
Bile salt no 3	1.5 gm
Sodium chloride	5 gm
Neutral red	0.03 gm
Crystal violet	0.001 gm
Agar	13.5 gm
Distilled water	1 Litre.
pH	7.1 ± 0.2

7.3.3 Directions

Dissolve 50 gm of powdered media in one litre distilled water. Boil to dissolve completely. Cool to around 60 degree centigrade & pour in sterile petri dishes. No need to autoclave as it may lead to over heating and degradation of some of the heat sensitive components. Let the media solidify. Store in fridge in sealed plastic bags.

7.3.4 Use

It is a moderately selective and differentiation media (solid media) used to isolate enteric bacteria like salmonella and shigella and E.coli etc (gram negative bacilli) characterized as either lactose fermenter (pink colonies) or non lactose fermenters (pale colonies).

7.4 BILE SALT AGAR (B.S.A)

7.4.1 Ingredients

Ingredients	gms / litre
Peptic digest of animal tissue	10.00 gm.

Meat extract	5.0 gm
Sodium chloride	5.0 gm
Sodium taurocholate	5.0 gm
Agar	18.00 gm

Ph - 8.2 + 0.2

7.4.2 Directions

Dissolve 43.0 gm of powdered media in 1 litre of distilled water. Boil to dissolve and autoclave at 15 lbs pressure for 15 min. Pour in petri plates

7.4.3 Use

For primary isolation of Vibrio cholerae from stool specimens.

7.5 SALMONELLA SHIGELLA AGAR (S.S.AGAR)

7.5.1 Ingredients

	gms / litre.
Peptic digest of animal tissue	5.0 gm
Beef extract	5.0 gm
Lactose	10.0 gm
Bile salt mixture	8.50 gm
Sodium citrate	10 gm.
Sodium thiosulphate	8.50 gm
Ferric citrate	1.00 gm
Brilliant green	0.00033 gm
Neutral red	0.025 gm
Agar	15.0 gm
pH ---- 7.0 $\pm$ 0.2	
Distilled water. ---- 1 litre	

7.5.2 Direction

Dissolve 63.0 gm of powdered media in 1 litre distilled water. Heat to boil, do not autoclave or over heat. Cool and Pour in petri dishes.

7.5.3 Use

A differential, selective medium for the isolation of SALMONELLA and some SHIGELLA species from stool/ rectal swab specimens, and suspected foodstuffs etc. Salmonella colonies grow as non lactose fermenting (NLF) pale colonies mostly with black head (due to H_2S production) while shigella grows as pale NLF colonies. E.coli - also grows though somewhat inhibited.

7.6 XYLOSE - LYSINE DEOXYCHOLATE AGAR (XLD AGAR)

7.6.1 Ingredients

Ingredients	gms / litre
Yeast Extract	3.0 gm
L.Lysine	5.0 gm
Lactose	7.50 gm
Sucrose	7.50 gm
Xylose	3.50 gm
Sodium chloride	5.00 gm
Sodium deoxycholate	2.50 gm
Sodium thiosulphate	6.80 gm
Ferric ammonium citrate	0.80 gm
Phenol red	0.08 gm
Agar	15.00 gm
pH	7.4 ± 0.2

7.6.2 Directions

Weigh 57.0 gm of dehydrated media and dissolve in 1 litre distilled water. Heat with agitation until medium dissolves. (do not overheat or autoclave). Cool to 50°C and pour in petri dishes.

7.6.3 Use

It is an enriched media recommended for the isolation and identification of Salmonella typhi and other Salmonella and Shigella species.

7.7 MUELLER HINTON AGAR (M.H.A)

7.7.1 Ingredients

Ingredients	gms / litre
Beef Infusion	3 gm
Casein acid hyrolysate	17.5 gm
Starch	1.5 gm
Agar	17.0 gm
Distilled water	1 Litre

7.7.2 Directions

Dissolve 38 gm of it in one litre of distilled water. Boil to dissolve. Autoclave at 15 lbs pressure for 15 min, mix well and pour in plates

7.7.3 Use

For carrying out antimicrobial susceptibility testing of pathogenic isolates, also used for cultivation of Neisseria.

7.8 CARY BLAIR MEDIUM (TRANSPORT MEDIUM WITHOUT CHARCOAL)

7.8.1 Ingredients

	gms / litre
Sodium thioglycolate	1.5 gm
Disodium hydrogen phosphate	1.1 gm
Sodium chloride	5 gm
Agar	5 gm
pH -------- 8.4 ± 0.2	

7.8.2 Directions

Suspend 12.6 gm of ingredients in 991 ml of distilled water. Boil to dissolve medium completely. Cool to 50°C and add aseptically 9 ml of 1% aqueous calcium chloride solution. Adjust pH to 8.4. Distribute in 3-5 ml amounts in small screw capped bijou bottles. Steam for 15 min.

7.8.3 Use

Recommended for transportation of stool / rectal swab specimens.

7.9 PEPTONE WATER

7.9.1 Formula

Peptic digest from Animal tissue	10.0 gm.
Sodium chloride	5.0 gm.
Final pH	7.2 ± 0.2

7.9.2 Methods

Suspend 15 gm in 1 litre distilled water. Warm to dissolve. Adjust pH to about 7.4. Pour into test tubes in about 5 ml amounts and plug with non-absorbent cotton wool, sterilize by autoclaving at 15 pounds pressure for 15 minutes.

7.9.3 Use

It is a commonly used liquid culture media used for a variety of purpose like sub culture, inoculation of sugar, indole test, antimicrobial susceptibility testing etc.

7.10 ALKALINE PEPTONE WATER

7.10.1 Formula

Peptic digest of animal tissue	10.0 gm.
Sodium chloride	10.0 gm.
pH	8.4 - 8.6

7.10.2 Method

Suspend 20 gm ingredients per litre of distilled water, adjust pH to about 8.6. Distribute into test tubes in about 5 ml amounts and plug with cotton wool. Autoclave at 15 pounds pressure for 15 minutes.

7.10.3 Use

It is used as an enrichment medium for Vibrio cholerae as number of vibrios increase after inoculation in this medium and chance of isolation improves.

7.11 NUTRIENT AGAR- STOCK MEDIA 1.5%

7.11.1 Formula

Beef extract	3.0 gm.
Peptone	5.0 gm.
Sodium chloride	8.0 gm.
Agar	15.0 gm.
pH	7.3 ± -0.2

7.11.2 Methods

Suspend 31 gm in one litre of distilled water. Boil to dissolve with frequent agitation. Pour in test tubes 12x100 mm for stock about 4 ml in each tube. Autoclave for 15 minutes at 15 lbs pressure. Cool and store in refrigerator prior to use.

7.11.3 Use

For stocking of bacterial isolates.

7.12 SELENITE BROTH (SELENITE - F BROTH)

7.12.1 Formula

Caesin enzymic hydrolysate	5.0. gm
Lactose	4.0.gm
Disodium phosphate	10.0.gm
Sodium hydrogen selenite $5H_2O$	4.0.gm
pH	7.0 ± 0.2

7.12.2 Methods

Suspend 23 gm in one litre of distilled water. Warm to dissolve. Distribute in tubes or screw capped bottles in 10 ml amount. Sterilize by steaming for 10 minutes. Amount of red precipitate should be slight. **DO NOT AUTOCLAVE.**

7.12.3 Use

Used as an enrichment medium for enrichment of Salmonella from faeces and some Shigella organisms.

7.13 VENKATRAMAN RAMAKRISHNAN FLUID (V.R. FLUID)

7.13.1 Formula

7.13.1.1 Prepare Stock preservative solution (A)as per the formula given below

Boric acid	12.4 gm.
Potassium chloride	14.9 gm.
Distilled water	800.0 ml

7.13.1.2 Prepare stock solution (B) i.e NaOH Stock Solution as per following manner

Dissolve 8-gm NaOH/ litre in distilled water.

V.R. Fluid the complete medium is finally made as follows:

Sea salt	20 gm.
Stock preservative **solution (A)**	250 ml.
NaOH stock **solution (B)**	133.5 ml

7.13.2 Methods

Take 250 ml of stock preservative solution (A) and add NaOH stock solution (B) 133.5 ml. Then add 20 gm of dried sea salt. Make volume upto one litre with distilled water. Adjust the ph to 9.2 with 1N-NaOH. Filter through double layer of filter paper put over funnel. Dispense in 5 ml amounts in bijou bottles (8 ml) bottles. Autoclave.

7.13.3 Use

It is a commonly used transport medium. Its also a holding medium for vibrios as it does not allow multiplication of vibrios.

7.14 BLOOD AGAR

7.14.1 Ingredients

Nutrient agar	31 gm
Sheep blood with anticoagulant (sodium citrate, 3.8% w/v)	60 ml.
Distilled water :	1 litre

7.14.2 Procedure

Dissolve 31gm of nutrient agar in1 litre of distilled water. Autoclave at 15 pounds pressure for 15 minutes.Cool to around 50^{0}C. Add sheep blood (with sodium citrate), 60 ml /1litre of nutrient agar and mix. Pour in plates quickly before agar solidifies. Remove air bubbles by flaming. Let the media solidify. Store in refrigerator.

Note : For chocolate agar preparation warm agar blood mixture over bunsen flame till colour of mixture turns brown. Pour in plates.

7.15 SUGAR (GLUCOSE, LACTOSE ETC)

7.15.1 Ingredients

1. Peptone water
2. Carbohydrates like glucose, lactose, sucrose, mannitol etc
3. Indicator stock solution (Andrades : NaOH - 4 gm, acid fuchsin-0.5gm, distilled water-100 ml or Bromocresol purple, 0.2 % solution)

7.15.2 Methods

Dissolve 15gm of peptone water in one litre of distilled water. Warm to dissolve.

Prepare 10% stock glucose solution as 10gm/100 ml.

Take 90 ml of peptone water and add 10 ml of 10% stock glucose solution.Put 2-3 drops of either bromocresol purple or andrades indicator stock solution as stated above.. Mix well. Sterlize it by subjecting it to open steaming for 50 minutes or alternatively Glucose solution may be filter sterilized as it is a heat sensitive component.Pour 3-4ml in sugar tubes with inverted durhams tube for glucose only. For other sugars like lactose, sucrose etc durhams tube is not needed.

7.16 DESOXYCHOLATE CITRATE AGAR (DCA)

7.16.1

Ingredients	gms/litre
Peptone	10 gms
Lactose	10 gms
Sodium desoxycholate	1 gm
Sodium chloride	5 gms
Dipotassium phosphate	2 gms
Ferric citrate	1 gm
Sodium citrate	1 gm
Bacto agar	15 gms
Neutral red	0.03 gm

7.16.2 Direction

Dissolve 45 gms of dehydrated medium in 1 litre distilled water, boil for 1 minute only with frequent agitation. Avoid over heating. **DO NOT AUTOCLAVE.**

Use

For isolating enteric bacilli, particularly Salmonella and many Shigella

7.17 TCBS AGAR (Thiosulphate Citrate Bile Salt Sucrose Agar)

7.17.1 Ingredients

	Per litre
Proteose peptone	10 gm
Yeast extract	5 gm
Sodium thiosulphate	10 gm
Sodium citrate	10 gm
Ox-Gall	8 gm
Sucrose	20 gm
Sodium chloride	10 gm
Ferric citrate	1 gm
Bromothymol blue	0.04 gm
Thymol blue	0.04 gm
Agar	15 gm
pH	8.6 ± 0.2

7.17.2 Direction

Dissolve 89 gms of dehydrated medium in 1 litre distilled water. Boil to dissolve completely. **DO NOT AUTOCLAVE.**

7.17.3 Use

It is a selective enriched -medium used for isolation of Vibrio cholerae from stool/ rectal swab. It is also used for cholera carrier surveys.

7.18 OXIDASE REAGENT

7.18.1 Procedure

Weigh 1 gm of tetramethyl para- phenylene diamine-di-hydrochloride. Dissolve it in 100 ml of distilled water. Store the reagent in ambered coloured bottle or in a stoppered flask with aluminium foil wrapped around it to prevent daylight exposure.

CHAPTER - 8

BACTERIOLOGICAL WATER QUALITY MONITORING

It is now well established that waterborne pathogens enter the drinking water systems through faecal contamination, and the list of such organisms (which include bacteria, viruses, protozoans and parasites) is a very long one. It would neither be practicable, nor is it necessary, to test a sample of drinking water for all the pathogens that might possibly be present in it, as that would involve many different procedures, a multitude of media and other materials, incubation conditions etc. It would, therefore, be sufficient if we can determine whether the water sample was recently contaminated with faeces. If we could establish that water was recently contaminated with faeces, such water is not fit for human consumption, as there would be a possibility that such water might harbour pathogenic organisms. Therefore, by establishing faecal contamination of water, we will be able to infer upon its potablility or otherwise.

8.1 IN THE LABORATORY THERE ARE TWO SIMPLE PROCEDURES OF TESTING WATER SAMPLES FOR FAECAL CONTAMINATION

8.1.1 Most Probable Number (MPN) method for coliform bacteria - using the multiple tube fermentation technique: In this method the MPN of total coliform bacteria, faecal coliform bacteria (or the thermotolerant coliforms) present in the water sample is determined along with the presence / absence of Escherichia coli. The coliform bacteria and E. coli are the universal indicators of faecal pollution.

8.1.2 H_2S-Strip method: This is a simple, reliable and easy-to-perform (by even untrained personnel), 'Presence / Absence' test for bacteriological quality, which works on the principle that there is a close correlation between faecal contamination and the presence of hydrogen sulphide (H_2S) producing bacteria and, that faecal pollution of water can be deduced by demonstration of H_2S production. It has been claimed, by various workers, that the H_2S-strip method shows >90% agreement with the conventional MPN test described above.

The two test procedures are described below in detail.

8.2 SPECIMENS

8.2.1 General instructions for collection, storage and transport of water samples for bacteriological examination

1 Care must be taken to ensure that samples are representative of the water being examined.

2 Care must be taken to see that no accidental contamination occurs during sampling; The sample collector must wash his / her hands well, using carbolic soap, just prior to collection of samples.

3 Test the water samples as soon as possible after collection. If delay of more than 3 hours is expected, transport samples to laboratory under cold-chain conditions.

4 A volume of water sample adequate to carry out all the tests should be collected; generally 200-250 mL of water sample should be collected.

5 Do not fill the sampling bottle up to the brim; a vacant space of 2-3 cm should be left for effective shaking of the sample, prior to testing in the laboratory.

8.2.2 Containers for sample collection

1. Samples for bacteriological examination shall be collected in :
   - Clean, sterilized, narrow mouthed neutral borosilicate glass bottles; OR
   - Autoclavable plastic (poly propylene) bottles with screw cap lids, of 250, 500 or 1000 ml capacity.
2. The glass bottle shall have a ground glass stopper having an overlapping rim. The stopper shall be relaxed by an intervening strip of paper between the stopper and the neck of the bottle. Glass bottles of suitable capacity with autoclavable screw cap lids may also be used.
3. The stopper and the neck of the bottle shall be protected by paper or parchment cover.
4. Collection bottles shall be sterilized :
   - In hot-air oven, at 160^{O}C for 1 hr (only glass bottles); OR
   - In an autoclave at 1.02 ± 0.03 kg/cm2 gauge pressure (15 ± 0.5 psi gauge pressure 120^{O}C temperature approximately), for 15 minutes, if plastic bottles are to be sterilized, they should be autoclaved for 30 minutes.

Note-1: Discard bottles which have chips, cracks and etched surfaces. Before use, bottles should be thoroughly cleaned with detergent and hot water, followed by a hot water rinse to remove all traces of detergent. Then rinse them 3 times with laboratory deionized water.

Note-2: A chelating agent should be added to sample bottles used to collect samples suspected to contain > 0.01 mg/L of heavy metals such as copper, lead, zinc, nickel etc. Add 0.3 mL of 15% EDTA-Na for each 125 mL of sample.

Note-3: Dechlorination - If the water to be sampled contains or is likely to contain chlorine, sodium thiosulphate shall be added to the clean, dry sampling bottles, before sterilization, in an amount to provide an approximate concentration of 100 mg/L in the sample. This can be done by adding 0.5 mL of 5% thiosuphate solution to a 250 ml bottle.

8.2.3 Collection of samples from taps:

1. Most samples will be taken from taps in treatment works, storage tanks, domestic premises (houses) or from public standpipes. When the distribution system is sampled, both domestic and community taps, including public fountains must be selected with care.
2. Samples should not be taken from taps which leak between the spindle and gland, as water from the outside of the tap may contaminate the sample.
3. External fittings such as filters, rubber or plastic nozzles and other anti-splash devices should be removed before collection.
4. Wiping of the tap surface with alcohol gauze /swab and flaming, using a spirit lamp (or even a cigarette lighter), should be considered an optional procedure.
5. The tap should be kept open, for free flow/run of water (at a moderate speed), for at least 1 minute prior to collection; this step will ensure flushing away of stagnant water in the pipe, before the sample collection.

8.2.4 Collection of samples from Stream/ river/ lake

1 Immerse the collection bottle, with its mouth closed by the stopper, a foot below the water surface, and facing the direction of the current.

2 Fill the bottle with water by opening the lid. Bring the bottle to the surface and replace the stopper.

3 Avoid collection of surface water, as it contains high organic matter.

8.2.5 Collection of samples from wells

1 Where the well is fitted with a hand pump or an electric pump:

   a. Flame the mouth of the pump (through which water is discharged on pumping); incase of plastic mouth, apply alcohol or rectified spirit and allow it to dry.

   b. Pump water to waste for four to five minutes, before the sample is collected into the bottle.

2 Where there is no pumping machinery:

   a. Sample can be collected directly from the well in a sterilized bottle fitted with weight at the bottom; however care must be taken to avoid contamination of sample by surface scum;

   b. Where it is not possible to collect the sample directly into the bottle, sample may be obtained by means of a suitable metal jug/ pot. Here, the jug/pot should be sterilized rinsing the inner surface of the container with a small quantity of methylated spirit and then igniting the same. Then the jug/pot

should be lowered to the required depth and then drawn up and down 2-3 times before it is brought to the surface. Avoid the jug / pot coming into contact with the bottom of the well or skidding along the surface, so that it may not collect the surface film. Once the jug / pot containing water is drawn up, samples from it should be poured into sterilized glass / plastic sample collection bottles and transported to the laboratory for testing. The samples should be properly labelled indicating source, date and time of collection.

8.3 MATERIALS

8.3.1 Equipment and Glassware / Plasticware

8.3.1.1 Equipment

- **Incubator:** Required for incubating the fermentation tubes, culture plates etc. Should be capable of maintaining uniform and constant temperature of 35-37°C (±0.5°C) at all times, in all parts. Incubator of water-jacketed type, with mechanical means of circulating air is preferable.

  Where the ordinary room temperatures vary excessively, it is desirable that the laboratory incubator be kept in a special room that can be maintained at a few degrees below the recommended incubator temperature. Accurate thermometers with bulb constantly immersed in liquid (glycerine, water or mineral oil) should be maintained within the incubator, and daily readings of temperature should be recorded.

- **Water-bath:** Required for carrying out the test for faecal coliforms by incubating the fermentation tubes at 44.5°C. Should be capable of maintaining constant temperatures between 44 and 45°C. Should be adequately insulated against heat loss, and should be provided with an accurate thermometer whose bulb is immersed in water at the level of medium in the fermentation tubes. Regular recording and monitoring of temperatures is a must.

- **Hot-air oven:** Required for dry sterilization of glassware. Should be of sufficient size to prevent crowding of the interiors, and constructed to give uniform and adequate sterilizing temperatures (in the range of 160-180°C). An accurate thermometer covering the range (160-180°C) and periodic monitoring of accuracy is a must.

- **Autoclave:** Required for wet sterilization of glassware / plasticware, media, fermentation tubes etc. Should be of sufficient size to prevent crowding of the interiors, and constructed to provide uniform temperatures within chambers up to and including sterilizing conditions of 1.02 (± 0.03) kg / cm^2 gauge pressure (corresponding to 15 (±0.5) lb / $inch^2$ gauge pressure or 120°C temperature approximately).

- **Refrigerator:** A refrigerator of sufficient capacity, depending on the work load, and capable of maintaining continuous temperature between 0°C and 5°C.
- **Colony Counter:** A Quebec or similar colony counter will be suitable (optional).
- **Gas burner:** Bunsen or similar burner or spirit lamp is adequate.
- **Inoculation loop and holder:** length of 24- or 26-gauge wire (7.5-10cm) should be used. Nichrome wire is acceptable, but platinum-iridium is better. The wire is set in a handle made of metal or glass, of diameter similar to that of a pencil. To make the inoculation loop, the wire is bent to form a circle 3-4mm in diameter.
- **Cleaning and maintenance equipment:** items such as brushes for cleaning tubes, bottles, etc., a waste bin, and a tool kit are required.
- **Safety equipment:** there should be an adequate first-aid kit and a fire extinguisher or other means of fire control in every laboratory.

8.3.1.2 Glassware / Plasticware

- **Pipettes:** Pipettes of various convenient sizes (2, 5, 10, 50 mL) will be required. Accuracy of volume delivered is very important and the error of calibration should not exceed 2.5%. Pipettes with unbroken tips and distinctively marked graduations should be used. Alternatively disposable (single use) pre-sterilized plastic pipettes may be used in place of glass pipettes. Rubber bulbs or automatic / semi-automatic pipetting device should be used to avoid mouth-pipetting.
- **Sample collection bottles:** As mentioned under the previous section on specimen collection and transport.
- **Petri dishes / plates:** Glass or clear plastic (disposable, pre-sterilized) Petri dishes of 100 mm dia., with side wall at least 15 mm high.
- **McCartney bottles, with metal (aluminium) screw cap lids and rubber washer** (required for preparing H_2S-Strip vials)
- **Test-tubes and racks:** tubes can be 20.3 x 150mm in size for 10-ml sample volumes plus 10ml of culture medium (screw caps are not recommended for fermentation media). The racks should be large enough to accommodate culture tubes of the largest diameter used.
- **Bottles:** used for the larger volumes consisting of 50ml of sample and 50ml of culture medium. They should have loose-fitting caps and ideally be calibrated with 50-ml and 100-ml marks. Conical flasks of 100-150 ml capacity may also be used.

8.3.1.3 Media preparation equipment: glass or stainless-steel containers (usually flasks) are required. Any heating equipment and stirrers used in the preparation of media should be clean and free from soluble toxic materials.

8.3.1.4 General laboratory equipment: various sizes of round and erlenmeyer flask, beakers, stands, glass or unbreakable plastic measuring flasks, spatulas, etc. are required.

8.3.2 Supplies (Dehydrated media, Chemicals, Reagents and Kits)

8.3.2.1 For MPN Coliform test:

- **MacConkey broth (double strength) with neutral red -** available commercially in ready-to-use, dehydrated form from various manufacturers;
- **MacConkey broth (single strength) with Neutral Red -** available commercially in ready-to-use, dehydrated form from various manufacturers;
- **Brilliant Green Bile broth (BGB) -** available commercially in ready-to-use, dehydrated form from various manufacturers;
- **Tryptone water / Peptone water** (for indole test) - available commercially in ready-to-use, dehydrated form from various manufacturers;
- **MacConkey agar without CV, with 0.5% bile salts -** available commercially in ready-to-use, dehydrated form from various manufacturers;
- **EMB Agar (Levine) -** available commercially in ready-to-use, dehydrated form from various manufacturers;
- **Kovac's Reagent -** available commercially in ready-to-use form from various manufacturers;

8.3.2.2 For H_2S- Strip Test

8.3.2.2.1 Chemicals

- Peptone, bacteriological grade I.P.
- Dipotassium hydrogen phosphate, A.R. Grade
- Ferric ammonium citrate
- Sodium thiosulphate, A.R. grade
- Teepol
- L- Cysteine hydrochloride, nonohydrate

Other miscellaneous items:

- Non-toxic, colourless tissue paper napkins (size 80 cm2)
- Forceps
- Scissors

8.3.3 Materials Required For QC

- Standard culture of Escherichia coli (ATCC 25922)
- Standard culture of Salmonella typhimurium (MTCC 98*)

OR

Standard culture of Citrobacter freundii (MTCC 1658*)

The standard and cultures are used for confirming the isolations made form water samples (E.coli) and for QC of H_2S strip vials prepared in the laboratory (S. typhimurium and Citrobacter freundii)

* Obtainable from Institute of Microbial Technology, Chandigarh.

8.4 PROCEDURE

8.4.1 Multiple Tube Fermentation Test For Coliform Bacteria

In the multiple-tube method, a series of tubes containing a suitable selective broth culture medium (lactose containing broth, such as MacConkey broth) is inoculated with test portions of a water sample. After a specified incubation time at a given temperature, each tube showing gas formation is regarded as "presumptive positive" since the gas indicates the possible presence of coliforms. However, gas may also be produced by other organisms, and so a subsequent confirmatory test is essential. The two tests are known respectively as the presumptive test and the confirmatory test.

For the confirmatory test, a more selective culture medium (brilliant green bile broth) is inoculated with material taken from the positive tubes. After an appropriate incubation time, the tubes are examined for gas formation as before. The most probable number (MPN) of bacteria present can then be estimated from the number of tubes inoculated and the number of positive tubes obtained in the confirmatory test, using specially devised statistical tables. This technique is known as the MPN method.

Different test portions to provide tenfold serial dilution steps may be used, the dilutions being based on the anticipated number of coliform bacteria in the water sample being tested. The reliability of the result obtained depends on the number of tubes inoculated with each test portion; in certain instances, the number can be reduced to three in each dilution step. Each combination of inoculated tubes will have its own MPN values table.

8.4.1.1 The following consumable items are required

Culture medium: The following culture media are required-

- MacConkey broth with neutral red (Double strength)
- MacConkey broth with neutral red (Single Strength)
- Brilliant Green Bile broth (BGB)

- Laboratory disinfectant: for cleaning laboratory surfaces and the pipette discard bin.
- Detergent: for washing glassware, etc.
- Sodium thiosulfate solution: required when chlorinated supplies are tested. Sodium thiosulfate neutralizes any residual chlorine in samples at the time of collection, preventing it from acting on any microorganisms present in water samples.
- Autoclave tape.
- Diluent: typical diluents include Ringer's solution and phosphate-buffered saline.

8.4.1.2 Culture media preparation

- Commercially available dehydrated media simplify the preparation of culture broths and are therefore recommended for laboratory work. Various manufacturers produce these media as powders, which can then be easily weighed out, dissolved in distilled water, and dispensed into culture tubes before sterilization.

8.4.1.3 Preparation of media (Refer to chapter 7)

Media should be prepared in accordance with the manufacturer's instructions, as follows:

(a) Dissolve the stated amount of the dehydrated medium in distilled water to obtain the double-strength or single-strength presumptive medium (for confirmatory analysis, only single-strength medium is used).

8.4.1.4 Procedure For Inoculation Of Samples

The procedure to be used for testing relatively unpolluted water, such as treated water from waterworks or distribution system or end user's tap, is described below:

1. Note down the details of the sample collected (from the label on the bottle) in the laboratory register.
2. With the stopper in position, shake the bottle vigorously to achieve a homogeneous dispersion of bacteria. (If the bottle is completely full, remove the stopper and aseptically discard about 20-30ml of water; then replace the stopper and shake. This ensures thorough mixing.)
3. With a sterile 10-ml pipette, inoculate 10 ml of the sample into each of five tubes containing 10 ml of presumptive broth (double strength). Add 50 ml of sample to a tube containing 50 ml of presumptive broth (double strength) and 1 ml sample into each of the five tubes containing 5 ml presumptive broth (single strength). It is advisable to shake the tubes gently to distribute the sample uniformly throughout the medium.

4. Incubate the tubes at 35°C or 37°C for 24 hours.

5. At the end of the 24-hour incubation period, examine each tube for the presence of gas. If present, gas can be seen in the durham tube. If none is visible, gently shake the tube; if any effervescence (streams of tiny bubbles) is observed, the tube should be considered positive.

6. Record the number of positive tubes after 24 hours.

7. Re-incubate negative tubes for a further 24-hour period. At the end of this period, check the tubes again for gas production as in 5 above. Gas production at the end of either 24 or 48 hours' incubation is presumed to be due to the presence of coliforms in the sample.

8. Record the number of positive tubes after 48 hours.

9. The confirmatory test should be carried out at the end of both the 24-hour and the 48-hour incubation. Using a sterile loop, transfer one or two drops from each presumptive positive tube into two tubes containing respectively confirmatory broth (BGB) and tryptone water. (Sterilize the inoculation loop before each transfer by flaming and allow to cool.)

10. To confirm the presence of thermotolerant coliforms, incubate the subculture tubes from each presumptive positive tube for 24 hours at 44.5 ±0.5°C.

11. At the end of 24 hours' incubation, examine each broth tube for growth and the presence of gas in the durham tube. Record the results, as done previously.

12. To each tube of tryptone water, add approximately 0.1 ml of Kovacs reagent and mix gently. The presence of indole is indicated by a red colour in the Kovacs reagent, forming a film over the aqueous phase of the medium.

13. Confirmatory tests positive for indole, growth, and gas production show the presence of E. coli. Growth and gas production in the absence of indole confirms thermotolerant coliforms.

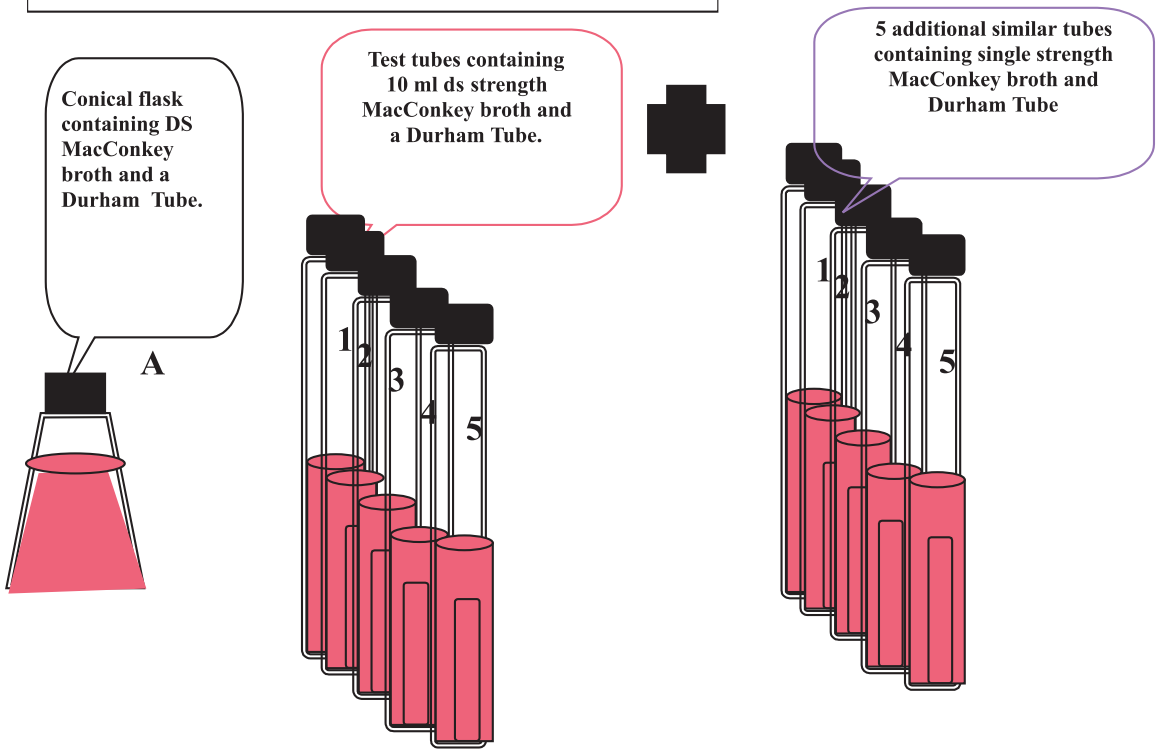
8.4.1.5 Determination of MPN

For treated water, where one 50-ml and five 10-ml portions are inoculated, the MPN can be found from the test results by refering to the MPN Table (also called McRady's Table) given in Table- 8.1.

8.4.1.6 Interpretation

Water samples are classified, based on the MPN coliform count test, in the following way:

Figure- 8.1: MULTIPLE-TUBE FERMENTATION TEST



Class	Grading	Coliform count / 100 ml (MPN)	E. coli count per 100 ml*
Class- 1	Excellent	0	0
Class- 2	Satisfactory	1-3	0
Class- 3	Suspicious	4-10	0
Class- 4	Unsatisfactory	>10	0 or more

* Presence of E. coli immediately places the sample in Class- 4

8.4.2 H_2S-Strip method

8.4.2.1 Description of the test device

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.

Preparation of H_2S- Strip medium

Composition

- Peptone ... 20 g
- Dipotassium hydrogen PO_4 ... 1.5 g
- Ferric ammonium citrate ... 0.75 g
- Sodium thiosulphate ... 1 g
- Teepol* ... 1 ml
- L- Cystiene HCl ... 0.25 g
- Water ... 50 ml

* If Teepol is not available, 0.2 g of Sodium dodecyl sulphate (SDS) (pure) may be used in its place.

Preparation

Dissolve the ingradients in 50 ml of water, over gentle heat, and resultant broth is used for soaking the paper strips in the McCartney bottles.

8.4.2.3 Preparation of the test vials

1. Take washed and clean McCartney bottles with aluminium caps lined with rubber washer.
2. Take tissue paper napkins (non-toxic, colourless) of size 80 cm_2 and fold them into strips of convenient size to be held in the bottle (approximately 5 cm x 2 cm)
3. Introduce folded tissue paper strips into the bottles with the help of forceps
4. Keeping/holding the vials in a slanted position, slowly pipette out 1 ml of the concentrated H_2S- Strip medium, taking care to see that the medium is totally absorbed by the paper strip as it is being added into the bottle.
5. Loosely cap the bottles, after soaking the paper strips with medium, and keep them in a dryer (hot-air oven) at 50°C, till the paper strips with medium become completely dry.
6. Sterilize the bottles containing dried paper strips in an autoclave at 15 lb/$inch^2$, for 15 minutes. Tighten the screw-caps of the bottles prior to removing from the autoclave.
7. Store the test vials in a cool place.
8. Each bottle of test vials prepared in the laboratory must be checked for performance using sterile water samples spiked by standard cultures of **Salmonella typhimurium** or **Citrobacter freundii,** which are known producers of H_2S. If the vials do not show good performance by turning black at the end of incubation period, the vials must be rejected and prepared freshly.

8.4.2.4 Test procedure: (see Figure- 8.2)

- Pour the water sample to be tested for faecal pollution into the bottle, upto the precalibrated level (20 ml).
- Incubate at 37°C or allow to stand at ambient temperature (30-37°C); for 24-36 hours. No incubator is necessary under field conditions, as the bottles can be held in the pockets and body temperature can be made use of.
- Faecal pollution is indicated if the contents of the bottle turn black.

