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Guidelines on Standard Operating Procedures for Microbiology

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Preface

Laboratory services have become an integral and inseparable component of modern medicine and public health. Laboratories play a decisive role in the diagnosis, treatment, prognosis and monitoring of both communicable and noncommunicable diseases. Intermediate and peripheral health facilities in developing countries are crucial to primary health care. Therefore, reliable, reproducible and rapid laboratory services, organized in a cost-effective manner, will go a long way in providing quality health services at the district hospitals and health centres.

Quality assurance in laboratory services, aimed at improving reliability, efficiency and facilitating inter-laboratory comparability in testing, is the backbone of quality health care delivery. The use of standard operating procedures in laboratory testing is one of the most crucial factor in achieving quality. This helps both in proper patient management and generates reliable disease surveillance data. This publication provides guidelines on standard operating procedures for diagnosing diseases of public health importance at intermediate and peripheral levels. Guidelines on early warning signals about epidemic-prone diseases based on laboratory data and on collecting and effectively transporting the appropriate clinical specimens to the referral/central laboratories for diseases for which diagnostic services are not as yet developed at the intermediate laboratories are also provided.

The publication contains guidelines on the use of conventional procedures which may be adapted as per local needs. The emphasis is on providing feasible, practical, easy-to-reproduce, specific, simple and cost-effective techniques for the diagnosis of communicable diseases. Necessary biosafety guidelines have been provided and situations where referrals to higher-level laboratories are indicated have also been identified.

It is hoped that this publication will be useful in achieving its objective of improving the quality of laboratory services at intermediate and peripheral levels.

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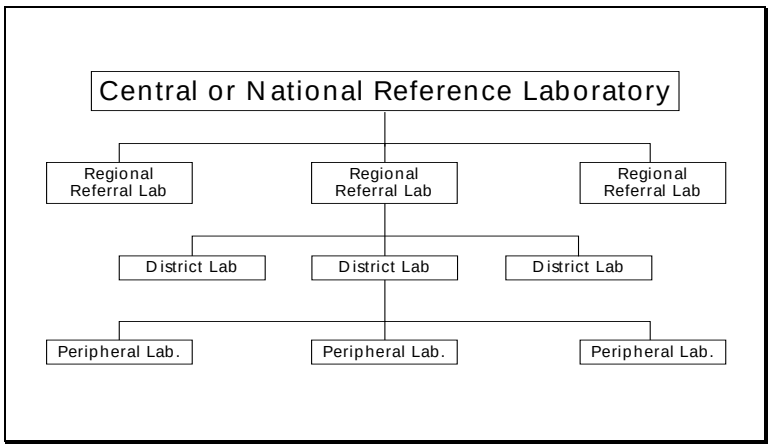
Section A
General Laboratory Practices

1. Organization and Functions of Laboratories

The organization of laboratories in any country is usually a three or four tier system with various possible functional linkages between them. One possible way of networking of laboratories is shown in Fig 1.

Figure 1: *Networking of*



Peripheral laboratory services

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

Staff

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

Space

The space available in peripheral laboratories should include at least one laboratory-cum-office/record room (approx. 5 meters x 3 meters) and one store-room which can be used for other services also (approx. 5 meters x 3 meters).

Other facilities

Other necessary facilities include

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

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

Equipment and supplies

Necessary equipment and supplies include good microscopes, centrifuges, autoclaves, refrigerators, balances, pH meters, incubators, water bath, transport media, glassware, sterile swabs, reagents for staining (eg. Gram, Albert, Ziehl Neelsen, Romanowsky), reagents for chemical examination of urine, kits and reagents for rapid diagnostic tests, sterilized syringes and needles, micropipettes and tips as well as sterile collection bottles for blood/serum and water analysis.

Tests to be performed

Peripheral laboratories are expected to undertake tests of public health as well as clinical relevance. Among the tests of public health relevance, diseases of greater epidemiological importance should be accorded priority. Testing of environment samples (especially water) also falls into the priorities of public health relevance. Certain rapid serological tests may be of use in studying epidemiological patterns of important diseases and the same can also be performed at peripheral laboratories.

The tests to be performed by peripheral laboratories are subject to the availability of resources, manpower, technology and prevalence of various diseases in the area catered to by the laboratory. A suggested list is provided in Table 1.

Table 1: ***Suggested tests to be performed at peripheral laboratories***

Procedure/Specimen	For detection/diagnosis of
Urine examination	Pus cells, RBCs Albumin Sugar
Stool examination	Ova and cysts
Stained smears	
Throat specimen	Diphtheria
Sputum	Tuberculosis
CSF (pyogenic and tubercular)	Meningitis
Peripheral blood smear	Malaria, filariasis

Rapid diagnostic tests	HIV Hepatitis B surface Ag Syphilis Meningococcal disease

Intermediate laboratory services

In most developing countries, intermediate laboratories are located at district or the regional headquarters and may act as clinical as well as public health laboratories. The following functions are expected to be performed by these laboratories:

1. Laboratory support to clinical diagnosis/public health
   Quality assurance
   Logistic and technical support
   Training of staff for peripheral laboratories

2. Supervision and monitoring of peripheral laboratories

Intermediate laboratories help in the diagnosis and treatment of the individual patient and are also used as public health laboratories for epidemiological surveillance and control of diseases in the community. These laboratories also serve as links between peripheral laboratories and the state/central laboratory for the following:

- ➢ Collection, storage and analysis of data.
- ➢ Distribution of reagents, media, laboratory manuals.
- ➢ Purchase of equipment.
- ➢ Supervision of peripheral laboratories.
- ➢ To conduct external quality assessment scheme (EQAS) for peripheral laboratories.
- ➢ To take part in EQAS organized by the state/central laboratories.
- ➢ To send samples to higher/reference laboratories for characterization of isolate/confirmation of diagnosis.

Staff

Qualified pathologist/ microbiologist (Doctor of Medicine/diploma in clinical pathology)	1
Technicians – DMLT (diploma in medical laboratory technology) with experience	2
Laboratory Assistants (DMLT)	1
Laboratory attendants	2
Cleaner	1
Clerk-cum-storekeeper	1

Since it may not be possible to have