8.4.2.5 Advantages of H_2S-Strip Test

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

8.4.2.6 Disadvantages of H_2S-Strip Test

- In principle, does not conform to the conventional standards of bacteriological testing of water samples.
- At the best, it is a screening test.

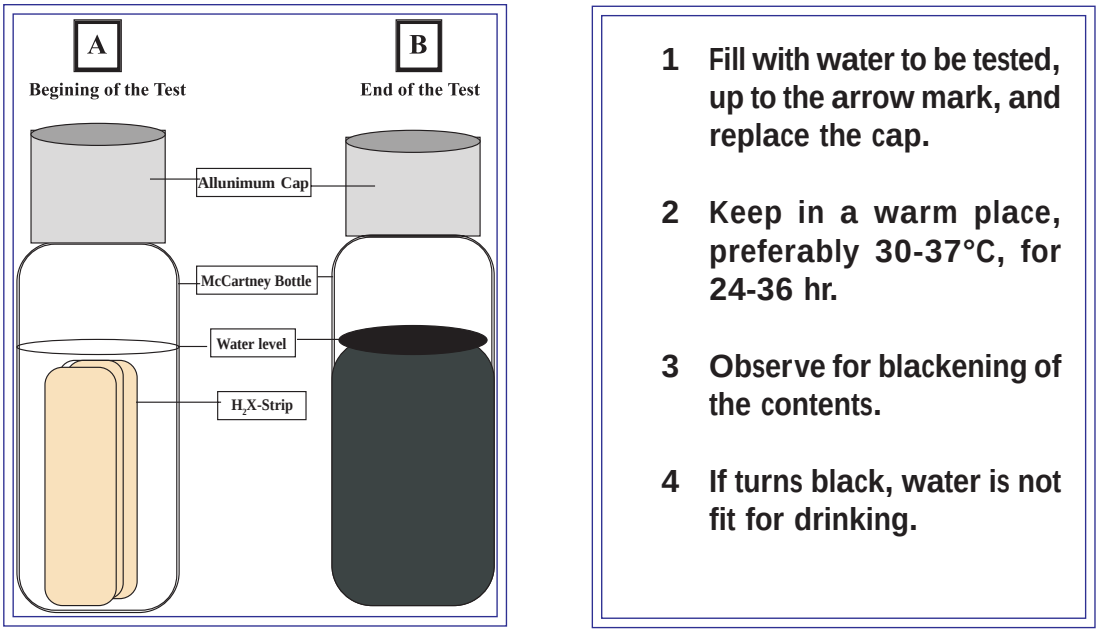


Figure- 8.2: H_2S - Strip Test

- Purely qualitative, **"PRESENCE- ABSENCE"** test.
- May not work in cold ambient temperatures.

8.5 RESIDUAL FREE CHLORINE TEST

The methods available for the determination of chlorine residual in drinking water are:

- Orthotolidine test (OT Test)
- starch-potassium iodide test (S-KI Test)
- N, N-diethyl-p-phenylenediamine test (DPD Test)

Methods employing orthotolidine and starch-potassium iodide are widely used. However, it should be noted that orthotolidine is a recognized carcinogen and hence its wide usage is questionable in recent years due to the hazardous nature of the chemical and also due to the possibility of environmental pollution with a carcinogen.

The method based on the use of starch-potassium iodide is not specific for free chlorine, but measures directly the total of free and combined chlorine in the sample. Hence this method is not recommended.

In our country the orthotolidine test (OT test) is still being used.

8.5.1 Ortho Tolidine (OT) test

It is widely used for field measurements, using simple colour-match comparators, so that testing can be done on site. Yellow colour (in different intensities, depending upon the level of residual chlorine) is generated following the addition of orthotolidine to the water sample and is matched against standard coloured discs or tubes / ampoules containing known standards of orthotolidine solution. The method can be used by staff without extensive specialized training. The reagent may be in the form of a solution. The solution (OT reagent) should be stored in a brown bottle.

Commercial visual comparator technique

Commercial comparators are of two basic types-the disc type, containing a wheel of small coloured glasses, and the slide type, containing liquid standards in glass ampoules. However, both consist of the same components: a box with an eye-piece in front and two cells; the hole is so arranged that both cells are in the field of vision of the eye-piece.

One cell, containing a water sample without the reagents, is placed in line with the rotating coloured glasses or the ampoules containing the standards. The water sample containing the reagent is placed in another cell. If free chlorine is present, a colour will develop. The concentration of chlorine is estimated by matching the colours in both cells, as seen through the eye-piece. Each colour of the disc or ampoule corresponds to a certain quantity of chlorine in the water.

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)

Sl. #	1x50 ml	5x10 ml	5x1 ml	MPN / 100ml	Sl. #	1x50 ml	5x10 ml	5x1 ml	MPN / 100ml
1.	0	0	0	<1	22.	1	2	1	7
2.	0	0	1	1	23.	1	2	2	10
3.	0	0	2	2	24.	1	2	3	12
4.	0	1	0	1	25.	1	3	0	8
5.	0	1	1	2	26.	1	3	1	11
6.	0	1	2	3	27.	1	3	2	14
7.	0	2	0	2	28.	1	3	3	18
8.	0	2	1	3	29.	1	3	4	21
9.	0	2	2	4	30.	1	4	0	13
10.	0	3	0	3	31.	1	4	1	17
11.	0	3	1	5	32.	1	4	2	22
12.	0	4	0	5	33.	1	4	3	28
13.	1	0	0	1	34.	1	4	4	35
14.	1	0	1	3	35.	1	4	5	43
15.	1	0	2	4	36.	1	5	0	24
16.	1	0	3	6	37.	1	5	1	35
17.	1	1	0	3	38.	1	5	2	54
18.	1	1	1	5	39.	1	5	3	92
19.	1	1	2	7	40.	1	5	4	161
20.	1	1	3	9	41.	1	5	5	>180
21.	1	2	0	5					

CHAPTER-9

LABORATORY DIAGNOSIS OF CHOLERA / GASTROENTERITIS

9.1 TITLE AND SCOPE

Gastroenteritis is defined as inflammation of mucous membrane of stomach and intestines resulting in frequent loose motions associated with or without vomitting. There may be passage of either mucus or blood or both accompanied by fever and sometimes pain abdomen.

Any gastroenteritis (also called Diarrhoea) should not be taken lightly, especially in children of small age (below 5 yrs), as it may rapidly lead to dehydration and other complications like acidosis, acute tubular necrosis leading to kidney failure / circulatory failure and death. More over, cholera should be suspected if there is history of passage of frequent watery / rice water stools and history of effortless vomiting. Similarly, passage of blood in stool should give clue to Shigella / Salmonella / Enteropathogenic (EPEC) or Enterohaemorrhagic E.coli (EHEC) dysentery.

Diarrhoea is one of the major causes of morbidity and mortality in India. In health institutions, upto one third of total admissions are due to diarrhoeal diseases and upto 17 % of all deaths in indoor paediatric patients are due to diarrhoea.

9.2 COMMON ETIOLOGICAL AGENTS THAT CAUSE DIARRHOEA ARE

9.2.1 Bacterial

- Vibrio cholerae
- Salmonella
- Shigella
- Escherichia coli (EPEC,ETEC,EIEC,EHEC)
- Staphylococcus aureus (food poisoning type)
- Bacillus cereus

9.2.2 Parasitic

- Giardia lamblia
- Entamoeba histolytica
- Helminthic infections
- Cryptosporidium

9.2.3 Viral

- Rotavirus

9.2.4 Fungal

- Candida albicans

9.2.5 Other Miscelaneous causes

- Antibiotic associated
- Chemical / non - infective cases (sometimes)

9.3 Equipments & Supplies

No special equipment or material is necessary for stool culture work, provided basic infrastructure for culture work exists. Hence a list of items is given below assuming that such facility does not exist at district level. The list is indicative only and not comprehensive.

9.3.1 Glassware

- Bijou bottle: 8 ml screw capped, with aluminum cap and washer, autoclavable.
- Glass slides : 75 mm by 25 mm size, 1.35 mm thick, 50 slides / box
- Coverslips: 18 mm by 18 mm size, no 1 size. 10 gm (about 50 coverslips) / packet, 20 packets / box
- Test tubes : 15 mm by 125 mm, glass tube with round bottom.
- Test tube (sugar tubes) : 12 mm by 100 mm, glass made with round bottom.
- Amber coloured storage bottles

9.3.2 Media

- Rectal swab: made up of absorbent cotton wool, sterilized
- Dehydrated culture media like bile salt agar, MacConkey's agar, Mueller - Hinton agar
- Transport media like Venkatraman - Ramakrishnan fluid (VR - fluid), Cary Blair's media

9.3.3 Plastic wares

- Disposable petri plates, 88 - 90 mm diameter, polypropylene, individually wrapped, pre -sterilized and disposable.
- Cotton absorbent: 500 gm pack size

9.3.4 Reagents and stains

- Crystal violet stain, ready made.
- Iodine stain, ready made.
- Safranin stain, ready made.
- Alcohol / acetone.
- pH paper: narrow range.

9.3.5 Antisera

- Vibrio cholerae antisera, Poly 01
- Vibrio cholerae antisera, 0139-Bengal

9.3.6 Equipments

- Autoclave.
- Hot air oven.
- Bunsen burner with gas supply (cylinder).
- Binocular microscope.
- Refrigerator
- Incubator.
- Table top centrifuge

9.3.7 Accessories

- Loop holder
- Nichrome wire
- Hand lens
- Discarding jar with disinfectant.

9.4 Specimen collection

Voided stool specimen is to be collected as far as possible, if the patient (infant/ small children) is passing loose stool then sterile plastic catheter (disposable) no 26 is to be used. Collect liquid stool in a Bijou bottle & transport to the laboratory within 2-3 hrs for culture & microscopic examination. In case of delay transport media) like Cary Blair's medium/ V.R. fluid (especially if cholera is suspected) should be used (for use of transport media, add about 1-2 gm of semi sold stool/liquid stool in 10 ml of transport media. It is preferable to keep, suspected cholera stool specimen at room temperature till it is transported to the designated laboratory. In practicality & during field situations, many times rectal swab are collected especially from infants and small children.

9.4.1 Rectal Swab

- Moisten the rectal swab in sterile normal saline
- Introduce the swab inside the anal sphincter and go upto 2-4 cm inside rectum.
- Rotate the swab upto 90 degrees and withdraw the swab.
- Put the swab in any of the transport media like VR fluid / Cary Blair medium by inserting the swab completely into the media.
- Break off the excess wooden portion of swab stick and screw cap the bottle of transport media.
- Store at room temperature till transported to the nearest lab (if cholera is suspected) or else keep it in fridge (4 deg centigrade) if salmonella / shigella is suspected.
- Label the bottle of transport media.

Vomitus is not a good specimen for collection, bed pan samples may be collected only if no other sample is available. For better isolation of pathogen there is no harm if stool samples are collected in duplicate, one in plain bottle (for microscopy) & other in transport media.

9.5 Transportation

Two transport media, **V.R. Fluid** (for cholera) and **Cary Blair's** are commonly used for isolation of common bacterial enteropathogens like Salmonella, Shigella, and Esch coli including Vibrios. If the specimens are collected in transport media like **Cary Blair's,** then it should reach nearby laboratory in 2-3 days time and samples can be kept at room temperature. If rotavirus or any other viral etiology is suspected then stool specimen can be kept in fridge (4°C-8°C) - till it is transported to the nearby laboratory.

9.6 Stool examination

9.6.1 Gross

Note the colour, (yellow/green, black etc), consistency (watery/semi-formed), and presence of blood/mucus.and odour etc. Offensive odour in stool specimen is suggestive of amoebic infections. Sometimes frank pus may be present in the stool specimen which is suggestive of severe dysentery.

9.6.2 Microcopy

This is a simple laboratory examination that yields meaningful information at low cost. One can not only see the presence of blood, mucus, parasites & their ova/cyst (ova of roundworm, hookworm, whipworm, H.nana and cysts/ trophozoites of Giardia lamblia and Entamoeba histolytica) but with some experience, darting motility suggestive of Vibrio cholerae can also be observed.

9.6.2.1 Methods

9.6.2.2 Preparation of Lugol's Iodine

Weigh	Iodine crystal (powder form)	: 5gm
	Potassium Iodide	: 10gm
Add	Distilled water (d/w)	: 100 ml
	Dilute the stain in d/w 1:5 before use.	

Potassium iodide is dissolved in distilled water and iodine crystals are slowly added. The solution is then filtered & kept in a stopper bottle of amber colour.

Shelf life of this solution is **2** weeks.

9.6.2.3 Procedure

- Take a drop of normal saline and a drop of Lugol's iodine with a nichrome wire loop and put it over a clean glass slide at 2 places.
- Take a minute portion of semi-solid faeces with nichrome wire loop and mix it with 0.9% saline. Similarly mix small amount of faeces with Lugol's iodine too. In Iodine stained preparation - Iodine kills the organisms (so motility of trophozoite forms of Entamoeba histolytica if any is lost) as well as iodine makes nuclear structure of amoeba stained, thereby making

it clearly visible.

- Mix the two with a wooden stick / toothpick so as to make an emulsion
- Put a cover slip no 0 or 1 over the normal saline and Lugol's iodine, stool emulsion
- For examination of the slide, first a low power (10x) objective should be used followed by high power (40x) objective.
- Record the findings.

9.6.2.4 Precautions

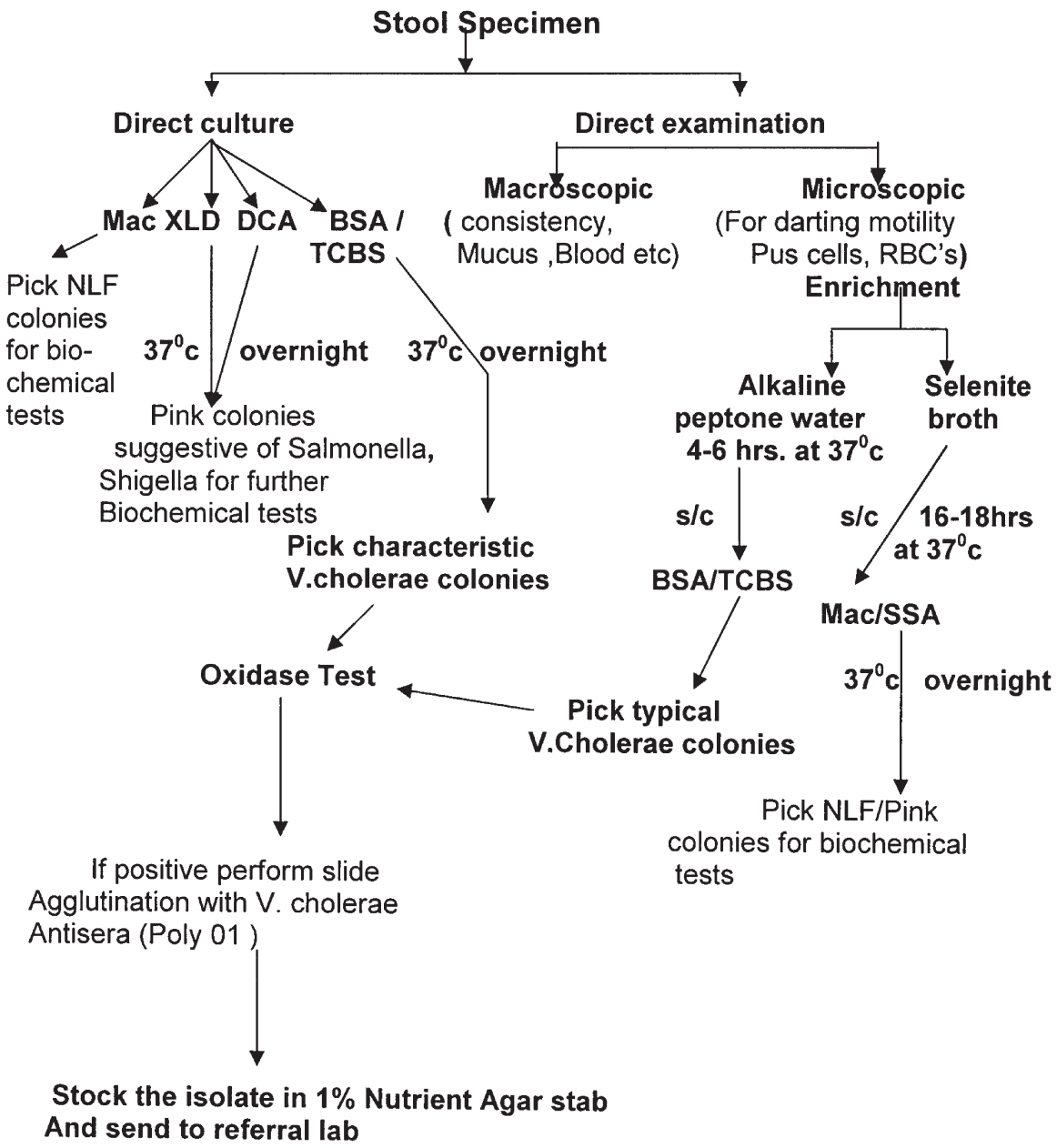
- While taking stool sample either for culture or microscopy select mucus or blood portion of it if present.
- If stool specimen is watery then there is no need to use normal saline for microscopy - it may be examined directly
- The emulsion should be neither too thick nor thin & air bubble entrapment should be avoided.
- Also there must not be excessive fluid while preparing the slides to prevent cover slip overflowing over the emulsion.
- While viewing normal saline preparation under microscope, condenser should be put down & diaphragm partially opened to control amount of light passing through the slide. For visualizing darting motility of Vibrio cholerae, diaphragm should be partially closed to reduce the amount of light passing through the slide.
- Motility of Entamoeba histolytica can be enhanced if normal saline used is slightly warm & stage of microscope is kept warm by microscope bulb / table lamp.
- Urine should not be allowed to mix with stool while collecting specimen.
- Rectal swab should be properly collected after putting it inside rectum so that proper rectal swab is collected - and not mere anal swab.

9.6.2.5 Interpretation of Microscopy

Following observations can be made:

1) Darting motility of Vibrio cholerae
2) Pus cell & RBC 's
3) Trophozoite or cysts of Entamoeba histolytica or Giardia lamblia.
4) Egg of Ascaris lumbricoides (roundworm), Ankylostoma duedenale, Necator americanus (hookworm), Trichuris trichiura (whip worm), Hymenolepis nana, Taenia saginata / Taenia solium (tapeworm) etc.

Flow chart for processing of stool specimens for V.cholerae and other enteropathogens



9.7 Stool culture

Direct inoculation of stool is done on to MacConkey's agar (Mac), XLD agar, Bile salt agar (BSA), (TCBS) Thiosulphate citrate bile salt sucrose agar and some times on blood agar depending upon availability of various media, resources and experience.

Enrichment culture

- Following 5-6 hrs. enrichment in alkaline peptone water (APW)and 12-18 hrs. enrichment in Selenite-F broth, repeat culture is done on to bile Salt agar and Salmonella Shigella agar (S.S. agar) respectively.

- After overnight incubation at 37°C non-Lactose fermenting colonies (NLF) at MacConkey's agar and pink colonies at Xylose -Lysine Dextrose agar (XLD agar) are picked up with straight wire for further processing.
- Preferably one should try to pick up single isolated colony as far as possible. In case of doubt it is preferable to first sub-culture suspected NLF / Pink colonies - purify the isolate - before putting up biochemical tests.
- There are many biochemical tests like glucose, lactose, sucrose, mannitol, urea, Indole, citrate, PPA, LIA, TSI, lysine, arginine, ornithine, oxidase etc, that are usually put up to biochemically characterize a stool pathogen. However at the district level simple biochemical tests may be put up for preliminary characterization of enterobacteriaceae that is glucose with durham's tube, lactose, triple sugar iron agar (TSI), Phenylalanine deaminase test (PPA), Indole, urease and motility test. No hard and fast rule can be applied - however number of biochemical tests that is required to be put up is ultimately determined by the experiences of the technician and resources available.
- For further characterization of isolates including sero -typing, phage typing etc the isolate may be stocked on to 1.5% nutrient agar slants and send to nearby state/ central reference laboratories.
- Antibiotic sensitivity of the isolate may be attempted at district laboratory or the isolate may be sent to reference labs.

9.7.1 Methods for enteropathogen isolation

- Select fresh culture plates (not older than 7 days).
- Dry culture plates at 37°C incubator for about half an hour.
- Use single culture plates for one specimen in the beginning. Later on two specimens may be plated in one plate.
- Label all the plates.
- Take little stool specimen (slight) with Nichrome wire loop & make a primary well on to BSA, MacConkey's agar plate or XLD as the case may be. (ref. fig. 9.14).
- Sterilize nichrome loop in blue portion of bunsen burner flame. Cool the loop.
- Heat sterilize the loop & re-streak in parallel lines after cooling the loop so as to isolate separate single colonies as far as possible.
- Also inoculate enrichment broth like selenite- F and APW after labeling the same.

- Incubate at 37 °C incubator for 24 hrs.
- Next day observe colony morphology on to various enriched / selective media plate.

9.7.2 Appearance of colonies for V. cholerae

BSA

Round, clearly translucent, oil drop like colonies suggestive of Vibrio cholerae. Sometimes one has to really hunt for cholera colonies if number of pathogenic bacteria excreted in stool is lesser or was destroyed due to improper collection / transportation of specimens. Sometimes samples are to be collected after patient has already started some empirical antibiotic treatment thereby leading to decrease in number of enterpathogens. Even a single suspected vibrio colony is significant & should be saved, sub cultured & identified. In outbreak situations, agglutination with poly '01' cholera antisera & later specific cholera antiserum (ogawa / inaba)or 0139-Bengal may be used to do slide agglutination test from solid culture plates (BSA). For further characterization, the representive isolates may be sent to designated reference laboratory.

9.7.3 Colonies on XLD: Look for pink colonies with black centres suggestive of Salmonella or Pink colonies without black centre suggestive of Shigella. Most Escherichia colonies are seen as yellow colonies.

9.7.4 Colonies appearance on MacConkey's Agar

Non-lactose fermenting, pale colonies with smooth consistency, either very minute suspected of shigella or slighly larger (1-2 mm) suspected of Salmonella sps. It is a good practice to carry out oxidase test for all types of NLF colonies so as to exclude Pseudomonas, Aeromonas. Pseudomonas and V. Cholerae are all oxidase positive. Enteric pathogen like salmonella, shigella and E.Coli are oxidase negative.

Any suspected Salmonella or Shigella colony should be subcultured on a Nutrient Agar Slope and subjected to biochemical tests for identification or sent to reference laboratory.

9.8 Tests for identification of organism

Biochemical Test

9.8.1 Oxidase Test

9.8.1.1 Method

- Take a piece of filter paper on a clean glass slide & add 1% freshly prepared oxidase reagent (tetra-methyl-para-phenylene diamine-di

hydrochloride-refer sec.7.18 for prep.). Dry the filter paper at room temperature.

- Pick a speck of colony using glass rod/ platinium loop & rub it on the filter paper. Development of purple colour within 30 seconds is (fig.-9.13) indicative of positive oxidase reaction. Do not use Nichrome wire for Oxidase Test.

9.9 After this the suspected V. Cholerae colony should be subjected to the following tests

9.9.1 Gram staining

Refer to chapter no. 6 for Grams staining

9.9.2 Motility test of organism in liquid medium

9.9.2.1 Method

- Take a clean glass slide & make a thin ring of plasticine (good quality of ISI make) & apply it over the slide. (refer to the Fig- 9.1).
- Thickness of the ring should not be more than 1mm, so that there is no difficulty in focusing the slide with 40x high power objective.
- Take a clean coverslip & put on small drop of liquid culture over the coverslip with the help of a small sized inoculating loop (about 1mm diameter). Fig.-9.1
- Put the slide containing plasticine ring over the coverslip containing the drop of liquid culture without touching the drop and then invert the slide so that the drop hangs (see Fig 9.3).
- Put the condenser low and focus the slide in low power (10x objective). And try to focus the edge of the drop.
- Examine next in high power i.e. 40 X objective for checking the motility against the stationary background (fig.-9.4)
- Motility can also be checked by inoculation in soft agar medium and observing the medium after 24 hrs incubation. (refer to the Fig 9.1, 9.4).

METHOD OF PREPARATION OF HANGING DROP

(For motility checking)

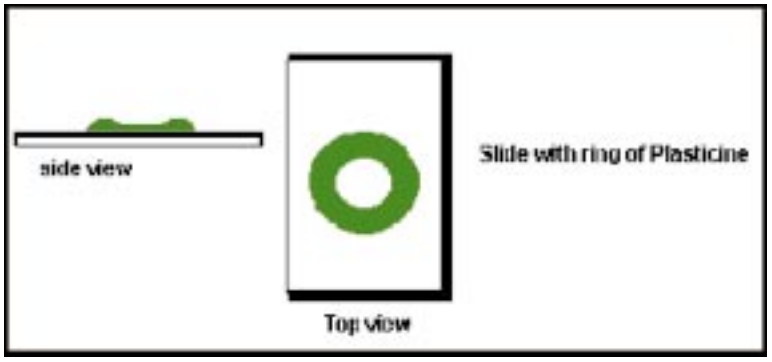


Fig 9.1

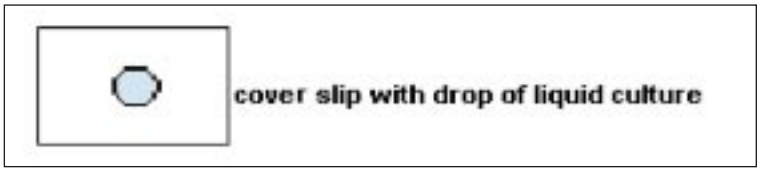


Fig : 9.2

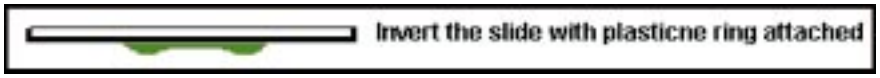


Fig : 9.3

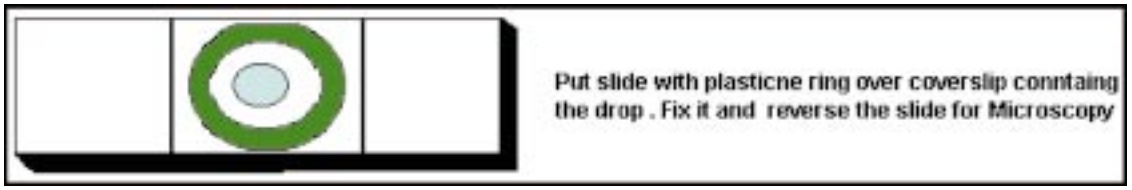


Fig: 9.4

9.10 Slide agglutination test for confirmation of Vibrio cholerae

9.10.1 Method

- Take out cholera antisera from the refrigerator and bring it to room temperature.
- For carrying out slide agglutination test take, 2-3 clean glass slides.
- Make them grease free by cleaning with cotton & then passing over flame several times. Cool the slide.

- All sera manipulation are to be done in an aseptic manner in clean area so as to prevent antisera contamination.
- Take two drops of normal saline with Nichrome wire loop and put over a clean glass slide - side by side.
- Pick up a suspected colony with a straight wire and emulsify on to both the saline drops one by one.
- Check for autoagglutinability i.e. formation of coarse granules without the addition of antisera.
- In case of rough strain auto agglutinability will be observed.
- In such a case, no further serotyping should be attempted. In case the strain is smooth & forming a smooth suspension, add a drop (small) of cholera antiserum (Poly O1).
- Look for clumping i.e. formation of coarse granules which indicate agglutination.
- In case there is no agglutination with Poly 01 V. cholerae antisera, repeat the steps with 0139-Bengal antisera.
- Inform laboratory incharge for further necessary action or public heath measure in case cholera is diagnosed at district level laboratories. **(Notification of cholera in the prescribed form)**
- In case no agglutination is found with any of the cholera antisera as stated above, then the strain is stated as non-agglutinable vibrios and preserved for further characterization.

To optimise the use of V. cholerae antisera it is advisable to carry out sero typing in representative isolates of V. cholerae in an outbreak situation

9.11 Other Biochemical tests

There are commonly two types of biochemical media available, one is liquid medium of sugar solutions (0.5 - 1.0 % carbohydrates added in peptone water base along with andrade's indicator) like glucose, lactose, sucrose, mannitol etc in test tubes i.e. sugar tubes and other is solid i.e. in the form of slopes. Inoculation procedure for some of the commonly used biochemical tests is given below: Refer to the diagrams **(Fig 9.5 to 9.8).**

For inoculation of glucose, lactose, sucrose, mannitol i.e. sugars take a single well isolated colony with a straight wire and inoculate by rubbing the wire on to the sidewall

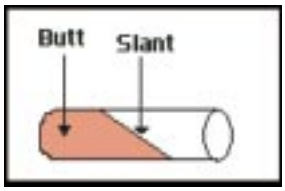
of the tube after tilting it slightly so that the point of rubbing remains underneath the liquid. (refer the Fig 9.7) If liquid peptone water culture is available then a small drop may be added in aseptic manner after flaming the neck of the sugar tubes.. For inoculation of slope media (solid) like urea and citrate, inoculate a colony or peptone water culture in wavy manner over the slope (see Fig 9.8). For TSI and LIA media one has to inoculate in similar manner as stated above and in addition a stab should be made using a straight wire. (refer to the Fig 9.6).For TSI single stab is enough and for LIA two stabs are required.

9.11.1 Observations of biochemical tests following overnight incubation

9.11.1.1 Glucose

Pink colour with or without gas production in durham's tube is suggestive of positive test & indicates that glucose has been utilized/fermented. Similarly lactose, mannitol, sucrose etc. change colour to pink on fermentation of respective sugars.

INOCULATION OF BIOCHEMICAL TESTS



TSI - Medium

Fig : 9.5

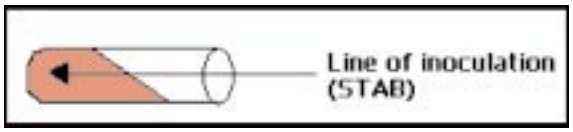


Fig : 9.6

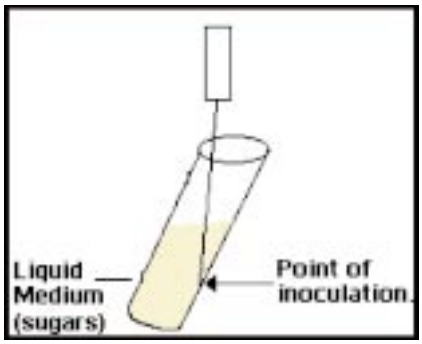


Fig : 9.7

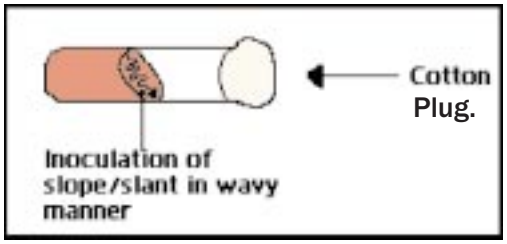


Fig : 9.8

- **Glucose / lactose / sucrose / mannitol:** Take a single well isolated colony with a straight wire loop and inoculate by rubbing the wire on to the side wall of the tube after tilting it slightly so that point of inoculation remains underneath the liquid. (fig : 9.7)
- **Urea / citrate:** Take with a straight wire and rub over the slope (Fig: 9.8)
- **TSI :** Inoculate over the slope and stab once (Fig: 9.8 & 9.6)
- **LIA :** Inoculate over slope and stab twice (Fig: 9.8 & 9.6)
- **NA Stab:** Stab twice (Fig: 9.6)

9.11.2 Triple sugar Iron agar test (TSI)

- The colour of uninoculated medium is orange red.
- Acid slant / acid butt: (colour remains as yellow / yellow) means lactose and or sucrose has been fermented along with glucose.
- Alkaline slant / acid butt : (pink slant / yellow butt) - glucose only has been fermented.
- Blackening of butt: H_2S has been produced usually shown by either Salmonella or Proteus species.
- Bubbles in butt or slant: Gas formed during fermentation
- Orange red: uninoculated TSI slant.

9.11.3 Phenylalanine deaminase test (PPA)

- Development of dark green colour on addition of few drops of 10% ferric chloride indicate a PPA positive test & s/o Proteus or Providentia sps.which are non pathogenic commensals in human intestine.

9.11.4 Urease test

- If colour changes to pink (pl refer to fig. 9.10): s/o Proteus or klebsiella sps.

9.11.5 Indole test

Development of red ring at the interphase of liquid culture and reagent on addition of p- diethyl amino benzaldehyde (Indole reagent) to overnight peptone water culture is suggestive of E.coli or other indole positive bacteria.

9.11.6 String test

This is one of the diagnostic test for cholera. Take a drop of 0.5% sodium desoxycholate on a clean glass slide. Then add a speck of culture with loop and lift the

loop. A string is formed if positive for cholera. (refer toFig.- 9.12).

9.12 Antibiotic Sensitivity testing

Antibiotic sensitivity testing of the pathogenic isolates should be done by Kirby Bauer's technique using Mueller Hinton agar plate. Appropriate quality control strain like E. Coli ATCC 25922 must be put up each time. Refer to the chapter 17 on antibiotic sensitivity.

9.13 Good Lab Practice (GLP)

- While taking rectal swab specimens, the swab made of absorbent cotton should be swirled into the rectum so as to have visible material on the swab.
- Swab stick should be properly made with cotton so that while taking rectal swab cotton should not come out of the stick.
- Use of freshly prepared dehydrated culture media is highly recommended. Only media less than one-week-old which is stored properly in fridge should be used.
- It is a good practice to check growth supporting quality of various solid media (batch wise if possible) like XLD - agar, MacConkey's agar etc by inoculation of known lactose fermenter / and lactose non fermenter / ATCC Strains / known pathogens etc so as to do quality control check of dehydrated culture media. Also after preparation of media one of two plates should be incubated at 37 degree centigrade so as to check sterility conditions.
- Distilled water should always be used while preparation of media.
- In case cholera is suspected, stool / rectal swab specimens should not be kept in freezer (as is usually the practice for suspected shigella / salmonella gastroenteritis) - rather keeping such specimens at room temp is a better option till the specimens are processed or transported to the nearby reference laboratory.

9.14 Quality control of media

Every batch of media should be checked for sterility and growth supporting properties of ATCC and locally available pathogenic isolates from local laboratories. Similarly every biochemical test should be checked with known positive and negative controls.

Table 9.1 Interpretation of Results

S. No.	Glucose	Lactose	Sucrose	Mannitol	Urea	Indole	Citrate	PPA	TSI	Provisional Identity
1.	AG	+	+/-	+	-	+	-	-	A/AG (-)	Esch.coli
2	AG	+	+/-	+	+	-	+	-	A/AG (-)	Klebsiella
3.	AG	-	+/ (-)	+	+	+/-	+/-	+	A/AG (+/-)	Proteus Providencia group
4.	AG	-	-	+	-	-	+	-	K/AG (+)	Salmonella
5.	A	-	-	+	-	-	-	-	K/A (-)	Shigella
6.	A	-	+	+	-	+	NA*	-	A/A (-)	V.Cholerae

Key: A: Formation of acid only due to fermentation of corresponding carbohydrate like glucose, lactose, mannitol, sucrose etc. pink colour develops.
AG: Formation of acid (colour changes to pink) and gas (in durhams tube)
+/-: Fermentation may be present or absent
TSI (-): H_2S not produced. Salmonella paratyphi A is H_2S negative.
TSI (+): H_2S produced leading to blackening of butt.
Motility (+): Motile bacteria
Motility (-): Non-motile bacteria.
* Not applicable

9.15 Interpretation of stool culture

Any pathogen isolated from stool culture should be interpreted in light of the fact that there are many commensals/normal flora (like Esch. coli, Klebsiella, Proteus, pseudomonas, streptococci) which are not to be reported as they are non-pathogens, unless in special circumstances like in infants and small children where E coli (EPEC AND EHEC- types) sometimes assumes pathogenic role. Hence all the results of stool cultures should be clinically correlated for proper interpretation.

9.16 Biosafety

Treat all stool specimens as potentially infectious material and observe proper handling procedure / biosafety procedure (refer to chapter on biosafety).In short all used laboratory cultures like used tubes., media, bijou bottles, sugar tubes, plates need to be decontaminated (autoclaved) prior to washing, sterilize and reuse.

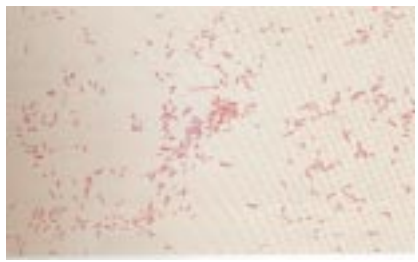


Fig: 9.9 Gram staining showing Gram negative bacilli (1000x)

Fig: 9.10 Pink colour development indicates positive usease test

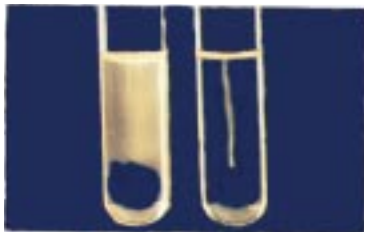
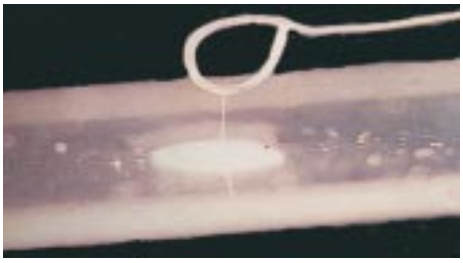


Fig:9.11 Motile bacteria : whole medium becomes hazy with growth of bacteria.
Non-motile bacteria: medium remains clear except growth at line of inoculation



Fig; 9.12 positive string test

Fig:9.13 Oxidase test: Development of purple colour indicates the test is positive

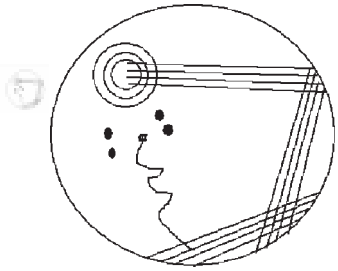


Fig: 9.14 Plating technique

CHAPTER -10

LABORATORY DIAGNOSIS OF INTESTINAL PARASITES

10.1 Protozoa

Protozoa are microorganisms consisting of a single cell. These can be found in stools in their motile form (Trophozoites) or as cysts. Some intestinal protozoa are pathogenic, other are harmless. The motility of trophozoite forms is either because of slow movement of the cell (Amoebae) or because of the presence of rapidly moving flagella or cillia. The common pathogenic human protozoa are as given below

1) Entamoeba histolytica
2) Giardia lamblia.
3) Balantidium coli.

10.2 Helminths

Helminthic infections cause a variety of clinical symptoms including abdominal cramps, fever, weight loss, vomiting, appendicitis etc. There are three groups of medically important helminths : i.e. Nematodes (round worms), cestodes (tape worms) and trematodes (flukes).

Helminthic infections are usually diagnosed by detecting eggs and larvae. Less frequently, infections are diagnosed by detecting adult worms (.e.g. Ascaris lumbricoides and Enterobius vermicularis) or proglottids of adult worms (e.g. T. saginata and T. solium).

The characteristics used to identify eggs of different helminths are the size, shape, colour, thickness of the egg shell and the other specific characteristics.

10.3 Examination of Stool Specimens For Parasites

Collect at least a spoonful quantity of faeces in a clean, dry, preferably screw capped transparent container without preservatives and send to laboratory immediately taking the following precautions.

- ⇨ Never leave stool specimens exposed to air in containers without lids
- ⇨ Never accept stool specimens mixed with urine.
- ⇨ Never examine stool specimens without putting on gloves.
- ⇨ Always examine specimens with in 1-4 hours of collection. If several specimens are received at the same time, examine the liquid stools and those containing mucus or blood first, as they may contain motile amoeba.

10.4 Examination

10.4.1 Naked eye: To look for color, consistency, presence of blood or exudates. The color can be black (occult blood), brown, pale yellow (fat) or white (obstructive jaundice), The consistency can be formed (normal), soft formed or watery (diarrhoeic). The presence of external blood or mucus, usually seen as streaks of red or white are indicative of either ulcerative colitis, schistosomiasis or amoebiasis.

10.4.2 Microscopic examination

- ⇨ Direct microscopic examination of stool/ faeces in saline or iodine is useful for the following reasons: i.e. to detect motile trophozoites (saline ppn) and cysts (Iodine ppn)
- ⇨ Select unformed or liquid portion of stool when looking for trophozoites, specially the blood & mucus containing portion.

10.4.2.1 Materials & Reagents

- ⇨ Microscope.
- ⇨ Microscopic glass slides
- ⇨ Cover slips.
- ⇨ Wooden applicators.
- ⇨ Grease pencils / Marker pens.
- ⇨ Normal saline (0.85 % Nacl) solution.
- ⇨ Lugol's Iodine solution.

10.4.2.2 Method

1) Take a clean, dry glass slide and label it with the specimen number.
2) Wear gloves
3) Put a drop, each of normal saline and Lugol's Iodine on the middle of the either end of the slide.
4) Using an applicator stick, take a small portion (about 2-3 mm diameter) of stool as described earlier.
5) Mix the sample with the drop of normal saline.
6) Using the same applicator, take a second similar portion of stool and mix it with the drop of lugol's Iodine solution.
7) Discard the applicator in a disinfectant solution. i.e. freshly prepared 1% sodium hypochlorite solution.
8) Place a cover slip on each drop taking care to avoid the formation of air bubbles.
9) Examine the preparation under the microscope. First, examine the saline preparation for trophozoites of protozoa and ova & larvae of helminths.

Reduce the amount of light using the condenser aperture or by lowering the condenser. Examine first using 10 x objective, starting at the top left hand corner and gradually covering the whole of the area under cover slip for presence of ova and larvae. Now, switch to 40x objective and again examine the whole area of cover slip for motile trophozoites.

10) Next, examine the Iodine stained preparation for the cysts of parasites using 40 x objective.

(Addition of Iodine kills the organism and therefore motility of trophozoites is lost but it stains the nuclei and glycogen mass of the cyst / trophozoites).

11) Report the findings after looking for the characteristic morphology of different parasitic trophozoites / cysts and helminthic ova as given below. (Fig 10.1, 10.2)

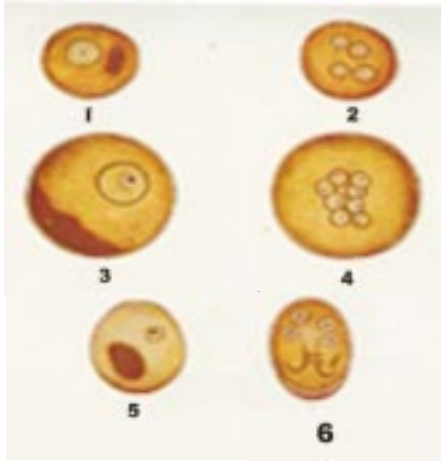


Fig: 10.1 Cysts of intestinal protoza of man (stained with Iodine) 1 and 2:Entamoeba histolytica (uni and quadrinucleate forms.3 and 4 Entamoeba coli. 5; Iodamoeba butchlii. 6: Giardia lamblia

Fig 10.2 : Helminthic eggs / ova in the stool of man. 1,2 and 4: Fertilised eggs of Asc.lumbricoides. 3: Unfertilized eggs of Ascaris. 5: Hookworm.6: E.vermicularis. 7: T.trichiura 8: Taenia solium / saginata. 9: H.nana

10.5 Dispatch of stool samples for detection of parasites

Stool samples may be sent to a specialized laboratory for the identification of rare parasites that are difficult to identify. In such cases, a preservative should be added to the specimens. e.g. 10 % formaldehyde solution.

- ⇨ Put one part of stool sample into 3 parts of 10 % formaldehyde solution in water / normal saline.
- ⇨ Mix the stool thoroughly using a glass rod.
- ⇨ Screw cap the container tight.
- ⇨ This solution preserves ova, cysts and adult worms indefinitely

10.6 Preparation of Lugol's iodine solution for wet mount

10.6.1 Requirements

A) 0.5 % lugol's iodine solution ….. **Soln A**

B) Acetic acid, 50% solution, diluted 1:1 with distilled water --- **Soln B**

Prepare a 1:1 mixture of lugols Iodine solution and acetic acid solution (diluted as above). Dilute this mixture with 4 volumes of distilled water and stir.

10.6.2 Preparation of solution 'A' (Lugol's Iodine 0.5 %)

Iodine	---------- 5 gms.
Potassium Iodide	---------- 10 gms.
Distilled water	---------- 300 ml.

Measure 300 ml of distilled water in a cylinder. First, dissolve the potassium iodide in about 30ml of distilled water. Add the iodine and mix until dissolved. Add the remainder of the water, mix well and store in a brown bottle. Label the bottle along with the date.

10.6.3 Preparation of solution 'B' (Acetic Acid 50% solution)

- ⇨ Glacial acetic acid ---------- 100 ml.
- ⇨ Distilled water ----------. q.s. 200 ml

Mix the two in a bottle. Label the bottle along with the date.

10.7 Kato-Katz Technique (for helminthic prevalence in community)

In order to find out the prevalence and intensity of helminthic infection, Kato Katz technique is used.

10.7.1 Materials and reagents

1. Kato-Katz kit

   The Kit contains

   i) A roll of nylon screen 80 mesh (20m)

   ii) 400 plastic templates with a hole of 6 mm on a 1.5 mm thick template, delivering 41.7 mg of faeces.

   iii) 400 plastic spatula.

   iv) A roll of Hydrophilic cellophane,34 µm thick (20m)

   These kits are available commercially

2. Microscopic slides
3. Flat bottom jar with lid.

4. Forceps.
5. Toilet paper or absorbent tissue.
6. Newspaper.
7. Glycerol-malachite green solution or glycerol-methylene blue solution. Method of preparation is as below:
   - (Glycerol…… ……..100ml
   - 3% aqueous malachite green, or

     3% aqueous methylene blue…… …… …….. …. 1 ml.
   - Distilled water…… …… 100ml.

     Grind some malachite green or methylene blue powder with a pestle in a clean,dry mortar, Weigh out 3 gm. Of the powder, pour it into a bottle and add distilled water to give 100ml. Seal and label the bottle: 3% aqueous malachite green or 3% aqueous methylene blue. Store in a cabinet away from light.

     To prepare the solution: pour 1 ml of the 3% aqueous solution into a 250 ml. bottle. Add 100 ml. of glycerol and 100 ml. of distilled water and seal the bottle; mix thoroughly before use).

10.7.2 Methodology

1. Place a small amount of faecal material on a newspaper/scrap paper and press the small screen on top of the faecal material so that some of the faeces will be sieved through the screen and accumulate on top of the screen.
2. Scrape the flat-sided spatula across the upper surface of the screen so that the sieved faeces accumulate on the spatula.
3. Place template with hole on the centre of a microscope slide and add faeces from the spatula so that the hole is completely filled.
4. Remove the template carefully from the slide so that the cylinder of faeces is left on the slide.
5. Cover the faecal material with the pre-soaked cellophane strip.
6. Invert the microscope slide and firmly press the faecal sample against the hydrophilic cellophane strip on another microscope slide or on a smooth hard surface such as a piece of tile.
7. Carefully remove slide by gently sliding it sideways to avoid separating the cellophane strip or lifting it off. Place the slide on the bench with the cellophane upwards. Water evaporates while glycerol clears the faeces.
8. The smear should be examined in a systematic manner and the number of eggs of each species noted separately.

10.7.3 Quality control for microscopy

1. Quality control is insured to verify the consistency of the microscopic readings. For this one day before the survey is spent on evaluating the consistency of

egg counting among the laboratory technicians. A simple method consists of preparing 10 slides and comparing the reading of each slide by each laboratory technician with that of the quality manager. A discrepancy of 5-10% for egg per slide count is normal, but if the discrepancy is larger, the testing is not valid and reasons must be identified and corrected. If one of the technicians presents readings, which are consistently different to those of others, he/she should be excluded from the team. An accurate egg per slide count is particularly important for the Kato-Katz technique for intensity assessment.

2. Besides this, on each day of the survey, one should read 10% of the slides of each technician without prior knowledge of the results independently. In the case of the discrepancy of more then 10%, the slides should be discussed by the two readers. And then further slides may be examined to avoid repeated errors.

10.7.4 Advantage of Kato-Katz technique are as follows

- In Kato-Katz technique a specified quantity of stool is examined, in contrast to STH survey carried out by other methods, where the quantity of stool examined may vary in each slide/sample. Hence any other concentration method is not suitable for comparison of worm load in the different community and further across country estimation.
- Kato-Katz technique is better than other technique for viewing STH.

10.7.5 Community categories for community diagnosis

Community category	Criteria for classification	
	% of children positive for worm	% of children with heavy intensity infection
I	>70%	10% or more
II	50-70%	Less than 10%
III	Less than 50%	Less than 10%

10.7.6 Community intervention for control of STH

Community category		Proposed intervention
I	High prevalence, High intensity	Treatment 2-3 times a year targeted to all school-age children, IEC, improvement in sanitation, water supply and appropriate waste management
II	High prevalence, low intensity	Treatment targeted to all school-age children at least once a year, IEC, supporting improvement in sanitation, and waste management
III	Low prevalence, low intensity	Case management, IEC, improvement in sanitation, water supply and appropriate waste management

CHAPTER-11

LABORATORY DIAGNOSIS OF DIPHTHERIA

Diphtheria is an acute bacterial infection of the upper respiratory tract especially in children. The disease begins with a sore throat and fever, followed by general malaise and swelling of the neck. The disease is caused by Corynebacterium diphtheriae, which is a gram positive, nonendospore forming pleomorphic rod. The dividing cells are folded together to form V- and Y shaped figures resembling chinese letters. Presence of polyphosphate metachromatic granules is a characteristic feature of this bacterium. With simple stains e.g. methylene blue, the bacilli often have a beaded or barred appearance. With Alberts stain, the metachromatic granules stand out purple black against the green stained protoplasm. Characteristically, C. diphtheriae causes a typical form of infection manifested by a greyish white membrane at the site of localization of organisms.

Though, the diagnosis of diphtheria is mainly clinical, laboratory is required to confirm the diagnosis, which is based on demonstration of the causative organism in the lesion. A presumptive diagnosis can be made on the basis of demonstration of the bacilli in the affected lesion by microscopic examination only, however confirmation is only done by the culture examination.

11.1 Collection and transportation of specimens

In suspected cases, swabs should be taken both from the throat and nose by a trained personnel as per the procedure given below.

11.1.1 Material Required

- Sterile cotton swab sticks in test tube.
- Tongue depressor.
- Adequate source of light.

11.1.2 Procedure

- Ask the patient to open his mouth without putting out his tongue and to say Ahhh-----.
- While the patient is saying Ahhhh--- press the outer two third of the tongue with the tongue depressor using the left hand, making the tonsils and back of the throat visible.
- Introduce the swab with the right hand avoiding touching the tongue.
- Locate the inflamed part of the throat which is red or white.
- Rub the swab firmly against the inflammed part, turning it round and collect membrane, if present.

- If nothing abnormal is seen, swab the tonsils, the fauces and the back of the soft palate.
- Collect two swabs and immediately place the same in sterile test tubes.
- Similarly, collect nasal swabs in duplicate.
- Specimens should be transported and processed as soon as possible preferably within 48 hours. In case of delay store the samples at 2-8°C

11.2 Processing of swabs

- In the absence of culture facilities, streak this swab on Loefflers serum slope which can be used as a transport medium. If feasible, this medium can be prepared in the district laboratory itself as described in subsequent pages, Alternatively, it can be prepared and supplied by the state laboratories. The inoculated transport media should be sent to state/ regional labs for culture and other tests.
- With the second swab streak two clean glass slides using a rolling movement to make sufficiently thick smears.
- If there is only one swab, it is mandatory to first inoculate the transport media and then prepare the smear to avoid contamination.

11.3 Staining of smears

- In all cases, stain one of the smear with gram stain as described in chapter -6.
- If gram stained smear shows morphology suggestive of C. diphtheriae, proceed to do Albert's staining (Refer to chapter - 6) which demonstrates the presence or absence of metachromatic granules (Fig- 11.1).

  Alternatively both staining may be carried out simultaneously.

These organisms have to be differentiated from commensal bacteria in throat called diptheroids which are thick and short in appearance and do not contain metachromatic granules. These also do not exhibit chinese letter arrangement. These exhibit a strong gram positive reaction as compared to C.diphtheriae organisms which are weakly gram positive. C. Diphtheriae show pleomorphism.

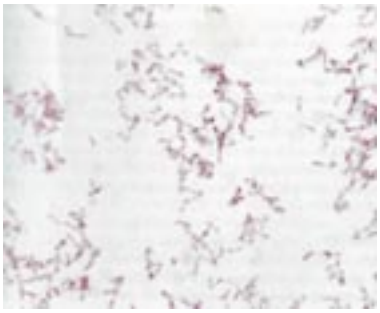


Fig- 11.1 Morphology of C.diphtheriae

11.4 Reporting of results

It could be one of the followings:

- ⇨ No C. diphtheriae like organism seen.
- ⇨ Morphologically suggestive of C.diphtheriae.

11.5 Quality assurance

Quality control checks on stains and media are to be performed with known positive and negative organisms. Use Staphylococcus epidermidis as the negative control and known C. diphtheriae as the positive control strain.

11.6 Other significant organisms isolated from throat

Throat swabs contain a variety of microorganisms including saprophytic Nesseria sps, Neisseria meningitidis, which is a gram negative diplococcus, an important organism which could cause disease or be present in a carrier state. Haemophilus influenzae is another important organism present in throat. Beta haemolytic streptococci, Staphylococcus aureus and candida sps. are also frequently encountered. Beta haemolytic streptococcus is the most common cause of sore throat in children.

11.7 Important points about diagnosis of diphtheria

- ⇨ Diagnosis of diphtheria is a laboratory emergency and deserves utmost priority.
- ⇨ Mild cases of diphtheria resemble pharyngitis and a pseudomembrane may be lacking.
- ⇨ Diphtheria is no longer diagnosed easily on clinical grounds.
- ⇨ Even non-toxigenic strains of C.diphtheriae are important. These have the potential to cause invasive disease in some patients and also the potential to undergo lysogenic conversion to toxin producers in vivo leading to disease.
- ⇨ Specimen should be transported and processed as soon as possible.

11.8 Biosafety

1. All work on suspected C.diphtheriae isolates which is likely to generate aerosols must be perfomed in a safety cabinet or in an inoculation hood.
2. All other laboratory biosafety procedures as outlined earlier should be adhered to.

11. 9 Preparation of Loefflers Serum slope

11.9.1 Requirements

- Sterile serum of ox / sheep---------- 300 ml.
- Nutrient Broth pH7.6 ---------- 100 ml.
- Glucose ---------- 1 gm.

11.9.2 Method

- Dissolve the glucose in the broth and sterilize by autoclaving at 120°C for 15-20 min.
- Add the glucose broth to the serum with sterile precautions, mix thoroughly and distribute 2.5 ml amounts into sterile screw capped 6 ml bijou bottles and apply the caps tightly.
- For inspissation / coagulation of the medium, lay the bottles on their side on a tray, sloped slightly to prevent the medium running up to the cap/ lid. Place the tray in a hot air oven. Raise the temperature slowly to 80°C and maintain it for 2 hours, till the serum would be coagulated to a yellow white solid matter.
- Allow the slopes to cool. They should have a moist surface and a small amount of condensation water at the bottom. Properly capped against drying, they can be stored for long periods.

CHAPTER - 12

LABORATORY DIAGNOSIS OF PYOGENIC MENINGITIS

Bacterial/pyogenic meningitis, an infection of the membranes (meninges) and cerebrospinal fluid (CSF) is a major cause of death and disability worldwide. Beyond the perinatal period, there are three principal organisms responsible i.e. Neisseria meningitidis (Menningococcus), Streptococcus pneumoniae (Pneumococcus) and Haemophilus influenzae. The etiology varies with age group and geographical location. The other less commonly involved agents are Staphylococci, Escherichia coli and Listeria monocytogenes. Meningococcus is the commonest etiological agent in young adults and responsible for outbreaks / epidemics of meningitis, whereas in extremes of age it is pneumococcus. H. influenzae causes meningitis exclusively in young children under 5 years of age, the vast majority of infections being caused by sero group B. Likewise in our country, commonest serogroup involved in Meningococcal meningitis is 'A' followed by 'C' out of the 13 serogroups known. Following the establishment of nasopharyngeal colonisation, illness results once bacteria invades the mucosal defenses, thus accessing the blood stream and eventually reaching the meninges and CSF. Early diagnosis of meningitis is very much essential to initiate appropriate intervention for the patient as well as the community.

12.1 Laboratory diagnosis

Mainly based on examination of the following clinical samples.

12.1.1 CSF for – Biochemistry for protein, sugar.

- Microscopic examination and cytology .
- Rapid antigen detection.
- Culture.

12.1.2 Blood for culture

Out of the above, examination of CSF is more informative and a much more sensitive investigation, and except for culture, all other tests on CSF can/ should be performed in a district laboratory.

12.2 Collection of CSF

- Should be collected by a physician / paediatrician or a specially trained nurse.
- Should be collected preferably before antimicrobial therapy.
- Using all aseptic precautions, the sterile lumbar puncture needle is inserted between the 4th and 5th lumbar vertebrae to a depth of 4-5 cms. The stylet is withdrawn to let the fluid flow freely.

- Collect 1-2ml of CSF each in 3 sterile screw capped containers (Bijou bottles) to be examined for Chemistry, Cytology and Microbiology respectively.
- Never pipette CSF by mouth.
- Never freeze or refrigerate the CSF in suspected pyogenic meningitis.
- Send the CSF to laboratory as early as possible preferably within an hour.
- In case the CSF is to be sent for culture, keep it in incubator at 37 ^{o}C or use a transport medium (Stuart's modified medium).

12.3 Stuart's Modified Medium

- Is commercially available
- Is supplied in 15-30 ml bottles containing 8ml solid medium. The bottles are filled with a mixture of air (90%) and carbon-dioxide (10%).

12.4 Method of Inoculation

- Unscrew the cap of bottle
- Holding the bottle as upright as possible, (to prevent the gas escaping), inoculate the whole surface of media with CSF using a sterile pasteur pipette.
- Replace the cap immediately. Dispatch the bottle at ambient temperature immediately.

12.5 Examination of CSF

12.5.1 Direct examination (Naked eye)

12.5.1.1 Describe the appearance of the CSF i.e. clear and colourless CSF (normal in aseptic meningitis), or cloudy CSF (due to presence of cells as in pyogenic meningitis)

12.5.1.2 Clot formation

Examine the CSF, 10 min after collection for the presence of clots. Normal CSF has no clots, but clots may be found in the following conditions:

- Pyogenic meningitis: (a large clot.).
- Tuberculous meningitis: single or numerous fine clots are formed.

12.6 Biochemical examination of CSF

CSF should be examined for levels of glucose and protein.

12.6.1 Glucose

- Glucose concentrations in the CSF are normally about 60% of those in the blood. i.e. 50-80 mg/dl.

- In patients with pyogenic meningitis and tuberculous meningitis, the concentration of glucose in the CSF is markedly reduced.
- As the glucose in CSF is rapidly destroyed, once the fluid is collected, it is important to carry out the estimation as early as possible. In case of delay, the CSF should be preserved in fluoride/ oxalate bottles.
- (Refer to chapter - 23 **(section 23.4)** for detailed procedure).

12.6.2 Protein

The normal concentration of protein in the CSF is 15-45mg/dl. The protein concentration is increased in bacterial meningitis.

12.6.2.1 Determination of Protein concentration : Refer to Chapter 23

The normal concentration of protein in the CSF is 15-45 mg/dl. The protein concentration is increased in bacterial meningitis, subarachnoid haemorrhage and cerebral malaria.

Table: 12.1 Characteristics findings on examination of CSF in different clinical conditions

Disease/Condition	Appearance of CSF	Glucose level	Protein level	Leukocyte concentration
Pyogenic meningitis	Cloudy yellowish	Markedly reduced	Highly elevated (100-1000 mg/100ml)	>3000 cells/cmm mainly polymorphs
Tubercular meningitis	Clear/Almost clear	Markedly Reduced	Elevated	30-300 cells/cmm mainly lymphocytes
Viral meningitis	Clear	Normal	Normal or slightly raised	10-300 cells/cmm mainly lymphocytes
Cerebral malaria	Slightly Cloudy	Reduced	Elevated	Elevated mainly granulocytes

12.7 Microscopic Examination Of CSF includes

12.7.1 Cytology : Determination of the leukocyte number concentration

12.7.1.1 Material and Reagents

- Microscope
- Fuchs-Rosenthal counting chamber (if not available, an improved Neubauer counting chamber may be used)
- Pasteur pipette with rubber teat.
- Coverslips (supplied with the counting chamber)
- Bottle, 2-5 ml.
- Turk's solution (described below).

Ppn of Turk's Solution

Glacial acetic acid	4 ml
Loeffler's methylene blue solution	10 drops

Distilled water q.s 200 ml

Dissolve the glacial acetic acid in 100 ml of distilled water. Add the methylene blue solution and mix. Transfer the mixture to a 200 ml volumetric flask and make the volume to 200 ml with distilled water.

12.7.1.2 Method

1. Cover the counting chamber with the coverslip supplied (Fig 12.2)
2. Gently mix the CSF and fill the chamber with the fluid (Fig 12.3)
   - Undiluted, if the CSF appears clear;
   - Diluted, if the CSF appears cloudy.

   Make a 1:20 dilution using 0.05 ml of the CSF and 0.95 ml of Turck's solution. Pipette into a small bottle and mix.
3. Leave the counting chamber on the bench for 5 minutes to allow the cells to settle. Place the chamber on the microscope stage.
4. Count the cells in 1 mm^3 of CSF, using the 10x objective. When reporting in SI units, report as "number x $10^6/L$", the value does not change.

   Example: 150 cells per mm^3 are reported as "150 x $10^6/L$

Fig: 12.1

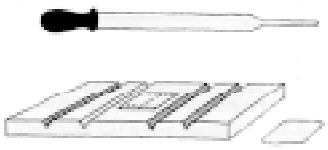


Fig: 12.2

Fig: 12.3

Fig: 12.4

Important: If undiluted CSF is used, examine the cells using the 40x objective to make sure the cells are leukocytes. If erythrocytes are present, make the count using the 40x objective.

12.7.2 Examination of a stained smear for bacteria

- Examination of a gram stained smear for bacteria causing meningitis.
- Examination of a Ziehl-Neelsen stained smear for AFB if tuberculous meningitis is suspected.

12.7.2.1 Gram stained smear

- ⇨ Centrifuge the CSF for 20 minutes at 1500-2000 rpm.
- ⇨ Prepare smear by placing 1-2 drops of sediment on a clean grease free glass slide, allowing drops to form one large drop. Do not spread fluid, nor use too heavy a concentration of sediment.
- ⇨ Air dry the slide.
- ⇨ Pass the slide quickly through a flame three times to fix the smear. Alternatively, fixation by methanol (95%) can also be done
- ⇨ Do not heat fix the smear until completely dry.
- ⇨ Stain the fixed smear by standard grams staining method as described earlier----chapter - 6)
- ⇨ Examine the stained smear microscopically using a bright field condenser and an oil immersion lens

N. Meningitis may occur intra or extracellularly in the polymorpho nuclear leukocytes and will appear as gram negative, coffee bean shaped diplococci, as given in figure 12.5. S.pneumoniae are lanceolate, gram positive diplococci sometimes occurring in short chains. H. influenzae are small, pleomorphic gram negative rods or coccobacilli with random arrangements.

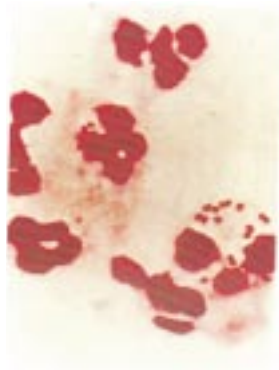


Figure 12.5 Gramstained smear showing intra/extracellular Meningococci

12.8 Antigen detection in CSF by particle agglutination.

12. 8.1 Several commercial tests based on Latex agglutination and Co-agglutination principle are available

Most of the available commercial tests are designed to provide diagnosis for meningitis caused by:

- N. meningitidis serogroup 'A'.
- Streptococcus pneumoniae.
- N.meningitidis serogroup 'C'.
- Haemophillus influenzae type 'B'.

Following are the general recommendations and instructions for detection of soluble bacterial antigens.

1) Store the reagents at 2°C to 8°C, latex suspensions should not be frozen.
2) Test the supernate of the centrifuged CSF sample as soon as possible.
3) In case of delay, refrigerate the sample at 2°C to 8°C, upto several hours or at -20°C for longer periods.
4) Follow the kit manufacturer's instructions precisely when using these tests.

12.8.2 Materials Required (But not supplied with the kit)

- ⇨ Pasteur pipettes (Sterile)
- ⇨ Rubber teats.
- ⇨ Container with disinfectant (for discard)

12.8.3 Performance of the test

- ⇨ Heat the supernatant of the CSF in a boiling water bath for 5 minutes.
- ⇨ Shake the Latex suspension gently until homogenous.
- ⇨ Place one drop of each latex suspension on a rinsed glass slide or a disposable card.
- ⇨ Add 30 - 50µl of CSF to each suspension.
- ⇨ Rotate by hand for 2-10 mts. Mechanical rotation at 100 rotations per minutes if available is recommended.

12.8.4 Reading the test results

Read under a bright light without magnification.

12.8.5 Negative reaction

The suspension remains homogenous and slightly milky in appearance.

12.8.6 Positive reaction

Visible clumping of the latex particles within 2 mins.

12.8.7 Interpretation

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

12.8.8 Controls : Periodically check for

a) That none of the latex reagents agglutinate in the presence of normal saline.
b) That each of the latex reagents do agglutinate with respective positive controls.

CHAPTER - 13

LABORATORY DIAGNOSIS OF ENTERIC/TYPHOID FEVER

Enteric /typhoid fever is a septicaemia caused by Salmonella sps. In our country it is caused mainly by Salmonella typhi and less frequently by Salmonella paratyphi 'A'. It manifests in the form of fever (step ladder rise) accompanied with other symptoms like loss of appetite, pain abdomen and constipation etc.

13.1 Laboratory diagnosis is based on

1) Isolation of the causal organism from the blood,faeces or urine of the patient by culture examination.
2) Demonstration of anti-salmonella antibodies in the patients serum by a serological test, which can be either the age old conventional widal test or the recently introduced typhidot test

13.1.1 Blood culture

Out of the many clinical samples that can be used for culture, blood culture is the most informative. Blood should be collected aseptically (ref. to chapter-4) in at least 5-10 ml quantity by venepuncture preferably during the first 7-10 days of illness before starting chemotherapy and added to a blood culture bottle containing 50-100 ml bile salt broth. Transport the inoculated broth as early as possible to the state laboratory for further processing. In case of delay, refrigerate the bottles.

Alternatively, clot culture can be performed as given below.

- Collect 5-10 ml of venous blood asceptically in a sterile screw capped container.
- Allow blood to clot.
- The serum is removed with a sterile pasteur pipette for carrying out the serological test.
- The clotted blood can be broken either with sterile scissors or glass rod and inoculated into bile salt broth.
- Alternatively, clot can be digested with sterile streptokinase solution (100 units/ml) and then inoculated into bile salt broth as before.

13.1.1.1 Preparation of Bile salt broth

The bile salt broth can be procured commercially in a dehydrated form or can be prepared in the laboratory as given below.

– Nutrient Broth ---------- 1 litre
– Bile salt (Sodium taurocholate)------- 5 gm.

Adjust the pH of the nutrient broth to 7.6 and dissolve the bile salt in it, autoclave for 15 minutes at 115°C, 10lbs pressure and distribute in 50-100 ml amount in presterilised blood culture bottles.

13.1.2 Serological Tests

There are two types of serological tests

- Rapid typhidot test
- Widal Test.

The kits are commercially available in our country and one should strictly follow the manufacturer's protocol.

13.1.2.1 Typhidot Test

Recently, with the availability of typhidot test,a reasonably sensitive (>95%) and specific (85-90%) diagnosis of fever caused by Salmonella typhi can be made in a short time (within 1 hour of collecting the sample). The test can be performed using a single sample of blood/serum. The test can be easily performed in a district laboratory without the need of any sophisticated equipments. Invented in Malaysia, typhidot is a qualitative antibody detection test designed for the rapid diagnosis of enteric (typhoid) fever. The presence of IgM and IgG antibodies made against a specific antigen on the outer membrane of salmonella typhi are detected by incubating nitrocellulose strips dotted with the specific antigen protein with the patients sera and control sera.

To visualise the antigen-antibody complex, the strips are simultaneously incubated with peroxidase-conjugated antihuman IgM & IgG upon addition of the chromogenic substrate, the results can be read visually. Positive reading is indicated by the blue colour as intense or more intense than that of the positive control. Total assay time is 1 hour.

13.1.2.1.1 Test Protocol

13.1.2.1.2 Reagents and Material

13.1.2.1.2.1 Provided with the kit

TYPHIDOT contains reagents and antigens dotted strips for detection of specific IgM and IgG antibodies to Salmonella typhi, inclusive of controls. Use of sterile disposable pipette tips is recommended. Do not use kit beyond expiration date and do not mix reagents from different batch numbers.

TYPHIDOT

Contents	28 Tests	56 Tests
Predotted antigen strips	56 Strips	112 Strips
Samples diluent (A1)	15 ml	30 ml
Washing buffer (10x) (A2)	10 ml	20 ml
Prediluted anti-Human IgM *HRP (B1)	7.3 ml	14.6 ml
Prediluted Anti-Human IgG *HRP (B2)	7.3 ml	14.6 ml
Colour reagents Substrate A (C1)	5.5 ml	11 ml
Substrate B (C2)	15.5 ml	31 ml
Positive control	60 ul	120 ul
Negative control	60 ul	120 ul
Worksheet	2	4

Note: Store kit at 2-8 ^{0}C. Allow kit to warm to room temperature (minimum 23 ^{0}C) before doing the test.

13.1.2.1.2.2 Materials required but not provided

- Measuring cylinder (100ml).
- Micropipettes (2-20 & 1000 ul) and sterile micropipette tips.
- Small conical flask.
- Forceps.
- Wash bottles.
- Filter paper.
- Distilled water.
- Rocker platform (optional).
- Gloves.
- Discarding jar containing suitable disinfectant.
- Aspirator.
- Aluminium foil.
- Conical flask covered with aluminium foil or a dark reagent bottle.

13.1.2.1.2.3 Preparation of Reagents

13.1.2.1.2.3.1 Washing buffer (10x)

Dilute washing buffer into 90 ml of distilled water to a final concentration of 1x. The diluted washing buffer is sufficient for the entire kit. Store it separately at 2-8°C and use when necessary.

10X (A2)	Distilled water	1X
10 ml	90 ml	100 ml

Store in a clean bottle at 2-8°C

13.1.2.1.2.3.2 Colour Development Reagents

Prepare 30 minutes before use. Substrate A (C1) and substrate B (C2) should be brought to room temperature before mixing. Avoid exposing these reagents to strong

light during incubation or storage. Based on the number of tests (including controls), add the recommended volume of substrate B into a reagent bottle of a flask covered with aluminium foil. Then add the recommended volume of substrate A and mix well, as given below:

No. of test (including controls	3	6	9	12	15	18	21	24	27	30
Substrate A (in ml)	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5
Substrate B (in ml)	1.25	2.5	3.75	5	6.25	7.5	8.75	10	11.25	12.5

13.1.2.1.3 Procedure

1) Divide the reaction tray into columns. Mark one column as 'M' and another column as 'G'.

2) Using forceps, remove the predotted antigen strips and place them with the marked slide up onto a filter paper. Align all antigen strips with the marked side on your right. Using a ball-point pen, for each serum sample, label one strip as M and other as G. Perform similarly for positive and negative controls.

   Note : Make sure that there is an IgM and an IgG strips (2 strips) for each patient or control serum.

3) Add 250 ul of sample diluent into the appropriate 'M' and 'G' reaction wells. Place the 'M' or 'G' strips into the appropriate reaction wells. Shake tray gently to allow strips to be thoroughly wet.

4) To the appropriate 'M' or 'G' reaction well, add 2.5 ul of either control or test serum to achieve a final serum dilution of 1:100. Gently aspirate the solutions to mix. To avoid cross-contamination, use a new sterile disposable piptette tip for each specimen. Make sure all membranes are with the marked side up, fully immersed in the first antibody solution.

5) Incubate at room temperature on a rocker platform (optional) for 20 minutes. Shake the tray gently, every 5-10 minutes if a rocker platform is not available.

6) Aspirate the first antibody solution into a discard jar containing a disinfectant. Add 250 µl of prepared washing buffer into each well and wash 3 times, each for five minutes.

7) Using sterile micropipettes tips, add 250 ul of prediluted anti-Human IgM (B1) into the 'M' well and 250 µl of prediluted anti-Human IgG (B2) into the 'G' well.

8) Cover the tray with aluminium foil and incubate for 15 minutes at room temperature (min 23^0C) on a rocker platform.

9) Aspirate the 2nd antibody solution and wash 3 times for 15 minutes as described previously in step '6'.

10) Add 250 µl of the colour development solution into each well. Cover the tray and incubator on the rocker platform.

11) Allow 15 minutes for colour development.

12) Stop the reaction by aspirating the solution and briefly rinsing the strips in distilled water (3times).

13) Place similarly coded strips for IgM and IgG for each patient and test control onto filter paper to dry. Interpret the results. (see guided interpretation charts). If interpretation cannot be done immediately, store the strips submerged in distilled water for up to 1 day.

14) After use, rinse the tray thoroughly with distilled water. Store dry for re-use.

15) For a permanent record, paste the dried membranes in the relevant position on the worksheet provided.

13.1.2.1.4 Interpretation

13.1.2.1.4.1 Basic Principles

To interpret the typhidot the colour intensity of the dots produced by the test sera must be equivalent to or greater than those of the positive control. Reading typhidot result depends entirely on your observation of the intensity of each dotted antigens after colour development. There are few very important principles to remember.

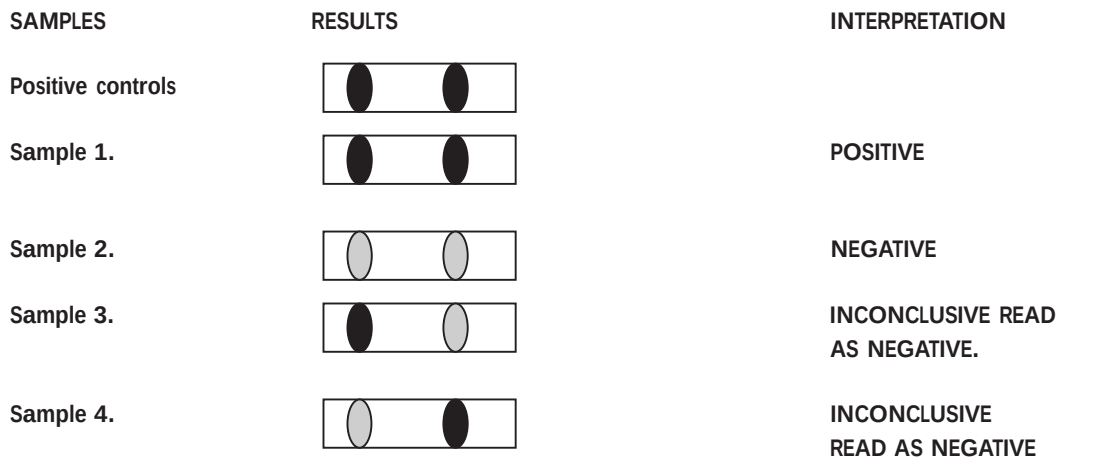
Always compare each IgM test with that of the positive IgM control strip. Similarly, compare the IgG test strips with that of the IgG positive control strips.

- When comparing colour intensity, compare the dot on the left of the test strips with the dot on the left of the positive control. Similarly, compare the colour intensity on the right of the strips (next to the line marked on the strips) with the colour intensity of the dot on the right of the positive control strip. Only when both dots on the test strips are as dark as or are darker than their corresponding dots on the positive control strips, the result is reported as POSITIVE (fig.-13.1).

- If one of the dots on the test strips is lighter compared to the corresponding dot on the positive control strips, the result should be reported as negative.

- If you are not sure i.e. borderline cases, ask for the repeat serum specimen one or two days later.

Figure - 13.1



Note: The strips should have a white background with only 2 dots appearing where applicable. The test should be repeated under the following conditions

a) If the strips show a blue background making interpretations difficult.

b) If the colour intensity of the negative control is similar to that of the positive control.

c) If the colour intensity of the test serum is high but not equal to the positive control. (Request for a second serum specimen at least 2 days later so that a higher serum titer would be available for detection).

13.1.2.1.4.2 Results and clinical interpretation

Possible results and their clinical interpretation include:

Result	Clinical interpretation
IgM positive only	Acute enteric fever
IgM and IgG positive	Acute enteric fever (in the middle stage of infection)
IgG positive	Implications for the presence of IgG antibodies include previous infection (in which case current fever may not be due to typhoid), or relapse or re-infection, therefore it is important that interpretation be made together with clinical symptoms.
IgM and IgG negative	Probably not enteric fever.

Limitation: when using typhidot, high IgG concentration may give false negative for IgM because specific IgG will drastically reduce binding of specific IgM to the antigen. We strongly recommend that in cases where Typhidot is IgG positive only, the test should be repeated using Typhidot-M to check for presence of specific IgM to Salmonella typhi. Tyhidot-M is a 3-hour dot EIA test in which the patients's serum is inactivated with a special reagent before the test is carried out for IgM detection

In case of nonavailability of typhidot test, widal test can also be performed in a district laboratory as given below.

13.1.3 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 fever.

13.1.3.1 Principle

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

In S.typhi and S.paratyphi A and B, two types of antigens are recognized as diagnostically important:

'O' antigen or 'Somatic' antigen.

'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' antigen 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 qualitative tests can be put up on suspected sera samples.

13.1.3.2 Material 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: S.Positive control	- 1 ml
Glass slide	- 1 No
Product Insert	- 1 No

13.1.3.3 Materials required, but not supplied in the kit

Small, dry and clean glass tubes

(For quantitative tube test)

Normal saline solution

Water bath

Micropipette / dropper

13.1.3.4 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 inactivate the specimen.

13.1.3.5 Test procedure

13.1.3.5.1 Qualitative slide test for screening

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

13.1.3.5.1.1 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.

13.1.3.5.2 Quantitative slide test

- 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

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

13.1.3.5.2.1 Interpretation

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

13.1.3.5.3 Quantitative tube test

- Take a set of 8 clean glass tubes, per specimen, per antigen.
- 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	1.0ml	1.0ml
Patient Serum	0.1ml							
Transfer diluted Serum	---✓	1ml---✓	1ml---✓	1ml---✓	1ml---✓	1ml---✓	1ml---✓ (discard 1ml)	1ml ---✓
Appropriate-antigen	1 drop	1 drop	1 drop	1 drop	1 drop	1 drop	1 drop	1 drop

- Mix well and incubate at 37°C for 16-20 hr, and observe for agglutination.
- Repeat steps (ii) and (iii) as shown in the table, 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 (matt).
  'H' antigen shows floccular appearance.
- Negative/saline control.No agglutination-button formation at the bottom of the tube.

13.1.3.5.3.1 Interpretation

Agglutination titre of ⩾ **1:160 (for 'O' and 'H')** is suggestive of infection. (These titres would depend upon prevalence of antibodies in the local population, and if possible, data about base-line titres should be generated and one should not go according to manufacturer's instructions).

13.1.3.6 Factors affecting Widal test

13.1.3.6.1 Effect of antibiotic administration

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

13.1.3.6.2 Effect of past infection or typhoid vaccination

- It hes been seen that the 'H' antibodies persist for a long time upto many years after typhoid vaccination. Also, many years after recovering from enteric fever, any gram-negative bacterial infection can trigger a Salmonella 'H' antibody production, thereby giving a false positive result in widal test.
- Cross reaction of 'O' antigen with other enteropathogens such as proteus species.

13.1.3.6.3 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 test result, whereas in the same patient, a sample collected during the second or 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 - 14

MICROSCOPIC EXAMINATION OF SPUTUM SPECIMENS FOR ACID FAST BACILLI (AFB)

Tuberculosis is caused by Mycobacterium tuberculosis, which is an acid fast bacillus (AFB). The highest priority for tuberculosis (T.B.) control is the identification and cure of infectious cases. i.e. patients with sputum smear positive pulmonary T.B. The highest priority in the diagnosis of T.B. is thus given to sputum microscopy. Acid fast bacilli are approximately 1-10 µm long, slender rod-shaped bacilli which may be curved or bent. These may be granular, isolated, in pairs or in groups. Stained bacilli may present a beaded appearance.

Diagnosis of pulmonary T.B. by sputum microscopy is simple, easy, inexpensive, rapid, technically not very demanding and more reliable than x-ray examination. The purpose of the sputum microscopy is two fold (a) Diagnosis of the patients with infectious tuberculosis (b) Monitoring the progress of treatment. For diagnosis, 3 sputum examinations are performed (Spot, Morning, Spot) and for follow up 2 sputum examinations (morning, spot) are performed.

14.1 Collection Of Sputum Samples

- Select a good wide- mouthed sputum container, which is disposable, made of clear thin plastic, unbreakable and leak proof material.
- Instruct the patient to inhale deeply 2-3 times, cough up deeply from the chest and spit in the sputum container by bringing it closer to the mouth.
- Make sure the sputum sample is of good quality. A good sputum sample is thick, purulent and sufficient in amount (at least 2-3 ml).

14.2 Storage And Transportation Of Specimens

If the specimen is collected in the field and can not be immediately processed, it should be transported to the laboratory within 3-4 days of collection. The specimen should be collected in the containers meant for the purpose and lid tightly secured, properly labelled and to be kept away from the sun and heat. These can be placed in a special box, which can withstand leakage of contents, shocks and other conditions incident to ordinary handling practices. These boxes should be kept in the cool conditions and then transported to the laboratory.

14.3 Ziehl Neelsen Staining

14.3.1 Materials

- Glass slides
- Bamboo sticks
- Bunsen burner / Spirit lamp / discarding jar

14.3.2 Reagents

- Carbol fuchsin ---- 1%
- Sulphuric acid ---- 25%
- Methylene blue --- 0.1%

14.3.3 Method

- Select a new, unscratched slide and label the slide with a laboratory serial number.
- Make a smear from yellow purulent portion of the sputum using the jagged end side of a bamboo stick. A good smear is spread evenly, 2cms x 3cms in size and is neither too thick nor too thin. The optimum thickness of the smear can be assessed by placing the smear on a printed matter, the print should be just readable through the smear.
- Let the smear air-dry for 15-30 mins.
- Fix the smear by passing the slide over the flame 3-5 times for 3-4 seconds each time.
- Place the fixed slide on the staining rack with the smeared side facing upwards.
- Pour filtered 1% carbol fuchsin over the slide so as to cover the entire slide. Do not leave the carbol fuchsin on the slide for a long time (not more than 5 mins.)
- Heat the slide underneath until vapours start rising. Do not let carbol fuchsin to boil or the slide to dry. Continue the process up to five minutes.
- Allow the slide to cool for 5-7 minutes.
- Gently rinse the slide with tap water to remove the excess carbol fuchsin stain. At this point, the smear on the slide looks red in colour.
- Decolour the stained slide by pouring 25% sulphuric acid on the slide and leaving the acid for 2-4 mins.
- Lightly wash away the free stain. Tip the slide to drain off the water.
- If the slide is still red, reapply sulphuric acid for 1-3 mins, and rinse gently with tap water.
- Counter stain the slide by pouring 0.1% methylene blue solution on to slide and let it stand for one min.
- Gently rinse the slide with the tap water and tip the slide to drain off the water.

- Place the slide in the slide tray and allow it to dry.
- Examine the slide under the microscope using 40 x objectives to select the suitable area of the slide and examine under the 100 x lenses using a drop of immersion oil for the characteristic acid fast bacilli as shown in figure (14.1). At least 100 oil immersion fields should be examined before declaring a smear as negative. In case of the scanty result, examine another 100 oil immersion fields.

14.3.4 Grading Of Smears

Record the result in laboratory form and laboratory register appropriately as per table given below:

Examination findings	Result	Grading	Minimum No of fields to be examined
More than 10 AFB per oil immersion fields	Positive	3+	20
1-10 AFB per oil immersion fields	Positive	2+	50
10-99 AFB per 100 oil immersion fields	Positive	1+	100
1-9 AFB per 100 oil immersion fields	Scanty	Record exact number seen	200
No AFB per 100 oil immersion fields	Negative	0	100

Fig.-14.1 **Clump of AFB seen in a sputum smear. (Ziehl-Neelsen stain. examined at × 1000.)**

14.4 Safety Precautions

- Treat all sputum samples as potentially infectious and use leak proof containers for collection and transportation of the samples.
- Use bacteriological safety hood while carrying out all procedures involving sputum and always wear mask.
- Disinfect the sputum cups/ containers by incineration, autoclaving or treating with 5% phenol or 1-2% freshly prepared hypochlorite solution, which ever is feasible and then disposed off.

- Wash hands with soap and water frequently especially after touching the sputum.
- Do not eat, drink or smoke in the laboratory area.
- Clean laboratory bench tops with a disinfectant (Phenol or hypochlorite solution) at the end of each day

14.5 Quality Control: To check the quality of staining reagents as well as the procedure, the following should be followed at least once a week

- Make a smear from H37Rv strain of Mycobacterium tuberculosis
- Stain the smear by Ziehl Neelsen method.
- Use this smear as a positive control (all the bacilli in the smear should be bright pink coloured).
- Make a smear from the growth of staphylococcus aureus.
- Stain the smear by Ziehl Neelsen method.
- Use this smear as a negative control (all the bacteria in this smear should be blue coloured).

CHAPTER - 15

COLLECTION, TRANSPORT AND STORAGE OF CLINICAL SPECIMENS FOR VIRUS ISOLATION

The demand for virus identification has greatly increased in recent years. Development in virology and refinements in identification techniques have introduced viral studies into many new laboratories. Quality laboratory examination of specimens for virus isolation and identification begins with the selection of patients for viral studies

The choice of specimen to be collected for diagnostic approaches, especially of viral origin, depends upon the nature of the symptoms of the patient and of the pathogenesis of suspected viral agent. The symptoms indicate the involvement of entire system and help in taking specimens. For example, throat swabs, nasopharyngeal swabs are required in upper respiratory tract infection, cerebro spinal fluid (CSF) for infection of central nervous system, and so on. When clinical symptoms are not clear, a number of samples namely faeces, urine, blood etc. have to be collected for virus isolation.

Table:- 15.1

Specimens	Transport Media if Required VTM	Storage condition		Purpose/ lab. Investigation
		Transport	Pending Testing	
Throat swab	Yes	2-8°C	-20°C	Isolation of virus
Nasopharngeal swab/ Aspirate	Yes	2-8°C	-20°C	- do -
CSF	No	2-8°C	-20°C	Serology, Isolation of virus
Faeces	No	2-8°C	-20°C	Isolation of virus
Urine	No	2-8°C	-20°C	Isolation of virus
Blood (clotted) /serum	No	2-8°C	Serum -20°C clotted blood 2-8°C	Serology, Isolation of virus
Whole blood (EDTA)	No	2-8°C	2-8°C	Serology, isolation of virus

15.1 Optimal time for collection of samples for virus isolation

For virus isolation, the specimen should be collected as early as possible during the course of illness.

15.1.1 Samples for serological tests

For IgM antibodies detection, collect blood samples between 3-7 days from the

onset of illness. However, for IgG antibodies detection- paired serum samples are to be collected at the interval of 15 days. Other clinical samples like CSF can also be used in certain conditions.

15.2 Material required for collection of samples for viral diagnosis

- Screw capped sterile bottle.
- VTM. (3-5ml in plain sterile vial)
- Screw capped stool sample collection bottle with spoon on cap and externally threaded.
- 50 ml urine sample collection tubes, sterile, externally threaded.
- Cold box/vaccine carrier
- Ice pack
- Sterile syringes/ needle
- Sterile saline
- Sterile cotton tipped swab sticks. (Dacron coated are preferred)
- EDTA to collect whole blood.

15.3 Nasopharyngeal wash/ aspirate

15.3.1 Material required

- Viral transport media/Normal saline
- Plastic catheter / tubing
- Sterile, screw capped external threaded. plastic vial

15.3.2 Procedure

- Make the patient seated with head tilted slightly back ward.
- Instill 1-1.5 ml of normal saline in one nostril.
- Flush a plastic catheter/ tubing with 2-3 ml of normal saline
- Insert the tubing into one nostril parallel to the palate
- Aspirate nasopharyngeal secretions
- Repeat this procedure with other nostril.
- Collect 1-2 ml of nasopharyngeal secretion/ wash / aspirate in VTM.

15.4 Oropharnyngeal / Throat swab

15.4.1 Material required

- Dacron coated cotton swabs.
- Tongue depressor.
- Viral transport Media (V.T.M)

15.4.2 Procedure (fig.-15.1)

- Allow the patient to be seated and ask him to open the mouth
- With the help of tongue depressor hold down the tongue
- Locate the area of inflammation using a strong source of light.
- Rub the area back and forth with cotton swab
- Withdraw the swab without touching cheeks, teeth or gums.
- Insert the swab into tube containing VTM.

15.4.3 Throat washing (Fig.-15.2)

- For obtaining throat washing let the patient gargle with about 5 ml. of sterile physiological normal saline/VTM.
- Collect the washing in a clean disposable paper cup.
- Transfer the content to a clean prescribed screw capped vial

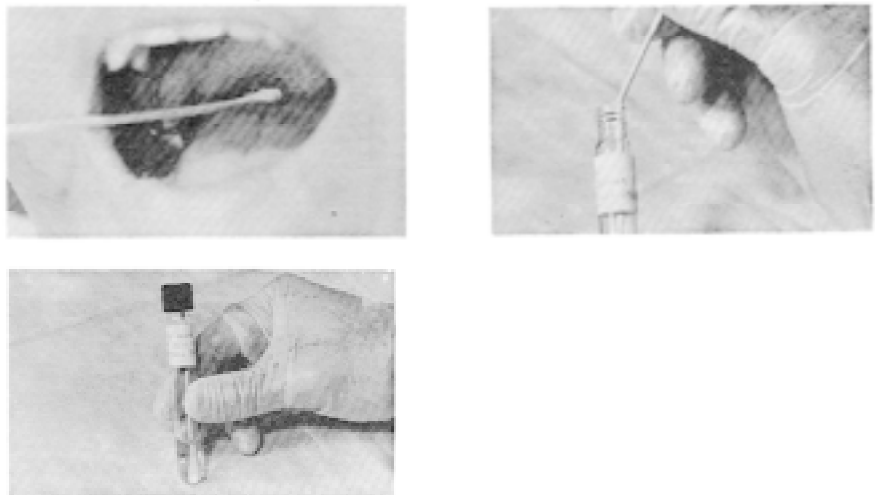


Fig15.1:- Throat swab sample collection

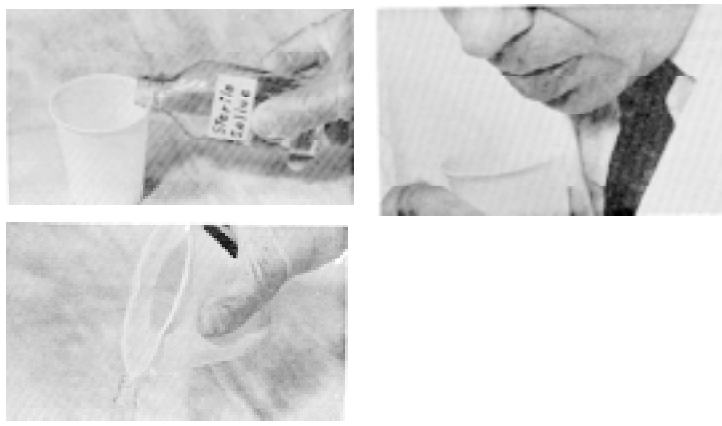


Fig:15.2 --- Throat gargles sample collection

15.5 Nasopharyngeal Swabs

15.5.1. Material

- Dacron coated swab.
- Viral transport medium
- Nasal speculum.

15.5.2 Procedure

- Seat the patient comfortably, tilt the head backward.
- Insert the nasal speculum
- Insert the Dacron swab through speculum parallel to the floor of the nose
- Rotate the swab on nasopharyngeal membranes for few seconds.
- Remove the swab carefully & insert in the vial containing VTM

15.6 Other Samples

CSF, Faeces (Stool), Whole Blood in EDTA/Clotted Blood/Serum can also be used for virus isolation. Refer to chapter -4 for collection, labelling, transportation and storage of these clinical specimens.

!5.7 VTM (Viral Transport Medium)/Hank's balanced salt solution (HBSS)

15.7.1 Composition for 10x

Solution 1

Sodium chloride (NaCl)	80.00 gm
Potassium chloride (KCl)	4.00 gm
Calcium chloride ($CaCl_2$)	1.40 gm
Magnesium sulphate.$7H_2O$ ($MgSO_4.7H_2O$)	2.0 gm

Solution 2

Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	1.2 gm
Potassium Dihydrogen phosphate (KH_2PO_4)	0.6 gm
Glucose	10.0 gm
Phenol red (1%)	16.0 ml

15.7.2 Method

- Weigh the chemicals for solution 1 and solution 2 separately and transfer them to clean/sterilized 1 litre flask each.
- Dissolve the content of each flask by adding less than 500 ml of double distilled **(DD)** water.
- Transfer solution 2 to the flask containing solution 1 while stirring.
- Make the volume upto 1000ml with double distilled (DD) water.
- The final concenteration of the solution is 10x.
- Sterilize the 10x solution by membrane filtration device using 0.22µ membrane filter.

  OR

- Dilute the 10x solution to 1x (1part conc. HBSS+9 parts DDwater) with DD water and sterlize it by autoclaving at 10lbs for 10 minutes.
- Before using it for collection of clinical samples, add presterilised 1.25 ml of 2.8% $NaHCO_3$ and 1 ml of presterilised antibiotic solution (Benzyl penicillin 20000 IU/ml and streptomycin 20mg/ml) in 100 ml of diluted (1x) HBSS.

VTM (HBSS) is also commercially available.

Note : If VTM is not available, MEM can be used.

CHAPTER - 16

RAPID SEROLOGICAL TESTS IN A DISTRICT LABORATORY

16.1 Tests For Syphilis Infection

Syphilis is caused by Treponema pallidum. There are four stages of syphilis infection: primary, secondary, latent and tertiary, and a special condition of maternal-fetal transmission termed as congenital syphilis. Immune responses to syphilis can be grouped into two categories: non-specific (or reaginic) and specific.

The non-specific reagin is of the IgM class and reacts with an alcoholic extract of beef heart known as cardiolipin (a phospholipid). Since, the reaginic antibody lacks specificity, it shows up in many other conditions and disease states unrelated to treponemal infection. In these cases false-positive reactions can occur. Specific antibodies to treponemes (both to T.pallidum and to nonpathogenic treponemes) of the normal bacterial flora of the oral or genital tract can also develop. These antibodies are of the IgG class and remain detectable throughout the life of the patient despite treatment. Routine tests for syphilis include the Rapid Plasma Reagin (RPR) test, the fluorescent treponemal antibody-absorbed (FTA-Abs) test and the T.pallidum haemagglutination (TPHA) test.

16.1.1 Rapid Plasma Reagin (RPR) test for diagonsis of syphilis

The RPR (Rapid Plasma Reagin) test is a non-treponemal test for serological diagnosis of syphilis. It is a reliable, economical, reproducible and rapid test which is easy to read without the help of a microscope. The specificity and sensitivity of the test are similar to those of VDRL test, the sensitivity being 85-90% in primary disease, 100% in secondary, 90-95% in latent disease and 75% in late or tertiary stage.

16.1.1.1 Principle of test

The RPR test has replaced the Venereal Disease Research Laboratory (VDRL) test, as a rapid screening test for the following reasons:

- There is no need for daily preparation of reagents.
- No microscope is required.
- Heat inactivation of serum is not required.

The RPR test uses the VDRL antigen modified with choline chloride to inactivate complement, and charcoal particles to allow the results of the reaction to be read without a microscope. The RPR test can also be applied as a semi-quantitative test.

RPR antigen suspension is a carbon coated non-treponemal cardiolipid antigen, which detects reaginic antibodies present in serum/plasma of patients suffering from treponemal infections. These antibodies may be occasionally found in sera of persons with other non treponemal conditions like rheumatoid arthritis, tuberculosis, Kala-azar,

etc. When a specimen contains antibody, flocculation occurs due to co-agglutination of the carbon particles of the RPR antigen, which appear as black clumps against the white background of the card, which is read with the naked eye.

16.1.1.2 Test Procedure

Follow the instruction of manual provided with test kits. Briefly the procedure is as follows:

- Place one drop of serum or plasma (50 ul) on the card with the help of a dropper.
- After thoroughly mixing RPR antigen suspension, place one drop (15-20ul) of the same alongside the drop of plasma on the card.
- Mix these drops well and spread out the pool of liquid uniformly within the entire area of the circle by using the applicator stick provided with the kit.
- Rock the card gently to and fro for 4 minutes and observe under a good light source for clumps.
- Use appropriate positive and negative controls provided with the kit.

16.1.1.3 Interpretation of test result

Positive result

Black aggregates which are deposited at the periphery of the liquid within 4-5 minutes.

Negative result

Complete absence of black aggregates with a uniform grayish background at the end of 4-5 minutes.

16.1.2 Rapid Specific Test For Syphilis

A rapid visual test for the qualitative detection of antibodies to Treponema pallidum (TP) in human serum / plasma is available, which is specific to T.pallidum.

16.1.2.1 Principle

It is a one step immunoassay for the detection of TP antibodies in serum or plasma. The syphilis antigen is immobilized on the nitrocellulose strip in a thin line. After a serum or plasma specimen is added to the sample pad, it flows laterally through an absorbent pad where it mixes with the Treponema pallidum antigen-colloidal gold conjugate. If the antibodies to Treponema pallidum are present in the sample, the antibodies will bind to the colloidal gold-antigen conjugate forming an antibody-antigen colloidal gold complex. The complex then migrates through the nitrocellulose strip by capillary action where it meets the immobilized antigen (test line) forming an antigen-antibody-antigen

colloidal gold complex. This forms a pink band indicating the sample is reactive for antibodies to Treponema pallidum. If antibodies to Treponema pallidum are absent, the Treponema pallidum antigen colloidal gold conjugate flows past the test line & no pink band is formed at the test line. To serve as a procedural control an additional line of anti-mouse antibody (control line) has been immobilized at a distance above the test line on the strip. If the test is performed correctly, this will result in the formation of a pink band upon contact with conjugate.

16.1.2.2 Storage & shelf life

Should be stored at 4-8°C in the coolest & driest area available. It has a shelf life of 12 months from the date of manufacturing. Do not freeze. It must be protected from exposure to humidity. Test strips should be used within one hour after removal from the plastic container. Plastic container containing strips should be tightly capped

16.1.2.3 Precautions

- For in vitro diagnostic use only.
- Handle all specimens as though they contain infectious agents. When the assay procedure is completed, dispose off specimen after autoclaving for at least 30 minutes at 121°C. Alternatively, it can be treated with 0.5 % sodium hypochlorite for 1 hour before disposal.
- Avoid repeated freezing and thawing of the sample to be tested.
- For best results, follow the given test procedure and storage instructions strictly.
- Do not use this product after the expiry.
- Clean & disinfect all spills of specimens using a suitable disinfectant, such as 0.5% sodium hypochlorite.
- Do not freeze the product.
- Do not open the zip lock foil pouch to remove the product until it attains room temperature and you are ready to perform the assay.
- Tightly zip seal the foil pouch after taking out required strips from the cylinder so that strips are protected from moisture.

16.1.2.4 Warning for user

DO NOT

1) Do not dip in human serum / plasma sample above the green line mark as the conjugate may dissolve into sample instead of migrating up.

2) Do not immerse holding end absorption pad in the serum / plasma sample.

3) Do not keep the strip under (a) working fan (b) heavy air circulation (c) table lamp bulb (b) high ambient temperature.

DO

1) Interpret the result at the end of 20 minutes only.

2) Take out the strip from the plastic container just before performing the test to avoid denaturation of the strips due to atmospheric exposure.

16.1.2.5 Specimen collection & storage

a) Patient sample performs best when tested immediately after collection. Fresh clear serum or plasma only may be used for testing. Do not use haemolysed, contaminated or lipemic sample for testing.

b) If not tested immediately, specimen should be refrigerated at 2-8°C for upto 3 days following collection or at -20°C if testing within 3 days is not possible.

c) Specimen containing visible precipitates or cloudy specimen may give inconsistent test result. Such specimen should be clarified prior to testing by high speed centrifugation i.e. 10,000 rpm for 10 minutes before testing.

d) Bring specimen to room temperature (25-30°C) prior to testing. Frozen specimens must be completely thawed & mixed well prior to testing. Specimen should not be frozen & thawed repeatedly.

16.1.2.6 Test procedure

1) Bring the specimen & zip lock pouch containing the strips to room temperature prior to testing.

2) Remove the required number of strips from plastic container.

3) Pipette 0.2ml of the specimen directly to the bottom of the labeled test tube. Avoid wetting of the inside walls of the test tube, as drops on the walls of the test tube may risk the test by wetting the strips above the filter area.

4) Hold the strip from the printed end and dip into the tube so that the end (as indicated by the arrows) contacts the specimen upto the green line mark. The fluid level of specimen must not be higher than the line indicated by the arrows.

5) Let the strip remain dipped in the sample. Allow the reaction to occur for 20 minutes.

6) At the end of 20 minutes take out the strip from the sample tube and read the test results.

7) Discard the strip immediately after reading the results as it is potentially infectious

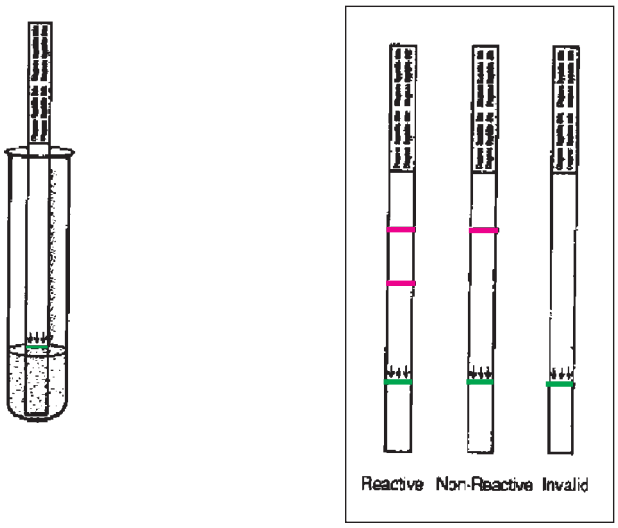


Fig: 16.1 Rapid Specific Test for Syphilis

Any change beyond the prescribed testing time (20 minutes) is of no diagnostic significance

Important: Do not dip the strip beyond the green line mark.

16.1.2.7 Interpretation of result

16.1.2.7.1 Reactive

If two distinct pink lines are formed, one near the dipping end and other near the holding end, the test should be interpreted as reactive (as shown in Fig.16.1(1). A difference of intensity in colour may occur between the test line and control line depending on the concentration of the antibodies in the specimen, but this does not affect the interpretation of the result.

16.1.2.7.2 Non Reactive

If only one distinct pink line is formed near the holding end of the strip (control line, as shown in fig. 16.1(2) the specimen is non-reactive. Interpret sample as non-reactive.

16.1.2.7.3 Invalid

If neither control line nor test line shows up (as shown in fig.16.1(3)) the test is considered to be invalid. This may be because of the following reasons.

1. If the strip is not immersed in correct direction.
2. If the specimen level is above the arrow mark of the strip.
3. If the conjugate is not migrating up at all.
4. If the specimen is not clear or is turbid.

In case of invalid result, the test should be repeated using a new strip and fresh sample.

16.1.2.8 Limitations of the procedure

1. The test is for in vitro diagnostic use only.
2. The test should be used for the detection of TP antibodies in serum or plasma only and not in other body fluids.
3. The test will only indicate the presence or absence of TP antibodies in the specimen and should not be used as the sole criteria for the diagnosis of TP infection.
4. As with all diagnostic tests, all results must be interpreted together with other clinical information available to the physician.
5. Additional follow up testing using available clinical methods (along with repeat test) is required, if the test is negative with persisting clinical symptoms.
6. This is only a screening test. All reactive samples should be confirmed by confirmation test. Therefore, for a definitive diagnosis, the patient's clinical history, symptomatology as well as serological data should be considered. The results should be reported only after complying with above procedure.

16.2 Tests for hepatitis virus infection

Routine tests for hepatitis include the use of markers for hepatitis A, B and C viruses. Hepatitis A is most common in children, especially in nurseries; however, it is not routinely tested for, except in cases of epidemics.

Hepatitis B and C viruses are transmitted through blood products, body fluids, contaminated needles and other contaminated materials.

Hepatitis B virus has several markers which include:

a surface antigen (HBsAg)

b. antibody to surface antigen (anti-HBs)

c. envelope antigen (HBeAg)

d. antibody to envelope antigen (anti-HBe)

e. antibody to core antigen (anti-HBc)

The concentrations of these markers vary during the course of an infection. The antigen markers appear first or earlier on exposure to the virus.

Sero-conversion (antibody production) often occurs several weeks or months after exposure.

Hepatitis testing is routinely done by solid-phase ELISA and radioimmunoassay methods. Commercial kits for detection of hepatitis markers are available and specific

criteria and instructions are provided with each kit.

16.2.1 Diagnosis of Hepatitis B Viral Infection

Diagnosis of Hepatitis B viral Infection is very important, not only in case of viral 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; 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 laboratories, is described below:

16.2.1.1 Latex Aggutination Test For Rapid Detection Of HBsAg

16.2.1.1.1 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.

16.2.1.1.2 Materials and reagents

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

Reagents 1:	HBsAg latex reagents	- 1 vial
Reagents 2:	Positive control serum	- 1 vial
Reagents 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.

16.2.1.1.3 Specimen

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

16.2.1.1.4 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 µL) 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.
- Add one drop (50 µL) of latex reagent on to each specimen drop in circles, using a disposable dropper.
- 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.
- Rock the slide gently, to and fro, for 5minutes, and watch for agglutination.

16.2.1.1.5 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 result
3) Vigourous rocking of slides may lead to impaired agglutination.

16.2.1.1.6 Interpretation

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

16.2.1.1.7 Limitations

- Probability of FALSE POSITIVITY = 1% of all samples, due to presence of other antigens. (Rheumatoid Factor).
- FALSE NEGATIVE results may be encountered with specimens containing very high titers 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 specimens 1:40, with normal saline.

16.2.1.1.8 Quality control

Positive and negative controls should be put up simultaneously as quality control measures.

16.2.1.2 Dipstick Test For Hepatitis B Surface Antigen

16.2.1.2.1 Principle

The dipstick test for the detection of hepatitis B surface antigen (HBsAg) takes advantage of the formation of a visible spot by precipitating immunocomplexes.

Conjugates of monoclonal antibodies against HBsAg coupled to colloidal gold particles are adsorbed to one area of a nitrocellulose strip (zone A in Fig.1)

Polyclonal antibodies against HBsAg are chemically fixed to another area of the strip, zone B. A drop of human serum is applied to zone A. The HBsAg antigen in the serum binds to the antibody conjugate and the gold-HBsAg immunocomplex migrates along the strip until it reaches the fixed polyclonal antibodies in zone B. The polyclonal antibodies precipitate the gold-HBsAg immunocomplex, and form a visible red band in zone B. No red band is formed if the serum does not contain HBsAg.

16.2.1.2.2 Materials and reagents

- Commercially available test kit containing dipsticks, reagents and controls.

16.2.1.2.3 Method

a. Label the test strip with the patient's name and/or number.

b. Add a drop of serum to zone A as recommended by the manufacturer.

c. Allow the serum fluid to migrate to zone B on the test strip.

d. Inspect zone B after 10-20 minutes for the appearance of a spot indicating a positive reaction.

16.2.2 Rapid Test For Hepatitis C Virus Antibody

Various rapid tests are currently available for detection of antibody to HCV (Hepatitis C Virus) in blood. We describe here an immunochromatic test (ICT) which has consistent results in various laboratories in recent past. It is a simple test for qualitative detection of antibodies to HCV in human plasma or serum.

16.2.2.1 Principle

HCV recombinant and synthetic peptide antigens are spotted on the membrane of the filter device. Antibodies to HCV in the serum or plasma bind with them. A procedural control is included in the form of spot or line of anti human immunoglobulin. HCV antibodies are visualized by reacting with conjugate. Appearance of two lines or spots, control and test indicate a positive test.

16.2.2.2 Components of Kit

- Antigen coated device
- Conjugate - Gold/colloidal gold protein A.
- Washing solution (ready to use).
- Positive and negative controls.

16.2.2.3 Precautions while handling kits and reagents

- Do not use kits or reagents beyond the expiration date.
- Do not mix reagents from different lots.
- Use all reagents (either as neat or reconstituted) strictly according to the instructions in the kit insert.
- Do not pipette by mouth.
- Take appropriate biosafety measure while handling the specimens, kits and reagents.

16.2.2.4 Storage

- Store kits as per the instructions on the kit.
- Bring the kit and its components to room temperature before use.

16.2.2.5 Test Procedure

16.2.2.5.1 Pre-assay preparations

16.2.2.5.1.1 Work bench preparation

- Spread filter paper sheets on the workbench.
- Keep a breadbox filled 1/4th with disinfectant (1% sodium hypochlorite) for disposing tips, vials, reagent bottles etc.
- Keep a biohazard bag ready for disposing dry waste.
- Keep disinfectant (70% alcohol) swabs ready in a closed container.
- Keep all accessories required for testing (micropipettes-single channel, marker pen, stop watch, thermometer) ready on the table.
- Keep sample deposition plan with kit details, proforma, and protocol on workbench.

16.2.2.5.2 Reagents

- Bring all specimens and reagents to room temperature (20-30°C).
- Immediately after use put back the reagents at 2-8°C
- Do not freeze reagents.

16.2.2.5.3 Assay procedure

16.2.2.5.3.1 Preparation of test samples

- Use plasma or serum as specimens.
- Do not use heat inactivated samples.

16.2.2.5.3.2 Preparation of reagents

- Bring all the reagents to room temperature for 15 to 30 minutes before testing if these test kits have been stored in refrigeration. But use them as such if already at room temperature.
- Take the required number of devices from the sealed case containing HCV antigen coated device.
- Reconstitute gold conjugate by delivering with micropipette 0.5 ml of reconstituted solution or add 2 volumes (about 0.5 ml) by attached dropper (circular line on the stem of the dropper indicate 0.25 ml) and mix gently for use.

16.2.2.5.3.3 Test method

- Take the required number of devices
- With each batch of test always use positive and negative control.
- Add washing solution first into a groove of device by using dropper.
- Add required amount of negative control, positive control/ test samples into each designated antigen-coated device.
- Add washing solution.
- Add gold conjugate and wait for its complete absorption.
- Again add washing solution and wait for its complete absorption.
- Read results within 10 minutes.

16.2.2.5.3.4 Quality control

- In positive control, test and control line/ spot appears.
- In negative control, only control line/ spot appears.
- Invalid, if no control line/ spot appears.

16.2.2.5.3.5 Interpretation of result

- Specimen is positive for HCV antibody if test and control line/ spot appear.
- Specimen is negative if only control line/ spot appear.
- Assay is invalid if no control line/ spot appears.

16.2.2.5.3.6 Post assay activities

- After the assay, discard the test devices in the box containing 1% sodium hypochlorite.
- Discard the used spreadsheets into bio-hazard bags.
- Swab the workbench and all equipments after use with 70% alcohol.

16.3 Anti HIV Antibody Detection

NACO guidelines are to be followed for Anti HIV antibody detection

CHAPTER - 17

IN VITRO SUSCEPTIBILITY TESTING OF BACTERIA TO ANTIMICROBIAL AGENTS.

Susceptibility testing is indicated for any organism that contributes to an infectious process warranting antimicrobial chemotherapy. The test is specially indicated when the causative organism is thought to belong to a species capable of exhibiting resistance to commonly used antimicrobial agents.

A variety of laboratory methods can be used to measure the in vitro susceptibility of bacteria to antimicrobial agents. An agar disk diffusion method is commonly used for testing common, rapidly growing and certain fastidious bacterial pathogens. The method involves placing filter paper disks impregnated with specific antimicrobial agents on agar plates pre seeded with the organism to be tested and judging the degree of sensitivity by the size of zone of inhibition resulting after overnight incubation. There are many methods of disc diffusion test, the most commonly used method is modified Kirby Bauer method also recommended by NCCLS (National Committee of Clinical Laboratory Standards)

17.1 Selection of the antibiotic discs

Selection of the most appropriate antimicrobial agent to test and report a given microorganism is best made by each clinical laboratory in consultation with the practicing physician. However, NCCLS recommended list of antibiotics based on the organism isolated is annexed **(Table: 17.1)**

To minimize confusion all antimicrobial agents should be reported using official nonproprietary (Generic) names.

17.2 Material / reagents required

17.2.1 Mueller-Hinton (M-H) agar

Of the many media available, NCCLS recommends the use of muellor hinton agar for the routine susceptibility testing of non fastidious bacteria.

Note: The recommended medium for testing S. pneumoniae and other streptococci is mueller hinton agar supplemented with 5% defibrinated sheep blood.

17.2.2 Preparation of Mueller-Hinton agar

1) M-H agar can be prepared from a commercially available dehydrated base according to the manufacturer's instructions. See Chapter 7.

2) Autoclave the reconstituted media at 120°C for 15-20mts, immediately thereafter, allow it to cool in a 45 to 50°C water bath.

Table 17.1 : -Suggestive list of antibiotic discs to be put up for different microorganisms

Sr. No.	Name of Antibiotics Discs	Enterobacteriacae (E.coli, Klebsiella, Salmonella,Shigella, Proteus, Citrobacter)			B.Pseudomonas aeruginosa / Acinetobacter			Staphylococcus species		
		Routine / primary	For special purpose / secondary	For urinary isolates	Routine / primary	For special purpose / secondary	For urinary isolates	Routine / primary	For special purpose	For urinary isolates
1.	Ampicillin	✓								
2.	Amikacin	✓			✓					
3.	Amoxycillin	✓								
4.	Cefazolin	✓								
5.	Cephalothin (Represents cephalothin, cephapirin, cephradine, cephalexin cefaclor and cefadroxil	✓								
6.	Cefuroxime (Represents cefamandole and cefonicid)	✓								
7.	Cefotaxime (Represents ceftriaxone and ceftizoxime)	✓				✓				
8.	Ciprofloxacin	✓			✓				✓	
9.	Co-trimoxazole									
10.	Ceftazidime		✓		✓					
11.	Chloramphenicol		✓			✓		✓		
12.	Cefoperazone			✓			✓			
13.	Carbenicillin						✓			
14.	Ceftizoxime						✓			
15.	Clindamycin							✓		
16.	Erythromycin (Azithromycin, clarithromycin)							✓		

Sr. No.	Name of Antibiotics Discs	Enterobacteriacae (E.coli, Klebsiella, Salmonella,Shigella, Proteus, Citrobactera)			B.Pseudomonas aeruginosa / Acinetobacter			Staphylococcus species		
		Routine / primary	For Special purpose /	For urinary isolates	Routine / primary	For special purpose /	For urinary isolates	Routine / primary	For special purpose	For urinary isolates
17.	Gentamicin	✓			✓				✓	
18.	Imipenem	✓			✓					
19.	Kanamycin		✓							
20.	Norfloxacin			✓			✓			✓
21.	Nalidixic Acid			✓						
22.	Nitrofurantoin									✓
23.	Oxacillin							✓		
24.	Penicillin							✓		
26.	Rifampicin								✓	
27.	Sulfisoxazole									✓
28.	Tetracycline (Represents doxycyclin, Minocycline)		✓			✓			✓	
29.	Tobramycine		✓		✓					
30.	Trimethoprim			✓						✓
31.	Vancomycin							✓		

3) Pour the freshly prepared and cooled medium into glass or plastic, flat bottomed petri dishes to give a uniform depth of approximately 4mm.

4) The agar medium should be allowed to cool at room temperature and stored at 2 to 8^0C in a refrigerator till used.

5) Agar plates should be used with in 7 days after preparation unless adequate precautions, such as wrapping in plastic have been taken to minimize drying of the agar.

6) A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35°C for 24 hours or longer.

7) PH: the PH of each batch of M-H agar should be checked at the time of preparation of the medium. The PH should be between 7.2 and 7.4 at room temperature.

17.3 Antimicrobial discs

Antimicrobial discs to be used for the drug sensitivity test can be obtained commercially or can be prepared in the laboratory.

17.3.1 Preparation of discs

1) Take whatman filter paper No.1, punch discs of 6mm diameter with the help of a punching machine.

2) Dispense the discs in a screw capped glass bottle and sterilize by dry heat in a hot air oven at 140°C for 60 minutes.

17.3.2 Preparation of antimicrobial solution

The antimicrobical solution is prepared depending upon the number of discs required and the amount of antimicrobial needed per disc. (Potency of the disc)

Tables 17.3 - 17.6 depict the concentration of different drugs per disc. To exemplify, if we require 100 discs of kanamycin with antibiotic content of each disc being 30µg, then the method shall be as follows :

a) Amount of drug required for 100 discs = 30µg x 100= 3000µg or 3mgm.

b) Accordingly, dissolve 3mgm of kanamycin powder in 1ml of sterile distilled water.

c) Take a sterile petri dish and spread the discs in the petri dish at a distance of around 2mm from each other.

d) Take a sterile syringe fitted with a needle that delivers 100 drops of solution per ml when syringe and needle are held vertically.

e) Drop a single drop of antibiotic solution on each disc.

f) Let the discs dry and store in a sterile container at 2-8°C.

17.4 Turbidity standards for inoculum preparation

To standardize the bacterial inoculum for the susceptibility test, a $BaSO_4$ turbidity standard equivalent to a 0.5 McFarland standard or its optical equivalent should be used. Macfarland standard may be prepared as per the procedure given below.

Preparation of McFarland Nephelometer Standards

Principle

A chemical induced precipitation reaction can be used to approximate the turbidity of a bacterial suspension.

Method

- Set up 10 test tubes or ampoules of equal size and of good quality. Use new tubes that have been thoroughly cleaned and rinsed.
- Prepare 1% chemically pure sulfuric acid.
- Prepare a 1.175% aqueous solution of barium chloride ($BaCl_2$. 2 H_2O)
- Slowly, and with constant agitation, add the designated amounts of the two solutions to the tubes as shown in table 17.2 to make a total of 10 ml per tube.
- Seal the tubes or ampoules. The suspended barium sulfate precipitate corresponds approximate to homogenous E. coli cell densities per milliliter throughout the range of standards as shown in table.
- Store the McFarland standard tubes in the dark at room temperature. They should be stable for 6 months.

Table 17.2 Macfarland Nephelometer Standards

TUBE NUMBER											
0.5	1	2	3	4	5	6	7	8	9	10	
0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	Barium chloride (ml)
9.95	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9	Sulfuric acid (ml)
1.5	3	6	9	12	15	18	21	24	27	30	Approx. cell density (x 10^8/ml)

Note: The turbidity standards should be vigorously agitated on a vortex mixer or manually done before use and inspected for a uniform turbid appearance. If large particles appear, the standard should be replaced.

17.5 Other minor requirements are

- Fine forceps
- Wire loop / pasteur pipettes.

- Peptone water / Trypticase Soy Broth.
- Sterile Normal Saline.
- Incubator
- Measuring scale.
- Bunsen Burner/ Spirit lamp.
- Standard ATCC strains.

17.6 Procedure for disk diffusion test

17.6.1 Inoculum preparation

17.6.1.1 Growth Method

1) At least 3-5 isolated colonies of the same morphology are selected from an agar plate culture. The top of each colony is touched with a wire loop and growth transferred into a tube containing 4-5 ml of suitable broth (peptone water/ nutrient broth/ trypticase Soy broth.)
2) The broth culture is incubated at 35-37°C for 4-6 hours or till it achieves or exceeds the turbidity of 0.5 McFarland standard
3) The turbidity of the broth culture can be adjusted using either sterile normal saline or sterile broth to match that of 0.5 McFarland. This results in a suspension containing approximately 1-2 x 10^8 CFU/ml of E. coli.

17.6.1.2 Direct colony suspension method

As a convenient alternative, the inoculum can be prepared by making a direct broth or saline suspension of isolated colonies selected from a 18 to 24 hours agar growth. The suspension is adjusted to match the 0.5 Mc Farland turbidity standard.

17.6.2 Inoculation of test plates

1) Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.
2) The dried surface of a Mueller Hinton agar plate is inoculated by streaking the swab over the entire agar surface. The procedure is repeated by streaking 2 or more times, rotating the plate 60° each time to ensure an even distribution of inoculum. Finally, the rim of the agar plate is swabbed.
3) The lid may be left ajar for 3 to 5 minutes but not more than 15 minutes to allow for any excess surface moisture to be absorbed before applying the drug impregnated discs.

Note :-

Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures.

17.6.3 Application of discs to inoculated plates

1) The predetermined battery of discs for a particular isolate are taken out of the refrigerator and brought to room temperature. Thereafter, discs are placed individually using the fine sterile forceps or with a dispensing apparatus on the inoculated agar plate. The discs should be distributed evenly so that they are no closer than 24 mm from center to center. No more than 12 discs should be placed on a 150 mm plate or more than 5 discs on a 100 mm plate.
2) The plates are inverted and placed in an incubator set to 35-37°C within 15 minutes after the discs are applied.

17.7 Reading plates and interpretation of results

After 16 to 18 hours of incubation, each plate is examined. There should be a confluent lawn of growth along with uniform circular zone of inhibition.

- Zones are measured to the nearest whole millimeter using a graduating scale held in back of the inverted petri plate. The petri plate is held a few inches above a black non-reflecting background and illuminated with reflected light.
- The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone should be ignored. However, discreet colonies growing with in a clear zone of inhibition should be sub cultured, reidentified and retested.
- The sizes of the zones of inhibition are interpreted by referring to tables17.3-17.6 and the organisms are reported as either susceptible, intermediate susceptible or resistant to the agents tested.

17.8 Quality Control

To control the precision and accuracy of the dics diffusion test, a set of standard bacterial strains as given below should be tested alongside the test cultures on a regular basis (once a week at least or when ever new batch of culture media and discs are used)

- Escherichia coli ATCC 25922.
- Staphylococcus aureus ATCC 25923.
- Pseudomonas aeruginosa ATCC 27853.

These strains can be obtained from Institute of microbial technology, Chandigarh. NICD, Delhi can also supply the same on request.

17.8.1 Storing quality control strains

For prolonged storage, stock cultures may be maintained at -20°C or below in a

deep freezer suspended in a suitable medium. e.g. 10 to 15 % glycerol in tryptic soy broth, or alternatively in a freeze dried state. Working cultures should be stored on nutrient agar / tryptic soy agar slopes at 2-8°C and subcultured weekly. New working cultures should be prepared at least monthly from the frozen / freeze dried stock cultures.

- Before testing the strains should be subcultured on to agar plates to obtain isolated colonies.
- The quality control strains should be tested by the standard disc diffusion test using the same materials and methods as for the test isolates.
- Acceptable zone diameters for different strains are listed in tables 17.3 – 17.6.

Table 17.3 Zone diameter interpretive standards for enterobacteriae

Sr.No.	Agents	Disc Content	Zone Diameter (mm)			Remarks
			R	I	S	
1.	Amipicillin*	10 µg	≤13	14-16	≥ 17	*Class drugs for Ampicillin and Amoxycillin.
2	Cephalothin**	30 µg	≤14	15-17	≥ 18	**Class drugs for Cephapirin Cephalexin, Cefaclor and Cefuroxime
3	Cefoxitin	30 µg	≤14	15-17	≥ 18	
4	Ceftriaxone	30 µg	≤13	14-20	≥ 21	
5	Nitrofurantion	300 µg	≤14	15-16	≥ 17	
6	Imipenem***	10 µg	≤13	14-15	≥ 16	***Also represents Meropenem.
7	Gentamycyin	10 µg	≤12	13-14	≥ 15	
8	Amikacin	30 µg	≤14	15-16	≥ 17	
9	Kanamycin	30 µg	≤13	14-17	≥ 18	
10	Streptomycin	10 µg	≤11	12-14	≥ 15	
11	Tobramycin	10 µg	≤12	13-14	≥ 15	
12	Tetracycline****	30 µg	≤14	15-18	≥ 19	****Representative for all tetracyclines
13	Ciprofloxacin	5 µg	≤15	16-20	≥ 21	
14	Norfloxacin / ofloxacin	10 µg 5 µg	≤12	13-16	≥ 17	
15	Nalidixic acid	30 µg	≤13	14-18	≥ 19	
16	Co-trimoxazole (Trimethroprim / sulfa)	1.25 / 23.75 µg	≤10	11-15	≥ 16	
17	Chloramphenicol	30 µg	≤12	13-17	≥ 18	

NOTE : R = RESISTANT, I = INTERMEDIATE, S = SENSITIVE

Table 17.4 Zone Diameter interpretive standard for Staphylococcus species

Sr.No.	Agents Antimicrobrial	Disc Content	Zone Diameter (mm)			Remarks
			R	I	S	
1.	Penicillin	10 units	≤ 28	—	≥ 29	
2.	Oxacillin	1 µg	≤ 10	11-12	≥ 13	
3.	Ampicillin	10 µg	≤ 28	—	≥ 29	
4.	Methicillin	5 µg	≤ 9	10-13	≥ 14	
5	Cephalothin	30 µg	≤ 14	15-17	≥ 18	
6	Ceftriaxone	30 µg	≤13	14-20	≥ 21	
7.	Cefotaxime	30 µg	≤ 14	15-22	≥ 23	
8.	Cefaclor	30 µg	≤ 14	15-17	≥ 18	
9.	Imipenem	10 µg	≤ 13	14-15	≥ 16	
10.	Vancomycin	30 µg	---	---	≥ 15	
11.	Erythromycin	15 µg	≤ 13	14-22	≥ 23	
12.	Azithromycin	15 µg	≤ 13	14-17	≥ 18	
13.	Gentamycin	10 µg	≤ 12	13-14	≥ 15	
14.	Amikacin	30 µg	≤ 14	15-16	≥ 17	
15.	Tobramycin	10 µg	≤ 12	13-14	≥ 15	
16.	Tetracycline	30 µg	≤ 14	15-18	≥ 19	
17.	Doxycycline	30 µg	≤ 12	13-15	≥ 16	
18.	Ciprofloxacin	5 µg	≤ 15	16-20	≥ 21	
19.	Ofloxacin	5 µg	≤ 12	13-15	≥ 16	
20.	Sparfloxacin	5 µg	≤ 15	16-18	≥ 19	
21	Nitrofurantoin	300 µg	≤ 14	15-16	≥ 17	
22.	Co-trimoxazole	1.25/23.75 µg	≤ 10	11-15	≥ 16	
23.	Chloramphenicol	30 µg	≤ 12	13-17	≥ 18	
24.	Rifampin	5 µg	≤ 16	17-19	≥ 20	

Table 17.5 Zone Diameter Interpretive standards for Vibrio Cholerae

Sr.No.	Agents	Disc Content	Zone Diameter (mm)			Remarks
			R	I	S	
1	Ampicillin*	10 µg	≤ 13	14-16	≥ 17	*Class representative for amoxycillin
2	Tetracycline**	30 µg	≤ 14	15-18	≥ 19	**Representative for Doxycycline also
3.	Co-trimoxazole	1.25/23.75 µg	≤ 10	11-15	≥ 16	
4.	Chloramphenicol	30 µg	≤ 12	13-17	≥ 18	

Note: Disc diffusion test not to be used for Erythromycin in case of Vibrio Cholerae

NOTE : R = RESISTANT, I = INTERMEDIATE, S = SENSITIVE

Table 17.6 Zone Diameter Interpretive Standard for Pseudomonas aeruginosa

Sr.No.	Antimicrobial agent	Disc Content	Zone Diameter (mm)			Remarks
			R	I	S	
1.	Tetracycline	30 µg	≤ 14	15-18	≥ 19	
2.	Doxycycline	30 µg	≤ 12	13-15	≥ 16	
3.	Ciprofloxacin	5 µg	≤ 15	16-20	≥ 21	
4.	Ofloxacin	5 µg	≤ 12	13-15	≥ 16	
5	Norfloxacin	10 µg	≤ 12	13-16	≥ 17	
6	Chloramphenicol	30 µg	≤ 12	13-17	≥ 18	
7.	Co-trimoxazole	1.25/23.75 µg	≤ 10	11-15	≥ 16	
8.	Gentamicin	10 µg	≤ 12	13-14	≥ 15	
9.	Amikacin	30 µg	≤ 14	15-16	≥ 17	
10.	Tobramycin	10 µg	≤ 12	13-14	≥ 15	
11.	Imipenem	10 µg	≤ 13	14-15	≥ 16	
12.	Carbenicillin	100 µg	≤ 13	14-16	≥ 17	
13.	Azlocillin	75 µg	≤ 17	----	≥ 18	
14.	Cefuroxime	30 µg	≤ 14	15-22	≥ 23	
15.	Ceftazidime	30 µg	≤ 14	15-17	≥ 18	
16.	Ceftizoxime	30 µg	≤ 14	15-19	≥ 20	
17.	Ampicillin/sulbactam	10/ 10 µg	≤ 11	12-14	≥ 15	

Note: Pseudomonas aeruginosa may develop resistance during prolonged therapy with all antimicrobials. Therefore, isolates that are initially susceptible may become resistant within 3-4 days of starting therapy.

Table17.7 Acceptable zone diameter of quality control strains

S.No	Antimicrobial agent	Disc Content µg	Zone Diameter (mm)		
			E.Coli ATCC 25922	Staph aureus ATCC25923	Pseudomonas aeruginosa ATCC 27853
1.	Amikacin	30 µg	19-26	20-26	18-26
2.	Ampicillin	10 µg	16-22	27-35	----
3.	Carbenicillin	100µg	23-29	------	18-24
4.	Cefaclor	30 µg	23-27	27-31	------
5.	Cefamandole	30 µg	26-32	26-34	------
6.	Cefixime	15 µg	23-27	------	------
7.	Cefotaxime	30 µg	29-35	25-31	18-22
8.	Cephalothin	30 µg	15-21	29-37	--------
9.	Chloramphenicol	30 µg	21-27	19-26	--------
10	Ciprofloxacin	5 µg	30-40	22-30	25-33

11.	Erythromycin	15 µg	-------	22-30	------
12.	Gentamycin	10 µg	19-26	19-27	16-21
13.	Imipenem	10 µg	26-32	--------	20-28
14.	Kanamycin	30 µg	17-25	19-26	-------
15.	Nalidixic acid	30 µg	22-28	---------	-------
16.	Nitrofurantoin	300 µg	20-25	18-22	------
17.	Norfloxacin	10 µg	28-35	17-28	22-29
18.	Ofloxacin	5 µg	29-33	24-28	17-21
19.	Penicillin	10 units	----------	26-37	------
20	Rifampin	5 µg	8-10	26-34	------
21	Streptomycin	10 µg	12-20	14-22	------
22.	Tetracycline	30 µg	18-25	24-30	------
23.	Tobramycin.	10 µg	18-26	19-29	19-25
24	Co-trimoxazole	1.25/23.75µg	24-32	24-32	-------
25.	Vancomycin	30 µg	-------	17-21	-------

CHAPTER - 18

LABORATORY DIAGNOSIS OF DENGUE AND DENGUE HAEMORRHAGIC FEVER

18.1 Case definition

18.1.1 Dengue fever

The clinical case description of dengue fever is an acute febrile illness of 2-7 days duration with 2 or more of the following :

Headache, retro-orbital pain, myalgia, arthralgia, rash, haemorrhagic manifestation and leucopenia.

18.1.1.1 Suspect case : A case compatible with the clinical description

18.1.1.2 Probable case : A case compatible with clinical description with one or more of the following:

- Supportive serology
- Presence of confirmed case in the area during the same period

18.1.1.3 Confirmed case : A case compatible with clinical description and laboratory confirmed

18.1.2 Case definition of DHF

A probable or confirmed case of dengue fever with haemorrhagic tendencies evidenced by one or more of the following:

- Positive tourniquet test
- Petechiae, ecchymosis or purpura
- Bleeding from buccal mucosa, gastrointestinal tract, injection site or others
- Haematemesis, malaena
- Signs of plasma leakage (pleural effusion, ascites, hypoproteinaemia)

18.2 Haematological criteria for diagnosis

Thrombocytopenia (100,000 cells or less per mm^3)

Haemoconcentration (>20% rise in average haematrocrit for age and sex)

$\geq$ 20% drop in haematocrit following volume replacement treatment as compared to baseline.

18.3 Collection, storage and transportation of samples

An essential aspect of the laboratory diagnosis is proper collection, processing, storage and transportation of the specimens.

Collect a blood sample as soon as possible after the onset of illness, hospital

admission or attendance at a clinic (This is called the acute serum, S1)

Collect a second blood sample shortly before discharge from the hospital or, in the event of a fatality, at the time of death (convalescent serum, S2)

Collect a third blood sample, in the event hospital discharge occurs within 1-2 days of the subsidence of fever 7-21 days after the acute serum was drawn (late convalescent serum, S3).

The optimal interval between the acute (S1) and the convalescent (S2 or S3) serum is 10 days. The above recommendations allow for the collection of at least two serum samples for comparison, and ideally will provide for an adequate interval between sera. Serological diagnosis are predicted on the identification of changes in antibody levels over time. Serial (paired) specimens are required to confirm or refute a diagnosis of acute flavivirus or dengue infection.

18.3.1 Blood collection in tubes or vials and transportation

Aseptically collect 2-10 ml of venous blood.

Use adhesive tape marked with pencil, indelible ink, or a typewritten self adhesive label to identify the container. The name of the patient, identification number and date of collection must be indicated on the label.

Use vacuum tubes or vials with screw caps, if possible. Fix the cap with adhesive tape, wax or other sealing material to prevent leakage during transport.

Transport specimens to the laboratory in an ice box as soon as possible. Do not freeze whole blood, as haemolysis may interfere with serology test results.

If there is more than a 24-hour delay before specimens can be submitted to the laboratory, the serum should be separated from the red blood cells and stored frozen.

18.3.2 Collection of samples for isolation of Dengue virus for transportation to referral laboratory

Clinical specimen for isolation of dengue virus diagnosis can be accomplished in a majority of cases provided the same is taken in the first few days of illness and processed without delay.

Specimens that may be suitable for virus isolation include:

(1) Acute phase serum; (2) plasma; (3) washed buffy coat from the patient; (4) autopsy tissues from fatal cases especially liver, spleen, lymph nodes and thymus, and (5)mosquitoes collected in nature.

These samples should be immediately transported (within 48 hours) to referral laboratory in an ice box.

18.4 Laboratory Diagnosis

Serological tests carried out for diagnosis of dengue are:

1. Dengue IgM ELISA test
2. Dengue IgM & IgG Rapid Strip Test

18.4.1 Dengue IgM ELISA Test (Commercial Kit)

The Kits are commercially available and the manufacturer's instructions as per kit insert should be strictly adhered to.

18.4.1.1 Principle

The qualitative immunoenzymatic determination of IgM-class antibodies against Dengue virus is based on the ELISA (Enzyme- linked Immunosorbent assay) technique. Microtiter strip wells are precoated with Dengue virus antigen to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled anti-human IgM conjugate is added. This conjugate binds to the captured Dengue virus-specific antibodies. The immune complex formed by the bound conjugate is visualized by adding tetramethylbenzidine (TMB) substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of Dengue virus-specific IgM antibodies in the specimen, sulphuric acid is added to stop the reaction. This produces a yellow endpoint color. absorbance at 450 nm is read using an ELISA microwell plate reader.

18.4.1.2 Materials and Equipment required

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450/62Onm
- Incubator 37°C
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000µl
- Vortex tube mixer
- Deionised or (freshly) distilled water
- Disposable tubes.
- Timer

18.4.1.3 Materials and Reagents Supplied

- Dengue virus coated wells (IgM): 12 breakapart 8-well snap-off strips coated with Dengue Virus antigen type 2; vacuum sealed, in resealable aluminium foil.
- IgM sample diluent
- Stop solution.
- Washing solution (20x conc.) for washing the wells.
- Dengue virus anti-IgM conjugate.
- TMB substrate solution
- Dengue virus IgM positive control
- Dengue virus IgM negative control
- Strip holder
- 2 Cover foils

18.4.1.4 Stability and Storage

The reagents are stable up to the expiry date stated on the label when stored at 2 to 8°C.

18.4.1.5 Reagent Preparation

It is very important to bring all reagents, samples and controls to room temperature (20 to 25°C) before starting the test run.

- **Coated removable well strips**

  The ready to use breakapart snap-off strips are coated with dengue virus antigen. Store at 2 to 8°C. The strips are vacuum sealed. Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2 to 8 °C, stability until expiry date.

- **Dengue virus anti-IgM conjugate**

  The bottle contains 20 m1 of a solution with anti-human-IgM horseradish peroxidase, buffer, stabilizers, preservatives and an inert red dye. The solution is ready to use. Store at 2 to 8°C.

- **Controls**

  The bottles labelled with Positive and Negative Control contain a ready to use control solution. It contains 0.1 % kaolin and has to be stored at 2 to 8°C.

- **IgM Sample Diluent**

  The bottle contains 100 m1 phosphate buffer, anti- human-IgG, stabilizers, preservatives and an inert green dye. It is used for the dilution of the patient specimen. The solution contains anti- human IgG class antibodies to eliminate competitive inhibition from specific IgG class antibody and to remove rheumatoid factor. This ready to use solution has to be stored at 2 to 8°C.

- **Washing Solution (20xconc.)**

  The bottle contains 50 ml of a concentrated buffer, detergents and preservatives. Dilute washing solution 1+19; e.g. 10 ml washing solution + 190 ml fresh and germ free redistilled water. The diluted buffer will keep for at least four weeks if stored at 2 to 8°C. Crystals in the solution disappear by warming up to 37 °C in a water bath.

- **TMB Substrate Solution**

  The bottle contains 15 ml of a tetramethylbenzidine / hydrogen peroxide system. The reagent is ready to use and has to be stored at 2 to 8°C, away from the light. The solution should be colourless or have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be discarded. After first opening it is stable until expiry date when stored at 2 to 8°C.

➢ **Stop Solution**

The bottle contains 15 ml 0.2 M sulphuric acid solution. This ready to use solution has to be stored at 2 to 8°C. After first opening stable until expiry date.

18.4.1.6 Test Procedure

(i) Sample Dilution

Before assaying, all samples should be diluted 1 : 100 with IgM sample diluent. Dispense 10µl sample and 1 ml IgM sample diluent into tubes to obtain a 1:100 dilution and thoroughly mix with a Vortex. Positive and negative controls are ready to use and must not be be diluted.

(ii) Test Performance

Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the protocol sheet. Select the required number of microtiter strips or wells and insert them into the holder.

➢ **Please allocate at least**

1 well for the substrate blank,

2 wells for the negative control and 1 well for the positive control.

It is recommended to test controls and patient samples in duplicate.

➢ Perform all assay steps in the order given and without any appreciable delays between the steps. A clean, disposable tip should be used for dispensing each control and sample. Adjust the incubator to 37° ± 1°C.

➢ **Methodology**

1. Dispense l00ml controls and diluted samples into their respective wells. Leave one well for substrate blank.
2. Cover wells with the foil supplied in the kit.
3. Incubate for 1 hour ± 5 min at 37°C.
4. When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300µl of washing solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be >5 sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step!

Note: Washing is critical. Insufficient washing results in poor precision and falsely elevated absorbance values.

5. Dispense l00µl Dengue virus anti-lgM conjugate into all wells except for the blank well. Cover with foil.
6. Incubate for 30 min at room temperature. Do not expose to direct sunlight.
7. Repeat step 4.
8. Dispense l00µl TMB substrate solution into all wells.
9. Incubate for exactly 15 min at room temperature in the dark.
10. Dispense 100µl stop solution into all wells in the same order and at the same rate as for the TMB substrate solution. Any blue color developed during the incubation turns into yellow.

Note: Highly positive patient samples can cause dark precipitates of the chromogen. These precipitates have an influence when reading the optical density. Predilution of the sample with physiological sodium chloride solution, for example 1 : 1, is recommended. Then dilute the sample 1 : 100 with dilution buffer and multiply the results by 2.

11. Measure the absorbance of the specimen at 450/620 nm within 30 min after addition of the Stop Solution.

➢ **Measurement**

Adjust the ELISA Microwell Plate Reader to zero using the substrate blank well.

If - due to technical reasons - the ELISA reader cannot be adjusted to zero using the substrate blank well then subtract the absorbance value of substrate blank well from all other absorbance values measured in order to obtain reliable results.

Measure the absorbance of all wells at 450 nm and record the absorbance values for each control and patient sample in the distribution and identification plan.

Dual wavelength reading using 620 nm as reference wavelength is recommended. Where applicable calculate the mean absorbance values of all duplicates.

18.4.1.7 Results

➢ **Run Validation Criteria**

In order for an assay to be considered valid, the following criteria must be met:

Substrate blank : Absorbance value lower than 0.100.

Negative control: Absorbance value lower than 0.300.

Positive control : Absorbance value equal to or greater than the cut-off value.

➢ **Calculation of Results**

The cut-off is calculated by addition of 0.35 absorbance units to the measured absorption of the mean value of the two negative control determinations.

18.4.1.8 Interpretation of Results

Samples are considered positive if the absorbance value is higher than 10% over the cut-off.

Samples with an absorbance value of 10% above or below the cut-off should not be considered as clearly positive or negative → grey zone

It is recommended to repeat the test again 2 - 4 weeks later with a fresh sample. If results in the second test are again in the grey zone the sample has to be considered negative.

Samples are considered negative if the absorbance value is lower than 10% below the cut-off.

18.4.2 Dengue IgM & IgG Rapid Strip Test (Commercially available)

18.4.2.1 Principle

Serum antibodies of the IgM or IgG class, when present, bind to anti-human IgM or IgG immobilized in two lines across the test strip. Colloidal gold-labeled anti-flavivirus monoclonal forms complexes with the dengue antigen that is captured by dengue specific IgM or IgG in the patient's serum. These complexes are visualised as pink/purple line (s).

18.4.2.2 Precautions

1. All human blood products should be handled as potentially infectious material.
2. Never pipette by mouth or allow reagents or patient sample to come into contact with skin.
3. Optimal results will be obtained by strict adherence to this protocol. Reagents must be added carefully to maintain precision and accuracy.
4. Care should be exercised to protect the reagents in the kit from contamination. Do not use if evidence of microbial contamination or precipitation is present.
5. The test should be performed on serum only. The use of whole blood, plasma or other specimen matrix has not been established.
6. Icteric or lipemic sera, or sera exhibiting haemolysis or microbial growth should not be used.
7. Do not heat inactivate sera.
8. Do not use reagents beyond the stated expiry date marked on the package label. Keep storage boxes dry.
9. Do not reuse test strips. Do not use test strips if foil pouch is damaged.
10. Testing materials should be disposed of in accordance with local, state and/or federal regulations.

18.4.2.3 Storage and Shelf life of Reagents

Store kit between 2^0C and 8^0C. The test kit may be used until the expiry date of the

kit. Constant storage temperature must be maintained for the reagents to be stable till the expiry period of the kit.

18.4.2.4 Material supplied

- Individually packed test strips. Each strip contains monoclonal antibodies to human IgM and human IgG immobilized in two lines. A control line is also included. Strips contain a pad with purified recombinant dengue proteins (DI, D2, D3, D4) and gold labelled monoclonal anti-dengue virus.
- 1 x 3 ml Bottle of Phosphate based buffer (contains O.I% Sodium azide)
- 30 x 1ml plastic loops

18.4.2.5 Materials required

- Timer - 0 to 60 minutes
- Glass or plastic tubes 12 x 75mm.

18.4.2.6 General procedure

- Allow all reagents to equilibrate to room temperature (20-25^{0}C) before running the assay. Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.
- **Steps**

1. A single glass or plastic tube is required for each sample to be tested.
2. Add 3 drops (75 µl) of buffer to each tube. Ensure that the buffer bottle is held vertically when dispensing.
3. Using the loop provided, add 1µl of serum to the tube.
4. Gently shake the tube to ensure adequate mixing of the serum in the strip buffer.
5. Remove the test strip from the pouch just prior to use.
6. Add the test strip to the tube containing diluted serum. Ensure that the red label coloured end marked "SAMPLE" is placed into the tube.
7. Read the results 15 minutes after adding the test strip. Any trace of a pink/ purple line in the test area indicates a positive result. If a pink line has not developed after 15 minutes, read the strip again after 30 minutes as some very weak results may require 30 minutes to develop.
8. The absence of a test band suggests a negative result. In some strips, a negative result may appear as a white "ghost' line if the background is pale pink.
9. The assay to be considered valid, the control line must appear. If it does not appear, the test must be repeated.

18.4.2.7 Expected values and test limitations

Primary dengue is characterised by the presence of detectable IgM antibodies 3-5 days after the onset of infection. Secondary dengue is characterised by the elevation of specific IgG 1-2 days after the onset of infection and in the majority of cases this is generally accompanied by an elevation of IgM.

18.4.2.8 Interpretation of results

1. IgM and Control Positive. The test is positive for IgM and is indicative of a primary dengue infection.
2. IgM, IgG and Control Positive. The test is positive for IgM and IgG antibodies and is indicative of a secondary dengue infection.
3. IgG and control positive. The test is positive for IgG antibody and is indicative of secondary dengue infection.
4. Control positive. No detectable IgG and IgM antibodies to dengue. The result does not exclude dengue infection. Retest in 3 - 4 days if dengue infection is suspected.

CHAPTER-19

LABORATORY PROCEDURES FOR DIAGNOSIS OF JAPANESE ENCEPHALITIS

Japanese Encephalitis (JE) is a zoonotic viral disease caused by group B arbovirus (flavivirus), involving the Central Nervous System. In nature, the virus is maintained in animal and birds, particularly pigs and ardied birds (e.g. cattle egrets, pond herons etc.). Although infection in human is incidental, the virus can cause serious neurological disease in human. The disease occurs with sudden onset and the common symptoms are headache, high fever, stiff neck, abnormal movements (coarse tremor, convulsions in children), impaired consciousness and coma. Case fatality rate in JE is high, ranging from 20-50%.

19.1 Case definition

19.1.1 Suspect case

- High grade fever of acute onset with at least two of the following:
- Decrease in level of consciousness independent of convulsions
- Significant change in mental status either in behaviour or personality
- Convulsions

19.1.2 Probable case

- Suspected case of Japanese encephalitis, and
- Usually not more than a few cases (1-2) in one village
- Presence of animal hosts and high density of vector

19.1.3 Confirmed case

- Presence of IgM antibody in serum.
- 4 fold rise in IgG antibody titre in paired sera collected at 14 days interval.

19.2 Laboratory diagnosis of JE

Laboratory diagnosis of JE is done by following methods:

19.2.1 Detection of antigen/isolation of virus

(i) Demonstration of viral antigen in the autopsied brain tissue by the fluorescent antibody test

(ii) Isolation and identification of the virus from CSF, occasionally from peripheral blood (within 3 to 4 days after onset of symptoms) or autopsied brain tissue.

19.2.2 Detection of antibody

The detection of antibodies to JE virus can be done routinely by haemagglutination

Inhibition Test (HI) test, IgM Capture ELISA test. The antigen and reagents for both the tests are available from National Institute of Virology, Pune.

The above tests cannot be performed at district laboratories. Hence the samples should be collected and properly stored and transported to referral laboratory for further processing. For details of collection, storage and transportation refer to chapter '4'.

19.3 Laboratories undertaking work on JE in India

1. National Institute of Virology, Ambedkar Road, Pune
2. National Institute of Communicable Diseases, 22-Sham Nath Marg, Delhi
3. Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raibareily road, Lucknow
4. King George's Medical College, Lucknow
5. Armed Forces Medical College, Pune
6. School of Tropical Medicine, 110 Chittaranjan Avenue, Kolkata
7. Veterinary Biological Institute, Hyderabad
8. King Institute of Preventive Medicine, Chennai

CHAPTER- 20

LABORATORY DIAGNOSIS OF KALA - AZAR (LEISHMANIASIS)

Leishmaniasis is a chronic parasitic disease caused by the parasite of genus Leishmania. Across the tropics three different diseases are caused by various species of genus Leishmania. These are:

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

In India, Visceral leishmaniasis or Kala azar is the major public health problem. It is endemic in Bihar, West Bengal and eastern Uttar Pradesh. The disease is transmitted by the bite of infected female sand fly of the genus Phlebotomus. There is no zoonotic reservoir. Man is the only reservoir of infection for Kala azar in India. The average incubation period of disease is 3 - 6 months. Patient presents with symptoms of fever, malaise, weight loss, anorexia, cough and diarrhoea. Liver and spleen are enlarged. In untreated patients, death ensures in as many as 90% cases which is usually attributed to the concomitant infections.

The diagnosis of Kala-azar comprises of demonstration or isolation of the parasite from blood or biopsy material and demonstration of leishmania specific antibodies in the serum.

20.1 Collection, storage and transportation of specimen

The collection, transportation and storage of specimens are extremely vital steps in laboratory diagnosis of any disease and hence, must be undertaken with utmost care. The following general rules should be observed while collecting these specimens:

- Collect sufficient quantity of specimen.
- Avoid contamination by using sterile equipment and aseptic precautions.
- Dispatch the specimen immediately to laboratory.
- In case the delay is inevitable, keep the specimen at 4°C in a refrigerator.

Label all specimens accurately and send all pertinent information to laboratory which will help in better interpretation of the laboratory findings.

The following specimens need to be collected:

- Blood
- Biopsy/aspirate from bone marrow and/or spleen.

20.1.1 Blood

It can be collected either through a venipuncture or by finger prick method.

20.1.1.1 By Venipuncture Method : See detail procedure in chapter 4

- Collect blood in a plain clean vial and allow it to clot at room temperature.
- Take specimen to the laboratory promptly. If there is a delay, store in refrigerator at +4°C.
- Separate serum in a clean test tube with pasteur pipette.
- Centrifuge the collected serum for 5 mts.
- Collect supernatant in another screw capped bottle or storage vial.
- Store at + 4°C or at - 20°C till used.

20.1.1.2 By Finger Prick method : See detail procedure in chapter 4

- Print 20 mm diameter rings on Whatman No 3 filter paper strips.
- Prick the finger using aseptic technique and soak the circles completely with the oozing blood.
- Allow the strip to dry at room temperature.
- Wrap the strip in polythene cover and store either at + 4°C or at - 20°C.

20.1.2 Bone Marrow Aspirate

Bone Marrow Aspirate should be collected by a qualified clinician using all asceptic precautions. It can be collected from:-

- Mid-sternal region, a little away from the middle line and at the level of second or third intercostal space.
- Posterior iliac crest puncture, 1 cm below the posterior superior iliac spine.

20.1.2.1 Material Required

Sternal puncture needle:- A short stout needle of the lumbar puncture type, provided with a stylet and a movable guard. The latter is adjusted at a distance of 1.5 to 2 cm from the tip according to the distance required by the needle to reach the marrow and therefore varies with the thickness of the skin and subcutaneous tissues of the thoracic wall. The needle is introduced with the stylet in position.

One 2 ml glass syringe for injecting xylocaine and one 10 ml syringe for taking the marrow.

2% solution of xylocaine.

Spirit, Tr. iodine, Tr. benzoin co.

Glass slides & culture bottle (Tobie's medium).

20.1.2.3 Procedure

- Ask the patient to lie on his back in the supine position.
- Paint the area with 1% Tr. Iodine. Clean with spirit wait for 30 sec.
- Inject local anaesthesia (xylocaine) upto periosteum.
- Wait for 5 minutes.

- Make a puncture by piercing through the skin and subcutaneous tissue upto the periosteum. When marrow cavity is reached, a loss of resistance is felt.
- Take out stylet and fit a 10 ml glass syringe (dry and sterilised).
- Aspirate about 0.25 to 0.75 ml of the marrow fluid by gentle suction.
- Withdraw the syringe along with sterile puncture needle.
- Seal the wound with Tr. Benzoin co.
- Inoculate 2-3 drops of aspirate in culture bottle and transport to laboratory immediately.
- Eject one drop on glass slide for making smear. Make 2-3 such smears.

20.1.3 Spleen Puncture

When spleen is considerably enlarged it is one of the valuable method for establishing the parasitological diagnosis of kala-azar. However, there is a risk of bleeding and it should be carried out in a hospital setting with suitable precautions under the supervision of clinician.

20.1.3.1 Material Required

- A 5 ml glass syringe having a 26 number needle. An absolutely dry sterile syringe is essential as water causes lysis of the parasite.
- Cotton wool bandage, Tr. Iodine, alcohol, Tr. Benzoin Co.
- Glass slides & culture bottles (Tobie's medium).

20.1.3.2 Procedure

- Ask the patient to lie on his back.
- Apply spirit and Tr. iodine to the area 1/2" to 1" below the costal margin on left side.
- Ask the patient to hold his breath.
- Introduce first the sterilised needle into the skin and then directly into the spleen.
- Aspirate and withdraw the needle quickly.
- Seal the area with Tr. benzoin Co.
- Apply an abdominal bandage with pressure pad on the splenic area.
- Inoculate 2-3 drops of the aspirate in culture bottle and transport to laboratory immediately.
- Eject a drop on glass slide for making a smear. Make 2-3 such smears.

20.2 Smear examination

The conclusive evidence in the diagnosis of Kala azar is the demonstration of the parasite. The amastigote forms of the parasite i.e. Leishman donovan bodies (LD bodies) can be demonstrated in stained smears of bone marrow or splenic aspirate and rarily in the lymph node aspirate smears. Because of the small number of parasite present in the

peripheral blood it is difficult to demonstrate the parasite in the peripheral blood smears. The highest positivity rate is in splenic aspirate followed by bone marrow aspirate.

20.2.1 Giemsa stain

Please refer to chapter No.9 for preparation of stain.

20.2.2 Procedure of staining

20.2.2.1 Giemsa staining

- Clean glass slide
- Put a drop of bone marrow/splenic aspirate on the slide
- Draw a thin smear on the slide and air dry
- Dilute Giemsa stain by adding 1 drop to each 1 ml of neutral or faintly alkaline (pH 7 - 7.2) distilled water.
- Pour diluted stain over the film (about 5 ml/film is required) and keep for 30 to 45 minutes
- Then flush the slide in a gentle flow of tap water. Keep the slide in an upright position to drain and dry.
- Examine the stained film under oil immersion and look for LD bodies.

20.2.2.2 Interpretation

The LD bodies or Amastigote form of the parasite look as pinkish oval outline with dark purple kinetoplast and nucleus (Fig-20.1)

L.D. Bodies

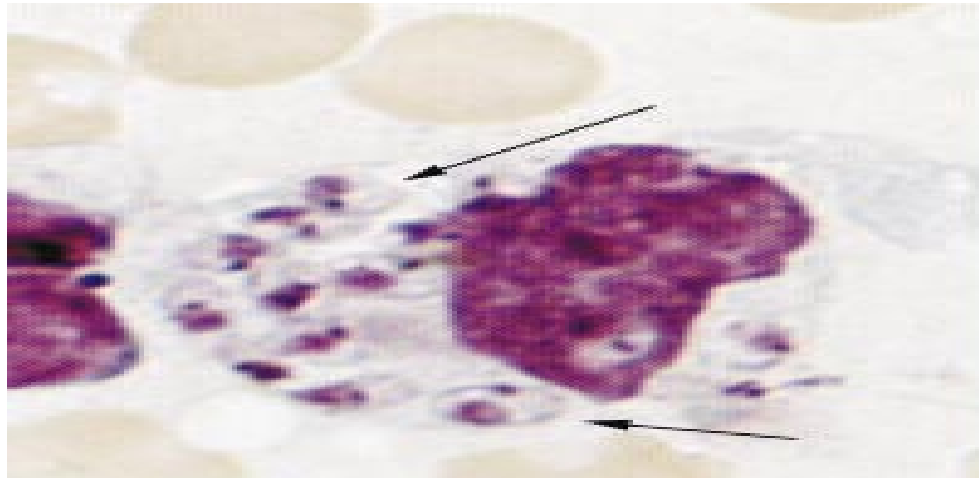


Fig: 20.1

20.2.2.3 Quality control

Leishman and giemsa staining of test specimen should be carried out with parallel staining of peripheral blood smear and known positive LD body slides to ensure proper staining results. Microscope lense and objectives should be kept dust free and oil free. The microscope mirrors should be free of dust and fungus growth.

CHAPTER - 21

LABORATORY DIAGNOSIS OF PLAGUE

Plague is a bacterial infection of small mammals transmitted from animal to animal by the bite of infected fleas. Humans acquire plague from the bite of infected fleas, from direct contact with contaminated tissue and by inhalation of bacteria laden droplets. Bubonic plague is most common form of infection resulting from the bacteria being taken up by the host macrophages in the lymph node closest to the flea bite site. The affected lymph node becomes inflamed (bubo), enlarged and painful as the bacteria replicate. From the infected lymph node the bacteria sometimes multiply and becomes blood borne (septicemic) and occasionally lodge in the lung (pneumonic). When plague infection becomes pneumonic, direct person to person transmission via respiratory droplet spread of bacteria becomes possible. Early diagnosis with prompt antibiotic treatment is effective against all forms of plague infection. WHO laboratory test criteria for diagnosis of plague are as follows:

21.1 Laboratory test criteria for diagnosis of plague

21.1.1 Suspect Plague

1. Clinical symptoms that are compatible with plague, e.g., fever, sepsis syndrome, lymphadenopathy and/or acute pneumonitis in a person who resides in or recently traveled to a plague-endemic area
2. If gram negative and/or bipolar-staining coccobacilli are seen on a smear taken from affected tissues, e.g.
   - Bubo (bubonic plague)
   - Blood (septicemic plague)
   - Tracheal/lung aspirate (pneumonic plague)

21.1.2 Presumptive

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

21.1.3 Confirmed

- Isolate identified as Y.pestis by phage lysis of cultures or a significant (equal or more than 4-fold) change in antibody titre to the F-1 antigen in paired serum specimens

21.2 Collection, Storage and Transportation of specimen

21.2.1 Specimen

21.2.2

- Bubo aspirate
- Sputum
- Blood
- Throat Swab

21.2.2.1 Materials and supplies

- 10 - 20 ml syringe
- 18, 20 gauze needle
- petridish
- sterile swab stick
- clean dry vials
- sterile normal saline
- Cary Blair Transport medium

21.2.3 Precautions for collection of specimens

21.2.3.1 Collection of Bubo aspirate

- Sterilize the skin surface of the bubo with tincture iodine.
- Draw a few ml of sterile physiological saline in a 10 - 20 ml syringe fitted with 18 or 19 gauge needle.
- Puncture the bubo and apply suction. If bubo fluid could not be aspirated, inject saline into the bubo and aspirate again.
- Make smears with the bubo aspirate.
- Transfer the bubo aspirate in the Cary Blair Transport Medium (CBT) for transportation to the laboratory.

21.2.3.2 Sputum

- Ask the patient to expectorate into a sterile, wide mouthed container such as petri dish or in bottle containing CBT medium.
- Put it in CBT medium for transportation to the laboratory.
- Make smears with the sputum.

21.2.3.3 Throat swab

Collect throat swab with sterile swab stick and put in Cary Blair Transport medium.

21.2.3.4 Blood (see chapte 4 for details))

- Collect 5 ml blood sample in plain vial. Allow the blood to clot.
- Centrifuge and separate serum in another vial.
- Store at $+4^0$C.
- Transport to the laboratory under cold chain for serological investigations.
- Collect acute and convalescent serum samples of the patients at an interval of at least 14 days.

21.3 Precautions in handling and collecting specimens

As these specimens are likely to contain infectious substances, the following precautions should be applied :

- Strict aseptic technique (gowns, gloves, masks)
- Wash hands before and after the collection of material
- Place the specimen aseptically in an appropriate sterile container
- Tightly close the container
- Label the container

21.4 Packaging/Transportation of specimen

The samples should be appropriately packaged in a triple packaging system as given in chapter-4 and should be sent to the designated referral laboratory for further processing. The processing of the samples for Y.pestis are not to be carried out at district level.

Since plague is notifiable disease, the sample should be collected and sent to referral laboratories.

21.5 Reference diagnostic laboratories

- Central Plague Laboratory, Zoonosis Division, National Institute of Communicable Diseases. Tel 011-23912901, 011-239123148
- Plague Surveillance Unit, NICD, NTI Campus, 8-Bellary Road, Banglore. Tel 080-23446723

CHAPTER-22

LABORATORY DIAGNOSIS OF LEPTOSPIROSIS

During the past decade many new zoonoses have emerged and resulted in epidemics causing significant morbidity and mortality in human beings in different parts of India's coastal belt due to the rapid ecological changes. Outbreaks of leptospirosis have been reported from coastal districts of Gujarat, Maharashtra, Kerala, Tamil Nadu and Andaman and Nicobar Islands. Cases have also been reported from Goa, Karnataka, Orissa, and Andhra Pradesh. However, the entire shoreline is extremely fragile in nature. States/union territories like West Bengal, Lakshwadeep from where the disease has not been reported, may be affected in future. The deltas of Godavari, Krishna, Mahanadi and Cauveri in addition to Ganga may also be future potential areas for Leptospirosis.

22.1 Mode of Transmission

Infection is acquired through contact with the environment contaminated with urine of an animal that is a carrier or is suffering from the disease caused by leptospires. Infection may also arise from bathing or accidental immersion in the fresh water lakes, rivers, or canals contaminated with the urine of the infected livestock that has been using the water for drinking or wallowing. Water may also get contaminated with rodent nests located on the banks especially after heavy rainfalls and inundations.

22.2 Clinical manifestations

Guidelines for collection, storage, transportation, and processing of clinical material for diagnosis of leptospirosis from suspected cases

22.3 Collection and transportation of blood, urine, and CSF for isolation of leptospires

The isolation of leptospires from clinical specimens is the backbone of diagnostic work and it confirms the clinical diagnosis of the disease. Leptospires can be isolated from a variety of clinical specimens such as blood, urine, CSF, aqueous humor, amniotic or peritoneal fluids or from autopsy tissues such as kidney or liver. Collection of biological material should be done taking recommended universal precautions.

22.3.1 Blood

22.3.1.1 Ideal time

7-10 days of the onset of the disease

22.3.1.2 Media

Ellinghausen-McCullough-Johnson-Harris (EMJH), Fletcher's and Stuart's, may be procured from Reference laboratory.

22.3.1.2 Procedure

- Swab the area with the spirit
- Draw the blood using sterile syringe and needle by vein puncture
- Inoculate two drops and four drops of blood into two tubes of 5 ml. Culture medium (two drops in the first tubes and four drops in the other).
- Transport the inoculated media to reference laboratory at room temperature.

22.3.2 Urine

22.3.2.1 Time: 10-30 days after the onset of the disease.

22.3.2.2 Media: Same as above

22.3.2.3 Procedure

- Collect the sample of midstream urine (freshly void midstream urine processed within 2 hours is the ideal specimen)
- Dilute the urine as follows using sterile test tubes and sterile phosphate buffer pH 7.2.
  - (a) Add 0.4 ml of urine to 3.6 ml of PBS pH 7.2 (1 in 10)
  - (b) Add 3 ml of (a) to 3 ml of PBS (I in 20)
  - (c) Add 2 ml of (b) to 2 ml of PBS (I in 40)
  - (d) Add I ml of (c) to I ml of PBS (I in 80)
- Each resulting 0.5 ml dilution of above is inoculated into 4 separate 5 ml volume of medium
- Label tubes with dilution

Transport the inoculated media to reference laboratory at room temperature

Note: Urine may have acidic pH in many cases. Therefore, urine should be collected in tubes containing equal amount of PBS with pH 7.2.

22.3.3 CSF

22.3.3.1 Time: 5-10 days of the onset of the disease.

22.3.3.2 Medium: As above

22.3.3.3 Procedure:

Inoculate 0.5 ml of CSF into 5 ml of culture media. Follow the same procedure as for blood collection.

22.4 Collection of serum samples

Refer to Chapter No. 4 i.e. sample collection and transportation. The serum sample

for serological diagnosis should be collected on 7th day of illness or onwards. A second serum sample should also be collected after a gap of 5 to 7 days.

22.5 Serological diagnosis

Tests available are :

- Leptospira agglutination test
- IgM ELISA / other IgM antibody tests

22.5.1 Leptospira agglutination test

22.5.1.1 Leptospira Antigens

Leptospira antigen is a whitish turbid homogeneous solution containing L.interrogans serovar icterohaemorrhagiae, L.interrogans serovar autumnalis, L.interrogans serovar hebdomadis, L.interrogans serovar australis and L.interrogans serovar canicola, which are inactivated and used for serodiagnosis of Leptospirosis.

22.5.1.2 Procedure

The serum under test is warmed at 56^{0}C for 30 minutes beforehand and is then diluted ten fold with physiological saline.

22.5.1.3 Screening Test

1. With 10 small test tubes, produce 5 series for 2 tubes each.
2. Pour ten fold diluted serum into one test tube of each series and physiological saline into the other test tubes in 0.25 ml quantities.
3. Add 0.25 ml of the antigen solution for each series and mix thoroughly.
4. Centrifuge at 2000 rpm for 5 minutes after warming at 50^{0}C for 2 hours.

22.5.1.4 Interpretation

1. Lightly shake each tube, and carefully visually check for the presence of any agglomerates while gently loosening precipitated bacteria. Check, however, that all control tubes are negative, have no agglomerate, and accordingly have a uniform suspensions.
2. Keep a record of the names of antigens that clearly produce agglomerates and, attach the names of the estimated pathogens to them.

22.5.2 Leptospira ELISA Test

ELISA test is an enzyme immunoassay for the detection of antibodies to Leptospira biflexa (serovar patoc 1) for the serological confirmation of infections in serum, and plasma. This test is intended to be performed by trained laboratory personnel only. **The kits are commercially available and manufacturer's instructions should be strictly followed.**

22.5.2.1 Reagents

- Test strips: Microwells containing leptospira antigen - 96 test wells in a test strip holder
- Enzyme conjugate: Anti-human IgM antibody conjugated to peroxidase enzyme.
- Positive control serum: Diluted positive IgM human serum.
- Negative control serum: Diluted negative human serum.
- Substrate : Tetramethylbenzidine (TMB).
- RF absorbent: Goat anti-human IgG.
- Wash concentrate (20X): buffer and surfactant.
- Dilution buffer: Buffered protein solution.
- Stop solution :1 M phosphoric acid.

22.5.2.2 Precautions

- Do not use solutions if they precipitate or become cloudy. Wash concentrate may show crystallization upon storage at 2-8^{0}C. Crystallization will disappear after dilution to working strength.
- Dilution buffer is a colloidal solution. It will appear opaque and have a precipitate form.
- Do not use serum that may have supported microbial growth, or is cloudy due to high lipid content. Samples high in lipids should be clarified before use.
- Do not add azide to any of the reagents or serum.

22.5.2.3 Storage

Store between 2 - 8^0 C as per kit insert.

Squeeze bottle containing diluted wash buffer may be stored at room temperature.

22.5.2.4 Materials required but not provided

- Pipettes
- Squeeze bottle for washing strips (narrow tip is recommended)
- Reagent grade water and graduated cylinder
- Tubes for sample dilution
- Absorbent paper
- ELISA plate reader with a 450 nm and 620 - 650 nm filter (optional if results are read visually)

22.5.2.5 Procedure

Test samples: Make a 1:40 dilution of patients sera using the dilution buffer (e.g. 10µl sera and 390µl dilution buffer).

22.5.2.6 Performance of Tests

1. Break off number of wells needed (two for controls plus number of samples) and place in strip holder.
2. Note: Negative and positive controls are supplied pre-diluted. Do not dilute further. Add 40µl of RF Absorbent in a tube and add 100µl of positive control and negative to each tube respectively (#1, #2). Transfer all 140µl mixture to well (#1, #2) after five minute incubation.

Dilute patient sera 1:40 in dilution buffer. To 100µl of diluted serum add 40µl of RF Absorbent. Mix well,. Incubate in tube for 5 minutes. Transfer all 140µl of test samples to the remaining wells (#3-#96).

3. Incubate at room temperature (15 to 25^0 C) for 10 minutes.
4. Shake out contents and wash 3 times with the diluted wash buffer.
5. Add 2 drops of enzyme conjugate to each well.
6. Incubate at room temperature for 10 minutes.
7. Shake out contents and wash 3 times with wash buffer.
8. Slap wells against paper towels to remove all liquid.
9. Add 2 drops of the chromogen to every well.
10. Incubate at room temperature for 5 minutes.
11. Add 2 drops of the stop solution and mix by tapping strip holder.
12. Read within one hour of adding stop solution.

22.5.2.7 Reading Results

Visually: Look at each well against a white background (e.g, paper towel) and record as clear or +,++ or +++ reaction.

ELISA Reader: Zero reader on air. Set for bichromatic readings at 450/620 nm.

22.5.2.8 Limitations of the Procedure

- Serologic results are an aid in diagnosis but cannot be used as the sole method of diagnosis.
- The ELISA has been tested against many serovars, but cannot guarantee that all strains will react equally.

22.5.2.9 Quality Control

The use of controls allows validation of kit stability. The kit should not be used if any

of the controls are out of range. Expected values for the controls are:

Negative - 0.0 to 0.3 OD units

Positive - 0.5 OD units and above.

22.5.2.10 Trouble Shooting

Negative control has excessive color development.

Reason: inadequate washings

Correction: wash more vigorously. Remove excessive liquid from the wells by tapping against an Absorbent towel.

Do not allow test wells to dry out.

22.5.2.11 Interpretation of the Test

22.5.2.11.1 Initially Non-reactive

Samples interpreted as non-reactive (0.0-0.3 OD units, or zero color) indicate antibody is not present in the sample. Since antibody may not be present during early disease, (5-8 days incubation), confirmation 2-3 weeks later is indicated for laboratory diagnosis. At this later time, patients showing weak reactions (0.5 - $\preceq$ 1.0 or +, ++) should be further tested by alternate methods or re-tested 10-14 days later. A convalescent serum with a significant reaction (>1.0 OD) indicates the formation of specific antibody against leptospira. An initially negative result followed by a positive result implies seroconversion.

22.5.2.11.2 Initially Weakly Reactive

Weakly reactive specimens should be cautiously interpreted. In normal populations, weakly reactive samples are infrequent but possible. Confirmation using a sample collected 2-3 weeks later (paired acute and convalescent sera) is recommended. >1.0 OD in the second sample confirms the presence of recent, specific antibody. [Caution: If this is a cross-reactive antibody, the convalescent serum sample may not show a higher antibody level than the acute sample.] If sample reading remains at $\geq$ 0.5 - $\preceq$ 1.0 OD, or +, ++, a second methodology should be considered, or the sample may be interpreted as taken beyond rising titer (titer declining).

22.5.2.11.3 Initially Reactive

Samples interpreted as strongly reactive (>1.0 OD or +++ or > may indicate the presence of specific antibody. Antibody presence alone cannot be used for diagnosis of acute infection, since antibodies from prior exposure may circulate for a prolonged period of time.

CHAPTER- 23

CLINICAL BIOCHEMISTRY

Introduction

Laboratory services play a key role in the primary health care system. Investigations done in a laboratory serve as an important adjunct to the clinical history based diagnosis. In fact it helps in the confirmation of diagnosis of the disease and hence lead to the exact/defined treatment.

Laboratory investigations/assays can be performed on various biological fluids and their products. Blood is the most important biological fluid and generally blood and its products are analysed in a clinical set up. Blood can either be venous or peripheral. Blood products include serum and plasma, which contain many metabolic substances like sugars, electrolytes, proteins and markers of liver function tests, kidney function tests and cardio vascular diseases. Information about the levels of these markers gives important preliminary indication about the liver, kidney and heart status of the individual. Besides blood, biochemical investigations can also be performed on CSF and urine.

Basically blood is composed of a liquid portion known as plasma which contains many metabolic substances such as proteins, urea nitrogen, glucose, creatinine, sodium, carbon dioxide, chloride and cholesterols etc. and a solid portion contains various cells like WBC, RBC and platelets.

23.1 Equipment and Glasswares

23.1.1 Following instrument and Glasswares are required

- Colorimeter/Spectrophotometer/ Semi autoanalyzer with micro and macro Cuvetts
- Electronic balance
- Incubator
- Hot air Oven
- Water distillation plant
- Centrifuge table top (up to 5000-6000 rpm)
- Vortex mixer
- Eppendorff pipettes and tips (20-1000µl)
- Glass test tubes, 10 ml capacity (Borosil/Corning)
- Glass pipettes (1, 2, 5, 10ml)
- Measuring cylinders, flasks, beakers, reagents bottles (50, 100, 250 and 500ml)
- Test tube stands

- Timer
- Vacutainer/blood collection tubes (1, 2, 5, 10, ml with screw cap)
- Syringes (1, 2, 5, 10ml)
- Needles (22, 23, 24G)
- Repetitive dispenser

23.1.2 Maintenance of Equipments

Spectrophotometer/Semi automatic analyser, are sophisticated, microprocessor controlled optical Instruments. It is advisable that the room should have adequate ventilation and free from chemical fumes for proper functioning of equipment.

For optical components to retain their quality, it is necessary to operate the instruments in dust free, non-corrosive environment. Optical surfaces must be cleaned or replaced periodically. With time, the output of UV source gradually decreases which results in decrease in the sensitivity of the instrument. In such condition, UV lamp should be replaced. Decrease output from the visible source is normally not that significant to cause decrease sensitivity. However, if decreased sensitivity is observed in the visible range, the visible lamp should be checked for a brown film coating inside the lamp. If the brown coating is noticed, the lamp should be replaced.

The cuvetts should always be washed properly and kept dried after use. Care of scratches on the cuvetts should be taken and if scratches are there, the cuvetts should be changed.

Such equipments should always be kept under Annual Maintenance Contract (AMC).

23.2 Quality Control

Internal and external quality controls (QC) are the two different monitoring procedures for the quality control in clinical biochemistry. Internal QC is essential for daily monitoring of precision and accuracy. Daily QC is of great value in detecting any error, large enough to invalidate the medical usefulness of the laboratory results. External quality assurance (EQA) is important for evaluating inter laboratory precision. The concepts of quality assurance has gained widespread acceptance today among Indian laboratories. Laboratory personnel must know that QC is an obligatory to the patient, that is designed to give the analyst confidence in the method used and that its purpose is not to find or punish mistakes. Please see detail in chapter 24.

23.2.1 Internal Quality Control Assessment (IQCA)

In clinical chemistry quality control programs are used to minimize random and systematic error. Through internal quality control, the laboratory can ensure that the results being issued by the laboratory are reliable to allow decision to be taken with

confidence.

23.2.1.1 Accuracy and precision are two terms of internal quality control assesment, which are used normally in clinical laboratory.

23.2.1.2 Accuracy is the degree of agreement between a measured value and its true value. Accuracy can also be defined as the difference between the measured value and the true value.

23.2.1.3 Precision refers to how close values are to one another or it is agreement between replicate values. More correctly precision refers to reproducibility. It is expressed in Standard Deviation (SD) or as a Coefficient of Variation (CV). A third term reliability is also used when a system is both precise and accurate.

Most clinical chemistry laboratories include at least one quality control sample. A quality control sample is a substance, which contains the same analyte that is being tested for, and has a certain nominal range associated with it. Substances that are often confused with QC material are standards and calibrators

A standard has a known exact value and purity associated with it.

A calibrator is a substance with a set of particular values that has been determined. A calibrator may also be a standard and vice versa. A calibrator is often used to adjust an instrument to certain values prior to using samples.

A QC sample is never used to set up an instrument or method because it is run in the same manner as the patient samples and does not allow manipulation of an instrument or method in order to achieve its associated values.Once a QC material is chosen, a laboratory should evaluate the control material during 20- 30 days period. This evaluation includes statistical as well as other performance characteristics such as stability and preparation time. The statistical evaluation generally includes calculation of the mean (X), Standard deviation (S.D).

23.2.1.4 Mean (X) = $\frac{\Sigma X}{N}$

Where Σ X is the sum of individual data, N is number of data point

23.2.1.5 Standard Deviation (S.D) = $\sqrt{\Sigma (X_1\text{-}X)^2 \ / \ N\text{-}1}$

Where X_1 is one of individual measurement, and X is the mean. Therefore the Standard Deviation is the deviation of data from the data's mean value. To calculate the standard deviation, we obtain the difference between the mean value and each measurement, square the resulting difference, and add them to determine the sum of the squares. The standard deviation is calculated by dividing the sum of square by N-1.

Table 23.1

Standard Deviation Limits for some common Biochemical Test chemistries

Name of test	Normal range	Test type	Curve type	Standard Deviation (SD) Limits
Blood Sugar (F) Blood Sugar (PP)	(60-110 mg/dl) (60-140 mg/dl)	End point	Blanked Linear	8.00
Total Cholesterol	(150-250 mg/dl)	End point	Blanked Linear	10.0
Triglycerides	(65-165 mg/dl)	End point	Blanked Linear	10.0
HDL Cholesterol LDL Cholesterol	(35-80 mg/dl) upto 140 mg/dl	End point	Blanked Linear	10.0
Serum Urea	(10-50 mg/dl)	Kinetic	Blanked Linear	3.0
Serum Uric Acid	(2.4 -7 mg/dl)	End point	Blanked Linear	0.5
Serum Creatinine	(0.4 - 1.4 mg/dl)	Kinetic	Blanked Linear	0.3
Serum Bilirubin (Total)	(upto 1.0 mg/dl)	End point	Blanked Linear	0.2
Serum Bilirubin (Direct)	upto 0.25 mg/dl)	End point	Blanked Linear	0.25
Total Protein	6.6-8.7 gm/dl	End point	Blanked Linear	13.0

It is recommended that each laboratory establishes its own reference range to reflect the age, sex, diet and geographical location of the population.

23.3 Standardization (Calibration)

Proper calibration and standardization of instruments, reagents and methods is essential in obtaining accurate results. The choice of a calibration and standardization technique is affected by the instrumental method, instrument response, interferences present in the sample, purity of regents and number of samples to be analyzed. The most commonly used standardization technique is the analytical or working curve.

23.3.1 Analytical Curve or calibration curve or standardization curve

In the analytical (working) curve technique, a series of standard solutions containing known concentrations of the analyte are prepared. These solutions should cover the concentration range of interest and have a matrix composition as similar to that of the sample solutions as possible. A blank solution containing only the solvent matrix is also analyzed, and the net readings of standard solution minus blank (background) are plotted versus the concentrations of the standard solutions to obtain the working calibration curve. When using a single standard of known concentration the standardization is called single point standardization. But the preferred approach to standardizing a method is to prepare a series of standards each containing different concentration. This is known as multiple - point standardization. In multiple - point standardization at least three standards should be used. A plot of measured value (OD) versus concentration of standards is known as calibration curve. A calibration curve shows us the relation ship

between the measured value and the analyte's concentration. The most useful calibration curve is a straight line. Since the method's sensitivity is the same for all concentrations of analyate. Results from calibration curve provide a systematic means of identifying source of error in analysis, such as depletion of test reagents, a defective instrument, or inaccurate standard solution.

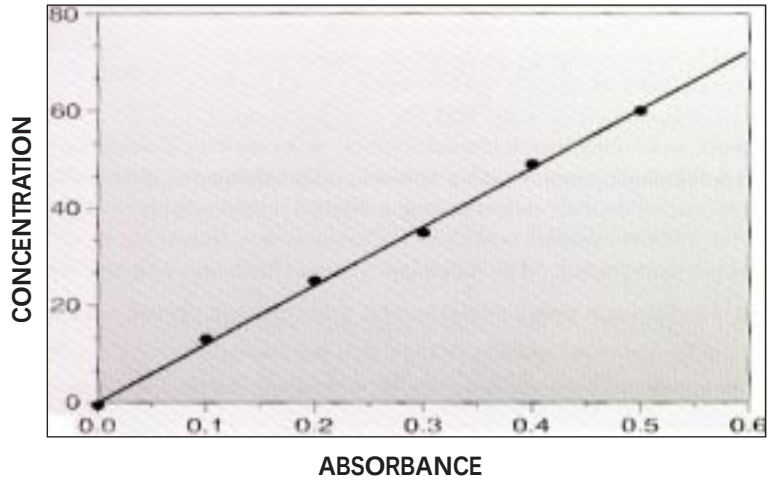


Figure: Normal calibration curve for the hypothetical data.

23.4 Blood Sugar

23.4.1 Principle: Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts under catalytic influence of peroxidase with phenol and 4- aminophenazone to form a red-violet quinoneimine dye as indicator.

23.4.2 Objectives/Importance of estimation

a) Blood sugar estimation is mostly required for detection of hyper & hypo glycemia.

b) Hypoglycemia i.e low blood sugar level, below 60 mg/dl occurs frequently by over dosage of insulin, hypothyroidism, hypopituitirism & hypoadrenalism (Addison's diseases)

c) Hyperglycemia i.e. high level of blood sugar upto 500 mg/dl is found in patient of diabetes.

23.4.3 Sample: Blood in sodium fluoride, heparinized plasma, EDTA Plasma, Serum, CSF. Fasting sample of blood is required and PP sample is taken exatly after two hours of the meal.

23.4.4 Reagents: The kit is commercially available, instructions therein should be strictly followed.

Glucose Reagent
Glucose standard (100 mg/dl)

23.4.5 Procedure: Pipette reagents into test tubes

	Reagent Blank (µl)	Standard (µl)	Sample (µl)
Reagent	2000	2000	2000
Standard	-	20	-
Sample	-	-	20
Distilled water	20	-	-

Mix, incubate for 5 min at 37^{0}C. Measure the absorbance of standard (A standard) and sample (A sample) against Reagent blank at 546 nm.

23.4.6 Calculation: Glucose (mg / dl) $= \frac{\text{A sample}}{\text{A standard}} \times 100$

23.4.7 Normal Range:

Serum /plasma (Fasting)	60-110 mg/dl
Post prandial (PP)	70-140 mg/dl
CSF sugar	50-80 mg/dl

23.4.8 Interference: Uric acid, ascorbic acid, glutathione, anticoagulants, bilirubin and creatinine interfere with the test if present above physiological concentrations.

Note: CSF sugar can be estimated in the same manner.

23.5 Total Cholesterol

23.5.1 Principle: The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4 - aminoantipyrine in presence of phenol and peroxidase.

23.5.2 Objectives/Importance of estimation

a) To assay serum cholesterol level which rises concomitantly with total lipid level.
b) Hyper cholesterolaemia i.e. high level of cholesterol is found in nephrotic syndrome, diabetes, obstructive jaundice & hypothyroidism.
c) Hypo cholesterolaemia i.e. low level of cholesterol is found in severe hepatic damage (cirrhosis), hyperthyroidism and malnutrition.

23.5.3 Samples: Serum, Heparinised plasma, EDTA Plasma, (Fasting sample is preferred).

23.5.4 Reagents: The kit is commercially available, instructions therein should be strictly followed.

Cholesterol Reagent

Standard (200 mg/dl)

23.5.5 Procedure: Pipette reagents into test tubes

	Reagent Blank (µl)	Standard (µl)	Sample (µl)
Reagent	2000	2000	2000
Standard	-	20	-
Sample	-	-	20
Distilled water	20	-	-

Mix, incubate for 5 min at 37^{0}C. Measure the absorbance of samples (A sample) & standard (A standard) against reagent blank at 540 nm.

23.5.6 Calculation: Total Cholesterol (mg / dl) $= \frac{\text{A sample}}{\text{A standard}} \times 200$

23.5.7 Normal Range: Total Cholesterol = 150 - 250 mg/ dl

23.5.8 Interference: Haemoglobin value above 200 mg/dl and bilirubin value above 5 mg/dl interfere with the test.

23.6 Serum Triglyceride (TG)

23.6.1 Principle: Triglyceride is determined after enzymatic hydrolysis with lipases. The quinoneimine indicator is formed from hydrogen peroxide, 4 -aminophena zone and 4 - chlorophenol under the catalytic influence of peroxidase.

23.6.2 Objectives: To assess the lipid profile status because high levels of triglycerides may be indicative of coronary artery disease (CAD).

23.6.3 Sample: Serum, heparinized plasma or EDTA plasma, (fasting sample is preferred).

23.6.4 Reagents: The kit is commercially available, instructions therein should be strictly followed.

- Triglyceride Reagent.
- Standard (200 mg/dl)

23.6.5 Procedure: Pipette reagents into test tube

	Reagent Blank (µl)	Standard (µl)	Sample (µl)
Reagent	2000	2000	2000
Standard	-	20	-
Sample	-	-	20
Distilled water	20	-	-

Mix, incubate for 5 min at 37^0C. Measure the absorbance of samples (A sample) and standard (A standard) against reagent blank at 546 nm.

23.6.6 Calculation: Triglyceride (mg / dl) $= \frac{\text{A sample}}{\text{A standard}} \times 200$

23.6.7 Normal Range: Triglyceride = 65 - 165 mg/dl

23.6.8 Interference: Haemoglobin concentration above 600 mg/dl or bilirubin concentration above 29 mg/dl interfere with the test.

23.7 High Density Lipoprotein (HDL) Cholesterol

23.7.1 Principle: Low-density lipoproteins (LDL & VLDL) are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation the cholesterol concentration in the HDL (high density lipoprotein) fraction, which remains in supernatant is determined.

23.7.2 Objectives/Importance of estimation

a) Determination of only total cholesterol alone is not sufficient to assess the coronary risk and hence the determination of HDL cholesterol which is inversely associated with the risk of coronary artery disease is needed.

b) Since high level of HDL has a protective role, as it is believed to remove cholesterol from tissues, its estimation is a useful parameter.

23.7.3 Sample: Serum, heparinized plasma, EDTA plasma. (Fasting sample is required)

23.7.4 Reagents : The kit is commercially available, instructions therein should be strictly followed.

Precipitant, cholesterol reagent, standard (200mg/dl).

23.7.5 Procedure

Precipitation: Pipette precipitant reagent and clinical sample into centrifuge tube

Precipitant reagent	1000 µl
Sample	500 µl

Mix and allow to stand for 10 min. at room temperature. Then centrifuge for 10 min. at 4000 rpm. Separate off the HDL rich clear supernatant within two hours.

HDL Cholesterol Procedure:

Pipette reagents into test tubes:

	Reagent Blank (µl)	Standard (µl)	Supernatant (µl)
Cholesterol Reagent	2000	2000	2000
Standard	-	100	-
Supernatant	-	-	100
Distilled water	100	-	-

Mix, incubate for 5 min at 37^{0}C. Measure the absorbance of sample (A sample), standard (A standard) against reagent blank at 540 nm.

23.7.6 Calculation: HDL Cholesterol (mg / dl) = $\frac{\text{A sample}}{\text{A standard}} \times 200$

23.7.7 Normal value: HDL Cholesterol = 30-80 mg/dl

23.7.8 LDL Cholesterol (mg/dl) = Total cholesterol – $\left(\frac{\text{Triglycerides}}{5}\right)$ - HDL cholesterol

23.7.9 Normal value: LDL cholesterol = upto 150 mg/dl

23.7.10 Interference: Haemoglobin concentration > 200 mg/dl or bilirubin concentration > 10 mg/dl interfere with the test. Ascorbic acid concentration >2.5 mg/dl will give lower values.

Note: Lipaemic sample with TG conc.>1200 mg/dl should be diluted with 1+ 9 with normal saline and the corresponding result should be multiplied by 10.

23.8 Aspartate Aminotransferases (AST/SGOT)

23.8.1 Principle: a- Oxoglutarate reacts with L-aspartate in presence of AST to form L-glutamate and oxaloacetate. The NADH consumption by oxaloacetate kinetically gives result of AST. Aminotransferases are a group of enzymes that catalyse the interconversion of aminoacids and a- oxo acids by transfer of amino group.

23.8.2 Objectives: To estimate levels of AST in serum as elevated levels of this can be indicative of both heart and liver diseases.

23.8.3 Sample: Serum, heparinized plasma, EDTA plasma

23.8.4 Reagents: The kit is commercially available, instructions therein should be strictly followed.

1. Enzyme/substrate
2. α- Oxoglutarate

23.8.5 Procedure: Pipette reagents into test tubes

	Macro method	Semi-micro method
1. Sample	200 µl	100 µl
2. Enzyme/substrate	2000 µl	1000 µl
Mix, incubate for 5 - 10 min. at 37^{0}C then add		
3. α- Oxoglutarate	100 µl	50 µl

Mix, read initial absorbance at 340 nm and start timer simultaneously. Read again after 1,2,3 min.

$A_2 - A_1 = \Delta$ A sample or Δ A standard

23.8.6 Calculation: AST (U/l) = 1817 X Absorbance Unit/min (Δ A/min)

23.8.7 Normal value: AST = upto 40 U/l

23.8.8 Interference: Avoid haemolysis as it interferes in the measurement of ALT.

23.9 Alanine aminotransferases (ALT/SGPT)

23.9.1 Principle: α- Oxoglutarate reacts with L-alanine in presence of ALT to form L-glutamate and pyruvate. The consumption of NADH kinetically by pyruvate gives concentration of ALT. The aminotransferases are a group of enzymes that catalyse the interconversion of aminoacids and α-oxoacids by transfer of amino group.

23.9.2 Objectives: ALT measurement are useful in diagnosis and treatment of certain liver diseases (e.g. viral hepatitis & cirrhosis) and heart diseases. Elevated level of transaminases can indicate myocardial infarction, hepatic diseases, muscular dystrophy and organ damage. Elevated level of ALT in serum are rarely observed except in liver diseases.

23.9.3 Sample: Serum, heparinized plasma, EDTA plasma

23.9.4 Reagents: The kit is commercially available, instructions therein should be strictly followed.

1. Enzyme/Substrate
2. a- Oxoglutarate

23.9.5 Procedure: Pipette reagents into test tubes

	Macro method	Semi-micro method
Sample	200 µl	100 µl
Enzyme/Substrate	2000 µl	1000 µl
	Mix, incubate for 5 - 10 min at 37^{0}C then add	
α- Oxoglutarate	100 µl	50 µl

Mix, read initial absorbance at 340 nm and start timer simultaneously. Read again after 1, 2, 3 min.

$A_2 - A_1 = \Delta$ A sample or Δ A standard

23.9.6 Calculation: ALT (U/l) = 1825 X Absorbance Unit/min. (Δ A/min)

23.9.7 Normal value: ALT = upto 40 U/l

23.9.8 Interference: Gross haemolysis will produce falsely elevated result. The affect of various drugs on ALT activity should be taken into consideration in the case of patient receiving large doses of drugs.

23.10 Serum Alkaline Phosphatase (ALP)

23.10.1 Principle: The substrate p-nitro phenyl phosphate is hydrolyzed by Alkaline Phosphatase from the sample in the presence of magnesium ions to form p-nitrophenol which is yellow in colour and can be read at 405 nm.

23.10.2 Objectives: Serum Alkaline phosphatase measurment are of diagnostic use in the investigation and treatment of hepatobiliary disease and bone diseases associated with increased osteoblastic activity. Also useful in diagnosis of parathyroid and intestinal diseases.

23.10.3 Sample: Serum, heparinized plasma

23.10.4 Reagents: The kit is commercially available, instructions therein should be strictly followed.

buffer/substrate

23.10.5 Procedure: Pipette reagents into test tube:

Reagent	3000 µl
Sample	50 µl

Mix, read initial absorbance at 405 nm and start timer simultaneously. Read again after 1, 2, 3 min.

23.10.6 Calculation: ALP (U/l) = 3300 X Absorbance Unit/min. (Δ A/min)

23.10.7 Normal value: ALP = 98 - 306 U/l

23.10.8 Interference: Avoid haemolysis as it interferes with the assay. Use only heparinized plasma that is free from haemolysis. Other anticoagulants interfere with the test.

Note: Alkaline phosphate increases slowly with storage of sample.

23.11 Total Bilirubin

23.11.1 Principle: Total bilirubin is determined in the presence of Dimethyl sulphoxide (DMSO) by the reaction with diazotised sulphanilic acid.

23.11.2 Objectives / Importance of estimation

a) Estimation of serum Bilirubin is of great value in investigation of suspected liver diseases.

b) In obstructive jaundice generally direct bilirubin rises while in hepatic and erythrocytic jaundice both direct and indirect bilirubin may rise.

23.11.3 Sample: Serum, heparinized plasma, EDTA plasma

23.11.4 Reagents: The kit is commercially available, instructions therein should be strictly followed.

Diazo reagent, bilirubin standard

23.11.5 Procedure

Pipette reagents into test tube

	Reagent Blank (µl)	Standard (µl)	Sample (µl)
Diazo Reagent	2000	2000	2000
Standard	-	40	-
Sample	-	-	40
Distilled water	40	-	-

Mix, incubate for 5 min at 37^0C and read absorbance of samples (A sample) and Standard (A standard) at 546 nm.

23.11.6 Calculation: Total bilirubin (mg / dl) = $\frac{\text{A sample}}{\text{A standard}} \times 2$

23.11.7 Normal Range: Total bilirubin = 0.2 - 1.0 mg/dl

23.11.8 Interference: Haemolysis interferes with the test. Fresh sample should be kept out of direct light. A sample blank should be carried out with the lipaemic and lysed samples. The absorbance of sample blank is subtracted from the absorbance of sample before calculating the concentration in the sample.

23.12 Direct Bilirubin

23.12.1 Principle: Direct bilirubin is determined in the absence of an accelerator by the reaction with diazotised sulphanilic acid.

23.12.2 Objectives / Importance of estimation

a) Estimation of serum Bilirubin is of great value in investigation of suspected liver diseases.

b) In obstructive jaundice generally direct bilirubin rises while elevated levels of direct and indirect bilirubin gives an indication of hepatic and erythrocytic jaundice.

23.12.3 Sample: Serum, heparinized plasma, EDTA plasma

23.12.4 Reagent: The kit is commercially available, instructions therein should be strictly followed.

Diazo Reagent

Standard

23.12.5 Procedure

Pipette reagents into test tube

	Reagent Blank (µl)	Standard (µl)	Sample (µl)
Diazo Reagent	2500	2500	2500
Standard	-	100	-
Sample	-	-	100
Distilled water	100	-	-

Mix, allow to stand for 5 min and then immediately read absorbance of samples (A sample), standard (A standard) against reagent blank at 546 nm.

23.12.6 Calculation: Direct bilirubin (mg / dl) = $\frac{\text{A sample}}{\text{A standard}} \times 2$

23.12.7 Normal Range: Direct bilirubin = upto 0.25 mg/dl

23.12.8 Interference: Haemolysis interferes with the test. Fresh sample should be kept out of direct light. A sample blank should be carried out with the lipaemic and lysed samples. The absorbance of sample blank is subtracted from the absorbance of sample before calculating the concentration in the sample.

23.13 Blood Urea

23.13.1 Principle: Urea is hydrolyzed in presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced combines with α-Oxoglutarate and NADH to yield glutamate and NAD^+ in presence of glutamate dehydrogenase.

23.13.2 Objective/Importance of estimation

a) To assess kidney function.

b) An increase in blood urea may occur in a number of diseases in addition to those in which kidney is primarily involved.

c) Decrease in blood urea may occur in pregnancy, severe liver disease, nephrotic syndrome.

23.13.3 Samples: Serum, heparinized plasma, EDTA plasma.

23.13.4 Reagent: The kit is commercially available, instructions therein should be strictly followed.

Reagent (working)

Standard (80 mg/dl).

23.13.5 Procedure: Pipette reagents into test tubes.

	Reagent Blank (µl)	Standard (µl)	Sample (µl)
Reagent	2000	2000	2000
Standard	-	20	-
Samples	-	-	20
Distilled water	20	-	-

Mix, read initial absorbance A_1 of standard and samples after 30 second at 600 nm and read A_2 of samples and standard exactly after 1 min.

$A_2 - A_1 = \Delta$ A standard or Δ A sample

23.13.6 Calculation: Urea (mg / dl) $= \frac{\Delta \text{ A sample}}{\Delta \text{ A standard}} \times 80$

23.13.7 Normal Range: Urea = 10 - 50 mg/dl.

23.13.8 Interference: Use glassware free from ammonium ions, to avoid interference with the test.

23.14 Serum Creatinine

23.14.1 Principle: Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of complex formed is directly proportional to creatinine concentration.

23.13.2 Objective/Importance of estimation

a) To assess renal function.

b) Creatinine level increases in renal diseases. It is more specific and sensitive indicator of kidney diseases than urea.

23.14.3 Samples: Serum, heparinized plasma, EDTA plasma.

23.14.4 Reagent: The kit is commercially available, instructions therein should be strictly followed.

Picric Acid / Sodium Hydroxide

Standard (2 mg/dl)

Working Reagent: Mix equal volume of picric acid and sodium hydroxide.

23.14.5 Procedure: Pipette reagents into test tube.

	Reagent Blank (µl)	Standard (µl)	Sample (µl)
Working Reagent	2000	2000	2000
Standard	-	200	-
Sample	-	-	200
Distilled water	200	-	-

Mix, incubate at 37^0C for 30 seconds and read the absorbance A_1 of sample and standard at 540 nm. Exactly 2 minutes later, read absorbance A_2 of standard and samples

$A_2 - A_1 = \Delta$ A sample or Δ A standard

23.14.6 Calculations

$$\text{Creatinine (mg / dl)} = \frac{\Delta \text{ A sample}}{\Delta \text{ A standard}} \times 2$$

23.14.7 Normal Value: Creatinine = 0.6 - 1.5 mg/dl (Male)
0.6 - 1.2mg/dl (Female)

23.14.8 Interference: Reaction rate and absorbance of the reaction product are very sensitive to temperature. The specified temperature must therefore be maintained.

Note: Haemolysis interferes with the test. Do not use lipaemic sera.

23.15 Uric Acid

23.15.1 Principle: Uric acid is converted by urease to allantoin and hydrogen peroxidase which under the catalytic influence of peroxidases, oxidize 3,5-dichloro -2-hydroxy benzene sulphonic acid and 4- amino phenazone to form a red-voilet quinoneimine compound.

23.15.2 Importance: Uric acid is a nitrogen containing organic acid. This is the end product of nucleic acid metabolism & is a component of urine. Crystals of this are deposited in joint of people suffering from gout.

23.15.3 Samples: Serum, heparinized plasma, EDTA plasma (fasting sample is required)

23.15.4 Reagents: The kit is commercially available, instructions therein should be strictly followed.

Enzyme Reagent

Standard (10 mg/dl)

23.15.5 Procedure: Pipette reagents into test tube :

	Enzyme Reagent µl	Standard µl	Sample µl
Enzyme Reagent	2000	2000	2000
Standard	-	40	-
Sample	-	-	40
Distilled water	40	-	-

Mix, incubate for 5 min at 37^0C. Measure the absorbance of standard (A standard) and samples (A sample) against reagent blank at 546 nm.

23.15.6 Calculation: Uric Acid (mg / dl) $= \frac{\text{A sample}}{\text{A standard}} \times 10$

23.15.7 Normal Value: Uric Acid = 2.4 - 7.0 mg/dl (Male)
2.4- 6.0 mg/dl (Female)

23.15.8 Interference: Haemoglobin concentration > 100 mg/dl or bilirubin concentration > 20 mg/dl, interfere with the test.

23.16. Total Protein

23.16.1 Principle: Cupric ions, in an alkaline medium, interacts with protein peptide bonds resulting in the formation of a coloured complex.

23.16.2 Objective/Importance of estimation:

a) Hypoproteinaemia i.e. low serum protein is most commonly associated with reduction in albumin synthesis as in malnutrition or in excessive loss of protein in case of nephrotic syndrome.

b) Hyperproteinaemia i.e. high serum protein is associated with increase in gamaglobulin e.g. myeloma and macroglobulineamia.

c) Cerabrospinal fluid (CSF) protein is increased in cases of pyogenic, tubercular and viral meningitis and syphilis.

23.16.3 Sample: Serum, Heparinized plasma, EDTA plasma, CSF.

23.16.4 Reagent: The kit is commercially available, instructions therein should be strictly followed.

Biuret Reagent,

Protein Standard (6.0 g/dl)

23.16.5 Procedure

Pipette reagents into test tube

	Biuret Reagent µl	Standard µl	Sample µl
Biuret Reagent	2000	2000	2000
Standard	-	20	-
Sample	-	-	20
Distilled water	20	-	-

Mix, incubate for 30 min at 37^0C. Measure the absorbance of sample (A sample) and of standard (A standard) against reagent blank at 546 nm.

Note: Take 20 µl of CSF in place of serum and follow the same steps as for serum total protein.

23.16.6 Calculation: Total Protein (g / dl) = $\frac{\text{A sample}}{\text{A standard}} \times 6$

23.16.7 Normal Value: Total Protein (Serum) = 6.6 - 8.7 g/dl

Total Protein (CSF) = 15 - 45 mg/dl

23.17 Chemical Examination of Urine

The main chemical pathological constituents in urine are sugar, protein, ketone bodies, bile salt, bile pigment and blood etc.

23.17.1Preprations of reagents for chemical analysis of urine

23.17.1.1 Benedict's qualitative reagent: For the preparation of benedict's qualitative reagent in the laboratory, dissolve 173 gm of sodium citrate and 90 gm anhydrous sodium carbonate in 300 ml of distilled water with constant heat, filter and make upto 850 ml with double distilled water (DD water). Dissolve 17.3 gm of copper sulphate solution slowly with continuous stirring into the above solution. The mixed solution is ready for use and is stable for long period. It is also commercially available.

23.17.1.2 Sulphosalicylic Acid (2% w/v): Dissolve 2 gm of pure crystals of sulphosalicylic acid in 100 ml double distilled water.

23.17.1.3 Sodium nitroprusside solution (5% w/v): Dissolve 5 gm of Sodium nitroprusside in 100 ml double distilled water.

23.17.1.4 Fouchet's Reagent: Dissolve 25 gm of trichloroacetic acid in about 50 ml of distilled water, add 10 ml of 10% ferric chloride solution and make the volume upto 100 ml with double distilled water.

23.17.1.5 Barium chloride solution (10%, w/v): Dissolve 10 gm of barium chloride in 100 ml of double distilled water

23.17.2 Test for Sugar

Take in a test tube 5.0 ml benedict's qualitative reagent and add to it exactly 8 drops of urine sample. Boil the content for 1 to 2 minutes and then allow to cool at room temperature. The precipitate appears varying from green, yellow, orange and brick red colour depending upon the amount of sugar present in the urine. A green precipitate is disregarded.

BLUE	Negative
GREEN	0.5%
YELLOW	1.0%
ORANGE	1.5%
BRICK RED	› 2.0%

23.17.2.1 Interference

Ketone bodies and streptomycin above 200µg/ml causes interference.

23.17.3 Test for protein

Centrifuge or filter the urine so as to prevent the interference in results due to the presence of pus cells or bacteria. Perform the following tests for protein in urine.

23.17.3.1 Heat Coagulation Test: Fill a test tube three-fourth with urine. Heat the upper half of the tube. Do not heat the lower half which serves as control. If turbidity develops in the upper half, it indicates the presence of protein or phosphate. If the turbidity is due to the presence of phosphate, it will dissolve when few drops of dilute (1%) acetic acid is added whereas turbidity due to protein will not dissolve by adding dilute acetic acid.

23.17.3.2 Heller's Test: Take 2 ml of concentrated nitric acid into a test tube and through the side of the tube add equal volume of urine in such a manner so that two layers develop. Formation of a white ring indicates presence of protein.

23.17.3.3 Sulphosalicylic Acid Test: Take 2 ml of urine in a test tube and add 3 to 4 drops of 2% sulphosalicylic acid solution. A cloudy precipitate appears indicating the presence of protein.

23.17.4 Test for ketone bodies (Rothera's Test)

Take 5 ml of urine into a test tube and saturate it with ammonium sulphate powder till a small quantity of ammonium sulphate settles at the bottom. To this solution, add 2 to 3 drops of freshly prepared sodium nitropruside (5% W/V) solution and add few drops of ammonium hydroxide solution. Mix the contents thoroughly and keep for sometime. A pink colour gradually develops indicating the presence of ketone bodies.

23.17.5 Test for Bile Salt (Hay's Test)

Take in a clean test tube about 5 ml of urine sample and sprinkle a little bit of sublimated sulphur powder over its surface. Sinking of sulphur powder from the surface indicates the presence of bile salt in the urine.

23.17.6 Test for bile pigment

23.17.6.1 Fouchet's Test: Acidify about 5 ml of urine by adding acetic acid in test tube. To this solution add 3.5 ml of 10% barium chloride solution and approximately 1-2 drops of saturated ammonium sulphate solution. The precipitate of barium sulphate appears to be green instead of white on account of bile pigment adsorption. Centrifuge the content and take the precipitate. Now treat the precipitate with one drop of Fouchet's reagent. A blue or green colour develops indicating the presence of bile pigment.

23.17.7 Test for blood haemoglobin (Benzidine Test)

Take 5 ml urine into a test tube, add 1 to 2 drops of freshly prepared benzidine solution, mix the contents thoroughly and then add hydrogen peroxide drop. A deep blue colour develops indicating the presence of blood haemoglobin.

Commercially available dip sticks can also be used

23.18 Washing Of Laboratory Glasswares

23.18.1 New Glassware: Usually new glasswares are slightly alkaline. Before washing these have to be neutralized. The method is as follows:

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

23.18.2 Dirty glassware

1. Rinse twice in lukewarm or cold water otherwise serum or blood may stick to them and may not be washed.
2. 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.
3. 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) and finally with distilled water.

4. Drain the water by putting each article on a wall draining rack.
5. Place the articles in a wire basket and dry in a hot air oven at 60^0C.
6. Plug each article with non-absorbent cotton wool or aluminum foil and store in a cupboard to avoid dust.

23.18.3 Pipettes

1. Immediately rinse glass pipettes in running tap water to remove blood, urine, serum and reagents etc.
2. If the pipettes were used for infected materials, soak them in a cylinder full of disinfectant solution (2% dettol or 2% phenol or 1% sodium hypochlorite) for 24 hours otherwise place in a large measuring cylinder full of water.
3. Soak in detergent solution for 2-3 hours and then wash in running tap water and finally with distilled water and put them in wire basket to air dry.
4. 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.
5. Dry in hot air oven and plug with non-absorbent cotton.

23.19 Biosafety Practices

Every Laboratory has its own select type of hazards and has general safety guidelines that should be adhered to in any laboratory and should be available at all times and clearly understood by all personnel see chapter 3 for details.

Biological hazards for laboratory personnel may be minimized by utilizing a disinfectant material on all bench tops and work areas and by frequent hand washing.

TEST REPORT FORMAT

Ref. No._____ Dated_____

Name_____ Age/Sex _____

Address_____

Clinical Details _____

Test Required: Blood Glucose/Lipid Profile/Liver Function/Kidney Function /Electrolytes

Samples: Blood/Serum/Plasma/CSF

(NORMAL VALUE)

1.	Blood Sugar (F)	mg/dl	(60-110 mg/dl)
2.	Blood Sugar (PP)	mg/dl	(70-140 mg/dl)
3.	CSF Sugar	mg/dl	(50-80 mg/dl)
4.	Serum Cholesterol	mg/dl	(150-250 mg/dl)
5.	Serum Triglycerides	mg/dl	(65-165 mg/dl)
6.	HDL Cholesterol	mg/dl	(35-80 mg/dl)
7.	LDL Cholesterol	mg/dl	(upto 150 mg/dl)
8.	Blood Urea	mg/dl	(10-50 mg/dl)
9.	Serum Uric Acid	mg/dl	(2.4 - 7 mg/dl)
10.	Serum Creatinine	mg/dl	(0.4 -1.4 mg/dl)
11.	Serum Protein	g/dl	(6.6- 8.7 g/dl)
12.	CSF Protein	mg/dl	(15-45 mg/dl)
13.	Serum Bilirubin (Total)	mg/dl	(upto 1.0 mg/dl)
14.	Serum Bilirubin (Direct)	mg/dl	(upto 0.25 mg/dl)
15.	SGOT/AST	U/l	(upto 40 U/l)
16.	SGPT/ALT	U/l	(upto 40 U/l)
17.	Serum alkaline phosphatase	U/l	(90-306U/l)

There can be minor variations in these values depending upon the Kit and methodology adopted.

Samples: Urine

	Constituents	Positive	Negative
1.	Sugar		
2.	Protein		
3.	Bile salt		
4.	Bile Pigment		
5.	Ketone Bodies		
6.	Blood		

CHAPTER-24

QUALITY ASSURANCE IN LABORATORY

This chapter is to introduce national external quality assessment schemes (NEQAS) for various laboratory testing. It is now widely accepted that quality assurance, quality control and quality assessment constitute as essential part of diagnostic testing In general quality assessment is one component of a total quality assurance programme.

24.1 Terminology

We need to draw distinction between three commonly used terms.

24.1.1 Quality assurance (QA) is the total process that guarantees that the final results reported by a laboratory are as accurate as possible. This involves inspecting specimens, reviewing transcriptional measures, using the most reliable assays and verifying final reports.

24.1.2 Quality control (QC) comprises those measures that must be included during each test run to verify that the test is working properly. This includes ensuring correct temperature conditions, kit controls, etc. This QC indicates whether the test run was valid and has produced acceptable results. QC does not, however indicate that the results are accurate, nor that they have been reported properly.

24.1.3 Quality assessment is a means of determining the quality of results. It is usually an external evaluation of a laboratory's performance using proficiency panels. Quality assessment is undertaken to evaluate the effectiveness of the quality control programe.

24.2 NEQAS

24.2.1 Objective / Importance of estimation

24.2.1.1 The primary objectives are

a) To assess the quality of laboratory performance.

b) To provide assurance to consumers (physicians and patients) that laboratory results are reliable.

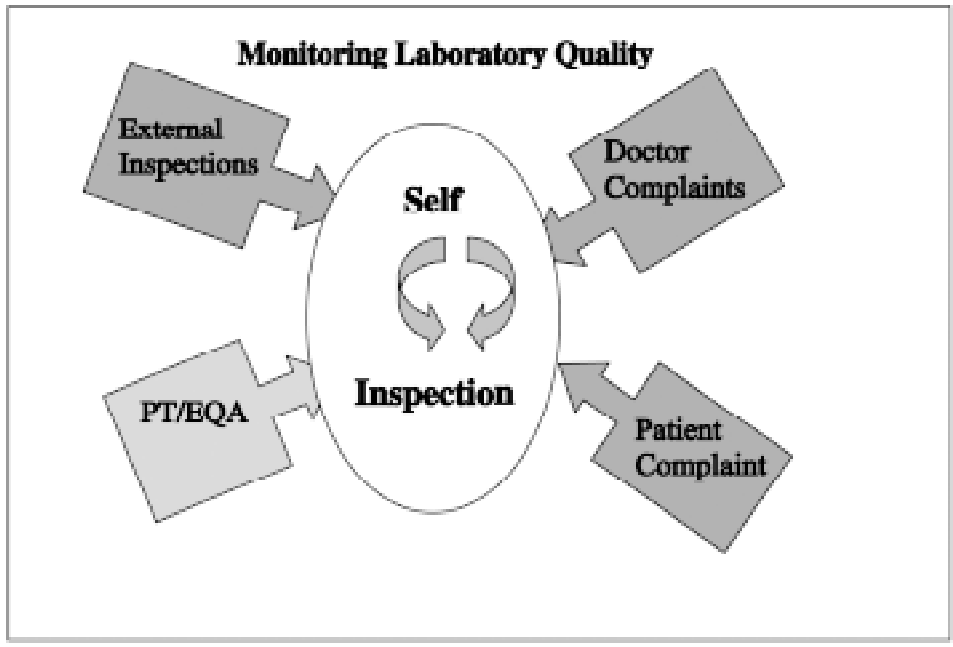
24.2.1.2 Secondary objectives achieved are

- To identify common errors and recommend corrective measures.
- To encourage the implementation of QA and control measures in the participating laboratories.
- To stimulate information exchange and networking among laboratories at national level.
- To provide updated information on new developments in diagnostics and related matters.

24.2.2 The laboratories should monitor quality to

- Ensure their information is accurate.
- Ensure their information is timely.
- Ensure their information is appropriate.
- Ensure their information is interpretable.
- Demonstrate that they provide quality patient care.

Monitoring Laboratory Quality



24.2.3 What is PT (Proficiency Testing)

- A quality monitoring scheme
- Delivery of samples that closely simulate clinical material of known composition.
- A technique to challenge a laboratory's routine methods and procedures.
- To assess laboratory performance and the ability to determine the "correct" result.
- To assess the overall ability and performance of a group of laboratories.

24.2.4 PT/EQ are for

Health Care System

- A valuable assessment tool
- An indicator of laboratory performance
- A mechanism to provide notice and education

The Laboratory

- A valuable tool for education
- Self assessment
- System confidence
- Public confidence

24.2.4.1 Consider following points before proficiency testing

- Is there a need?
- Are the laboratories ready for PT?
- Is there a demand?
- Is there already an existing, operating satisfactory PT program?
- Is the demand specific?
- Which analytes need to be challenged?
- Does the demand have limitation?
- Cost, resources, time, number of samples.
- Can you meet those demands and limitations?

Answer above before initiating proficiency testing

24.3 Proficiency testing by inter laboratory comparisons steps to be followed

24.3.1 Development and operation of proficiency testing schemes

- The test items or materials to be distributed in the scheme should generally be similar in nature to those routinely tested by participating laboratories.
- Participants should be advised to treat proficiency test items as if they were performing routine tests (unless there are some special requirements in the design of the proficiency test which may require departure from this principle).

24.3.2 Minimum organizational requirements for EQA

24.3.2.1 Staff:

- Experience and understanding of the clinical laboratory
- A commitment to quality management
- A commitment to detail
- A commitment to confidentiality and privacy.

24.3.2.2 Facilities and equipment

- A safe and secure facilities which may include on-site laboratory equipments.
- Safety appropriate to the materials being handled.
- Reliable, versatile, and flexible communication capabilities.

24.3.2.3 Network

- Viable and stable client group
- Reference groups and individuals.
- Licensing and accrediting bodies.
- Regulatory authorities.
- Funding source.

24.3.3 Challenge Selection Issues

1. Which analytes to challenge.
2. Challenges per send-out survey.
3. Send-outs surveys per year.
4. What range to consider.
5. What complexities to incorporate.

24.4 Quality Control and Validation

24.4.1 Quality controls are integral part of program credibility and it should have the following issues for decision

- Are we sending what we think we are sending?
- Have the PT samples been altered during preparation?
- Did we send the same thing to all laboratories?
- Did all laboratories receive the same thing in the same condition?

24.5 Post Analytic Data Management is an important aspect

One must do following :

- Results recieving
- Results analysis
- Results summation
- Results reports generation.
- Reports transport to laboratories.
- Reports transport to requisite authorities.
- Correspondence and communications
- Development of education materials.

24.6 The EQA programme also provide educational opportunities

PT programs have the unique platform to provide real-time and continuous laboratory education in a variety of formats.

24.6.1 The organizing laboratory needs to have

- Technical competence
- Necessary facilities (staff, space, equipment, computer, etc.) and access to suitable biological material.

24.6.2 Participating laboratories

The aim of every national scheme should be to secure the participation of every district laboratory under IDSP, this programme will be launched in a phased manner.

24.7 Operating EQA scheme: Launch the scheme as follows

- Stability during transportation.
- Dispatch of panels.
- Frequency of distribution of specimens. Initially we should plan for once in a year with each distribution, depending on the disease for which it is being done we should have at least 5-10 specimens.
- Instruction sheet should always be there, where general information on how to process the specimen and how to record should be there.
- One must record the EQA results on a designed report form provided with EQA panel.
- The participating laboratory should get feed back report, analysis and intended results in a timely manner.

24.7.1 The feed back should highlight performance as

a) Poor performance and reasons for improvement.

b) Good performance.

This target for performance should be evaluated from time to time.

24.8 Proficiency testing scheme

Inter-laboratory comparison designed and operated to assure laboratory performance in specified areas of testing, measurement or calibration.

24.9 Check list for Testing programme development under QA by laboratory

- Determine the need
- Garnering support
- Status of laboratory testing
- Design of the programme
- Distribution of samples
- Data collections, scoring criteria analysis.
- Distribution of reports.
- Quality improvement.

24.10 In Summary

24.10.1 EQA is a program that provides

- Quality management
- Education

- Research and development
- Social responsibility.

24.10.2 For being a EQA provider one must have

- Knowledge and Expertise
- Commitment
- Good Communication Skills
- Respect for confidentiality.

The detail of quality control check for each test is given in individual chapter.

CHAPTER-25

BIOTERRORISM AGENTS: - LABORATORY ASPECTS

Biological weapons are devices used intentionally to cause diseases or death through dissemination of microorganisms or toxins in foods and water, by insect vectors or by aerosols. Bioterrorism differs from other types of terrorism (chemical, radiological or nuclear). In fact this would impose particularly heavy demand on the nation's public health and health care system as ultimately it is the public health system that will be called on the mitigate and ameliorate the consequences of bioterrorism attack.

Table: 25.1 Agents which can be used and their mode of transmission

S.No	Diseases	Agents used	Mode of Transmission in Biological warfare
1	Anthrax	Bacillus anthracis	Powder aerosol spray in vents/ tunnels
2	Plague	Y. Pestis	Inhalation by limited people who in turn produce secondary cases. Transmission into rodents in field
3.	Smallpox	Variola virus	Droplet/ skin contact by introduction to small group. It is highly contagious and spreads fast.
4.	Viral Haemorrhagic fever	Marburg / Ebola	Aerosol
5.	Yellow fever	Virus of flaviviridae	Introduction of virus through infected vector mosquito. Aerosol transmission achieved in lab.
6.	Dengue / DHF	Dengue virus1,2,3	Introduction of infected mosquito by dengue virus.
7	Tularemia	Francisella tularensis	Transmission by aerosol/ contamination of food and water/ enzootic reservoir among wild animal.
8.	Q Fever	Coxiella burneti	Aerosol route most favoured as it infects large number.
9.	Botulism	Cl. botulinium	Contamination of food with toxins or spores.
10.	Cholera	V. cholera	Contamination of public drinking water sources/ food supplied to large population.
11.	Shigellosis	S.dysentery	"

Laboratories will be critical in identifying the etiologic agents and assist public health system for

- Effective response
- Appropriate treatment

- Control measure implications

25.1 Sample collection and transportation

25.1.1 Collection

Remains same as for natural infection and one should follow the guidelines as given in chapter-4.

25.2 Remember

- Bioterrorism agents are highly infectious and all guidelines for biosafety to be strictly followed.
- Needs quick diagnosis as need is for specific & urgent action.
- Send through courier on urgent basis to designated laboratory.
- Laboratory needs preparedness to handle large number of samples & transportations.
- Inform CMO on an urgent basis.

25.3 When to suspect that a disease out break is due to bioterrorism

- Drastic increase in microbiological culture request.
- Appearance of unusual sample
- Cluster of sample with same characteristics.
- Diseases out break of same illness occurring in non contiguous area.

District laboratory needs to upgrade and develop rapid diagnostic techniques for such diseases which they can handle.

25.4 Preparedness for laboratory capacity

- Information regarding laboratory network should be available with district, so that there is improvement in the diagnosis of agents potentially used in bioterrorism.
- Biosecurity in the laboratories should be strengthened to prevent the misuse, contamination or improper handling of agents.
- The quality control in laboratory diagnosis is to be inbuilt component.
- Transfer of infectious samples should be done according to guidelines in the previous chapter.

CHAPTER-26

MAINTENANCE OF EQUIPMENTS

Of the large number of factors which affect the sensitivity/specificity of the test results generated by the laboratory, role of equipments being used cannot be ignored in final reporting. So vigilance is required at different level as far as the equipments are concerned. Some of them are listed below.

- Procurement of equipments.
- Proper maintenance of equipments during use.
- Timely calibration of equipments to ensure the uniformity in the results.

26.1 Procurement of equipments

Following points should be kept in mind while procuring the equipments.

- Equipments should be cost effective
- It should be easily available.
- Manufacturer should provide a good after sale service for the proper maintenance of the equipments.
- Supplier/manufactures should have good stock of spare parts pertaining to the equipments.
- Supplier and manufactures should provide warranty/guarantee for a longer duration.
- The equipments supplied should be as per the user's specifications.
- An operative manual should be provided along with an equipment.
- Equipments should be safe and easy to handle.

26.2 Some of the equipments required at district laboratory

26.2.1 Water Bath

It is important that the water bath maintains a constant temperature within a narrow range (± 0.5^0C) when used. Inadequate adjustment and insufficient stabilization of the temperature will strongly affect the result of kinetic measurements.

26.2.1.1 Use of Water Bath

1) The level of water in the water bath must be above the level of the solution to be incubated.
2) Open containers, vials or tubes must be incubated in a water bath with an open lid to avoid contamination and dilution of the incubated material by condensed water.
3) The water bath must be cleaned regularly to prevent growth of algae and bacteria.

26.2.1.2 Few applications of water bath are

37^0C Water Bath -	For bringing laboratory material before testing to 37^0C when ever needed during performance of test
44^0C Water Bath-	required in faecal coliform count (water bacteriology) test.
56^0C Water Bath-	For inactivating complement in the serum.

26.2.2 Autoclave

Bacteria and viruses cannot survive in such an environment of sterilization. It should be borne in mind that autoclaves need careful handling and must be regularly inspected. They can be hazardous and can seriously injure a person with hot steam accidently evaporating from the instrument.

26.2.2.1 The main factors influencing perfect steam sterilization are

- Saturated steam.
- Temperature.
- Time.

26.2.2.2 Use of autoclave

Refer to chapter-5.

26.2.3 Maintenance of Refrigerators

The following general advice may be helpful for maintenance.

- Refrigerators must be placed where sufficient air can pass the condenser (at the back of the refrigerators) for exchange of heat and also facilitate for cleaning of the condenser.
- The refrigerator door must seal perfectly to prevent warm outside air from entering the cool chamber.
- For photovoltaic refrigerators the collector must be optimally positioned to ensure production of enough electricity.

26.2.3.1 Daily checks

- Check temperature daily. It should not exceed 12^0C. Maintain record of daily temperature.
- Check gas bottles or kerosene tank for sufficient fuel in gas or kerosene fed refrigerators.

26.2.3.2 Monthly checks

- Clean cool chamber and defrost monthly.
- Clean refrigerators from outside.

- Make condenser dust free.
- Clean door gasket.
- Periodically clean burner and check for gas leakage.
- In photovoltaic refrigerators, check level of electrolyte solution of batteries and fill up with pure distilled water, if necessary.

26.2.4 Centrifuge

Two types of centrifuge are used, mechanical and electrical. The major aspects in maintenance of different types of centrifuges used in laboratory are as follows :

- The centrifuges must be positioned exactly horizontally to prevent the instrument moving away from its place when out of balance during centrifugation. Check if the rubber buffer are in the buckets.
- It is critically important that the centrifuge's load is balanced at all times. Therefore, buckets should be loaded in matched pans and tubes should be arranged so that balanced tubes oppose each other in the centrifuge head. This is necessary to maintain the same forces of gravity in the opposite positions of the tubes. This arrangement involves placing a "dummy", i.e. tube filled with the appropriate volume of water corresponding to the weight of the volume, in the oppositely positioned test tube, when an odd number of specimens must be centrifuged.
- Turn the speed control slowly up and down.
- If infectious material is being centrifuged, cover the centrifuge completely with plastic sheet during centrifugation. After use disinfect sheet and discard; buckets should be appropriately disinfected and dried.
- Stop the centrifuge immediately if it makes an abnormal noise.
- After use the buckets should be inverted to drain dry.
- After any sample spillage, wipe and disinfect immediately.
- Clean the centrifuge at short intervals (preferably daily) because it is one of the most frequently used instruments.
- Check mounting and replace if necessary.
- Check brushes and bearings every 3 months. Replace if necessary.
- Check for corrosion and repaint if necessary.

26.2.5 pH meters

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

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

26.2.5.1 Calibration of pH meters

To obtain a precise measurement of pH, the pH meter must be calibrated with two different buffers at pH 4 and pH-7 every day (two-point calibration). Before measuring the pH of unknown solution, the pH meter should be calibrated with standard buffer of pH near to the targeted pH of the solution. For the calibration of a pH special buffer solutions must be used, the pH of which should be near the pH of the solution to be measured. Phosphate buffer and acetate buffer are preferable. Calibration measurements should be done using plastic containers.

A pH meter needs to be standardized before each run with a standard buffer of pH 7.0. However, when the work is related to a pH range of less then 6.0, it is advisable to use a standard buffer of pH 4.0. The buffer should be discarded if the pH deviates more than 0.4 or if the buffer is contaminated with microrganisms.

26.2.6 Incubator

Incubators should be subjected to continuous recording of temperature. However, if it is not possible, the temperature must be recorded on a sheet or log book every day and before opening the incubator.

- ⇨ Incubators are used for bacterial culture by laboratories working in microbiology.
- ⇨ The incubators must maintain a constant temperature of $35^0C \pm 2^0C$.
- ⇨ Temperature should be daily recorded in incubators.
- ⇨ Like all laboratory instruments, incubators must be cleaned routinely at short intervals (at least every fortnight) and after spillage of infectious material.
- ⇨ The actual temperature must correspond to the thermometer control when the instrument is used.
- ⇨ Fumigation of the incubator is done occasionly

26.2.7 Hot Air Oven

Hot air ovens are mainly used for drying laboratory equipments and medical devices in dry air. Hot air ovens are also used for sterilization. Sterilization in dry air is only effective when the material is exposed for 60 minutes at 160^{0}C or for 40 minutes at 180^{0}C. It is important to remember that the timing of sterilization is sufficient when the holding period begins after the air in the oven has reached its expected temperature.

26.2.7.1 Use of Hot air ovens

1. Set up the thermostat at the required temperature prior to sterilization.
2. If there is a fan, check if it is working.
3. Allow to continue heating for an additional required holding time after the temperature reaches the pre-set degree.
4. Switch off the heat when the time is up.
5. Wait until the temperature falls to 40^{0}C before opening the door.

26.2.8 Maintenance of balances

The following guidelines are worth remembering

- The balance should be placed on a solid vibration-free surface, free from air currents and at room temperature away from sunlight.
- The instrument must be placed in an exactly horizontal position.
- The balance should be zeroed prior to each use.
- Use the smallest possible vessel for weighing. Avoid weighing in vessels made of plastic, because they can become electrostatically charged. Use, instead glass vessel or weighing paper, as applicable. The weighing vessel and the sample to be weighed should be at the ambient temperature. Hands should never be inserted into weighing chamber as it may give working effect.
- Place the sample to be weighed in a weighing vessel in the middle of the weighing pan, to avoid corner-load error. Liquid or powder should never be directly weighed on the pan. The weight of the weighing vessel needs to be determined prior to placing the substance to be weighed. A tweezer is useful as a substitute for hands in the weighing chamber.
- Use clean weighing vessel. Keep the working place, weighing chamber and weighing pan clean. To avoid any possible corroding effect of chemicals, any spillage must be cleaned immediately. Biological material may be a source of infection. Disinfection can be done with 70% alcohol.
- After completing weighing return the balance to zero weight.
- Keep the working place near the balance as clean as possible.

26.2.9 Deep freezer (-20^{0}C)

- Should have visible display unit (Temp).
- Temperature should have back up support (UPS).
- Check temperature daily and maintain written record (sheet/log book).
- Temperature should not exceed -20^{0}C.
- Make sure that door is air tight.
- Defrost every month after taking care of stored samples.

26.2.10 Microscope

- Place a slide on the stage, specimen side up and center of the section to be examined as accurately as possible over the hole in the center of the stage.
- 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 ¼" 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 the eye piece. Bring the specimen to sharp focus with the fine adjustment. Adjust the iris diaphgram and substage, condenser 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 objective from the side carefully, lower it into the oil. Do not allow the objective to touch the slide. Look through the ocular and slowly focus upwards with the fine adjustment until the image appears. Once it appears do the fine adjustment and adjust the mirror and iris diaphragm to obtain optimum illumination.

26.2.10.1 Maintenance

⇨ Never touch the lenses if they become dirty, wipe them gently with lens paper/lint-free smooth cloth.

- ⇨ Always remove oil from the oil immersion objective after its use. If by accident, oil gets on either of the low powers, wipe of the objective immediately with the lens paper. If oil becomes dry or hardens 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 oculars of different microscopes.
- ⇨ Store the microscope in its cabinet when not in use

TABLE 26.1

Quality Control Surveillance Procedure of Commonly Used Microbiology Equipments

Equipments	Procedure	Schedule	Tolerance Limit
Refrigerators	Recording of Temperature	Daily or continuous*	2^0C to 8^0C
Freezers	Recording of Temperature	Daily or continuous*	-8^0C to -20^0C -60^0C to -75^0C
Incubators	Recording of Temperature	Daily or continuous*	35.5^0C ± 1^0C
Water Bath	Recording of Temperature	Daily	36^0C to 38^0C 55^0C to 57^0C
Autoclaves	Test with spore strip (Bacillus stearothermophlius)	At least weekly	No growth of spores in subculture indicates sterile run.
PH meter	Test with pH-calibrator	With each run	± 0.1 pH units of Standard being used.
Centrifuge	Check revolution with Tachometer.	Monthly	Within 5% of dial indicator setting.
Safety hoods	Measure air velocity Across face opening.	Semiannually or Quarterly.	50 feet of air flow per minute ± 5 feet/min.
Microscope	Clean optics and stage	Daily	.....
Balance	Service and recalibrate	Annually	.....
Hot air Oven	Check temperature	Daily	160^0C, 180^0C

* Continuous by thermographs.

Temperature monitoring worksheet

Name of Equipments:_____ Location : _____

Model: _____ Month : _____

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Tech-nician																															

CHAPTER-27

DATA MANAGEMENT IN DISEASE SURVEILLANCE

The district laboratories are an important functionary in the health setup and are the point of first contact of patients with the public health system. Inputs from this level are the key to the effective functioning of any surveillance system. The success or failure of any public health or disease control initiative depends on establishing and maintaining a good information exchange system, with accurate and timely data being provided for appropriate action. Therefore, the importance of good laboratory data management cannot be overstated. The "laboratory data management" includes:

- **Recording** the details of the **specimen tested**
- **Recording** the **results** of testing
- **Analysis** and **interpretation** of results
- **Looking** at epidemiological **patterns** and **trends**
- Summarizing the results in the form of **regular reports**

27.1 Guidelines for data management

27.1.1 Recording of data

27.1.1.1 Establish the set of minimum information to be recorded on each specimen

- Name, age, sex, in-patient/ outpatient status of the patient, broad geographical area to which the patient belongs, syndromic diagnosis.
- Nature of the sample/site of the sample, date of receipt of sample, date of processing of the sample, quality of the specimen received (cold chain condition, quantity, leakage etc.), inpatient/ outpatient source, investigation requested, result of the investigation such as nature of the pathogen isolated, resistance pattern, any other significant observation.
- Design a standard format for laboratory request, data recording, and data reporting. Reporting format for laboratories is annexed (Annexure I).

27.1.2 Data recording system

- For laboratories with smaller workload, manual maintenance of records can suffice
- For laboratories with larger workload, establish a computerized record system (Microsoft Excel, Epi-Info, FoxPro, Access, any other developed by IDSP) depending upon the type of data and the type of analysis required.
- Features of the computerized reporting system:
  - User and programmer friendly
  - Rapid and accurate access to chosen/selected records

- Able to perform simple calculations e.g. frequency and time interval
- Create graphs and tables
- Well documented installation procedures, user manual etc.

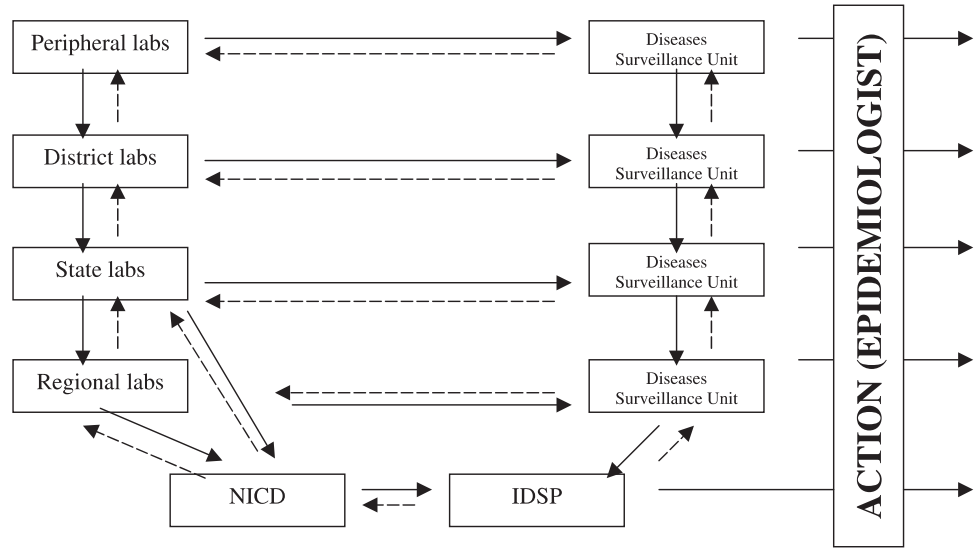
o Components of the computerized laboratory record system:

- Data entry
- Data cleaning and routine back up of data
- Routine analysis and reporting
- Feedback
- Feed forward

o Record the results (manual or computerized) in the form of specimen based database i.e. each line of information relates to one specimen. Therefore for a case with two specimens collected and processed, there will be two lines of information.

27.2 Data flow

- What information is reported, where it is reported from and where it is reported to must be clearly agreed upon by all parties involved in the system
- All information flow must be hierarchical, going from one level to the next, without missing levels. Information can also be broadcasted i.e. sent to several sources at the same time.



Data / Information flow ——▶

Feedback ·····▶

Fig : 27.1

- A diagram of information flow should be made including frequency of reporting and distributed to all parties so that everyone involved understands the reporting system. The system must be followed without exception.
- Review the system from time to time to make any improvements.

27.3 Data analysis

- Regular analysis and reporting followed by reporting has a direct effect on the surveillance system. It helps in:
  - Providing feedback to the programme managers for follow-up and supplementary planning
  - Coordination activities
  - Monitoring of laboratory results and performance to identify problems and constraints.
  - Generating early warning signals for an outbreak.
  - Monitoring the changing trends of prevalent pathogens
  - Monitoring the emergence / re-emergence of pathogens including agents of bioterrorism
  - Establishing networks of laboratories & therefore preparedness for unusual activity round the year
  - Development of laboratory database
- Analysis may be done on daily (during outbreaks), weekly, monthly, annual basis and also communicated according to the set protocols as per the guidelines laid down by the IDSP.
- Analysis may be done on the basis of various parameters such as:
  - Age / sex distribution of patients
  - Time distribution of cases
  - Inpatient / outpatient status of the patient
  - Geographical distribution of cases: clustering of cases
  - Syndromic diagnosis
  - Nature of the sample
  - Nature of the pathogen
  - Results of the investigation such as titers, resistance patterns.
  - Correlation between different parameters
  - Correlation with the epidemiological data.
  - Unusual activity in laboratory results as compared to the last observation e.g. last month or the same month of the last year or as compared to

previous years. This will help in not only picking up early warning signals but also contribute to monitoring the quality of results of the laboratory.

- Analysis and interpretation must be supported by baseline studies, which need to be performed from time to time to make the data generated more meaningful.
- Data analysis must also be presented as graphs, charts and maps (wherever possible) for better comprehension and decision-making.
- Wherever possible, take the help of the software for data analysis.
- The interpretation generated from the analysis must be regularly conveyed to the programme manger (disease surveillance units) for necessary action.
- Analysis and interpretation must be coupled with a feedback and feed forward component for all the partners involved in the system.

Monthly IDSP District Laboratory Report

Name of District:		Month / Year:	
Investigation	Samples Processed (no.)	**Results**	
		Positive	Negative
Wet preparation for cholera (M/E)			
P/S for MP (M/E)			
Any other microscopy (Specify)*			
Typhoid serology / Widal Test			
Hepatitis markers (specify)			
Any other Serology (specify)**			
Water Bacteriology (specify method)		Unsatisfactory	Satisfactory

Bacterial cultures:			
Sample	No. Tested	Isolate	Antibiogram***
Stool culture for enteropathogens****			
		Total	
Blood culture (for Salmonella, any other isolate of clinical significance)		Isolate	Antibiogram***
		Total	
Any other (specify)		Isolate	Antibiogram***

*Sputum for AFB, throat swab for diphtheria, bubo aspirate/sputum for plague, P/S for microfilaria, Bone Marrow/Peripheral Blood For LD bodies, CSF for pyogenic meningitis. ** Dengue, JE, measles, leptospirosis etc. *** Mention the common antibiotics used & provide the antibiogram as S- for sensitive, IS- for intermediate sensitive & **R**- for resistant. **** Salmonella, Shigella, Vibrio cholerae, Diarrheagenic Esch. coli (EPEC, ETEC, EHEC etc.). NOTE: Details may be attached on a separate sheet

ACRONYMS

AFB	:	Acid Fast Bacilli.
ALP	:	Alkaline Phosphatase
AMR	:	Anti microbial resistance
AMR testing	:	Antimicrobial resistance testing
ATCC	:	American type culture collection. (U.S.A)
Bacto	:	Proprietory item name of manufacture "DIFCO", "USA" now re-named as Becto Dickinson (BD)
BSA	:	Bile Salt Agar (Plate)
C.diptheriae	:	Cornybacterium diptheriae
Cary Blair	:	Cary blairs transport medium.
Cl. bohilinium	:	Clostridium bohilinium
Cm	:	Centimetre.
CMO	:	Chief Medical Officer
CSF	:	Cerebro spinal fluid.
DHF	:	Dengue Haemorrhagic fever
E. Coli	:	Escherichia coli.
EHEC	:	Entero haemorrhagic. Escherichia coli
ELISA	:	Enzyme Linked Immunosorbant Assay
EPEC	:	Entero pathogenic Escherichia coli.
EQA	:	External quality assurance.
GLP	:	Good Lab Practice.
Gm	:	Gram
HDL	:	High Density Lipids
H.Nana	:	Hymenolepis nana
Hrs.	:	Hours
IDSP	:	Integrated Disease Surveillance Programme.
IQCA	:	Internal Quality control assessment.
JE	:	Japanese Encephalitis
J.S.B.stain	:	Jaswant Singh and Bhattacharjee stain.
KFT	:	Kidney function tests
L	:	Litre
Lab	:	Laboratory
lbs	:	Pounds
LD bodies	:	Leishman donovan bodies

LDL	:	Low Density Lipids
L. donovani	:	Leishmania donovani
LF	:	Lactose fermenting colonies.
LFT	:	Liver Function Tests
LIA	:	Lysine Iron agar (medium)
L.tropica	:	Leishmania tropica
MAC	:	MacConkey's agar (plate)
MDR	:	Multi Drug Resistance
min	:	Minutes
ml	:	Milliliter
mm	:	Millimeter.
MPN	:	Most probable number. (of coliforms present in or fixed volume of)
MTCC	:	Microbial type culture collection (India)
Myc. tuberculosis	:	Mycobacterium tuberculosis
NA	:	Nutrient Agar
NCD factors	:	Non communicable disease factors
NCTC	:	National collection of type culture (U.K)
NEQAS	:	National External Quality Assesment Scheme
NCCLS	:	National Committee of Clinical Laboratory Standards.
NLF	:	Non-lactose fermenting colonies
ORS	:	Oral Rehydration fluid/solution
P. Falciparum	:	Plasmodium falciparum.
PHLab	:	Public Health Laboratory
P. Vivax	:	Plasmodium vivax.
PPA	:	Phenyl Pyruvic Acid
Ppm	:	part per million
ppn	:	preparation.
PT	:	Proficiency Testing
QA	:	Quality Assurance
QC	:	Quality Control.
S/O	:	Suggestive of
SGOT	:	Serum Glutamic Oxaloacetic Transaminase
SGPT	:	Serum Glutamic Pyruvic Transaminase
SOP	:	Standard Operating Procedures

Sps	:	Species.
SSA	:	Salmonella Shigella agar (plate)
S. dysentry	:	Shigella dysentry
TG	:	Triglyceride
Tr. iodine	:	Tincture iodine
Tr. benzoinco	:	Tincture benzoin co.
T. solium	:	Taenia solium
T.saginata	:	Taenia saginata.
TB	:	Tuberculosis
TCBS	:	Thiosulphate Citrate Bile Salt Sucrose Agar (Culture medium).
TSI	:	Triple sugar Iron agar (medium)
TP	:	Treponema pallidum
V.Cholerae	:	Vibrio Cholerae
VR-Fluid	:	Venkatraman Ramakrishan Fluid.
VTM	:	Virus Transport Medium
XLD	:	Xylose- Lysine Dextrose agar.
Z.N.stain	:	Ziehl Neelsen stain.

Blanks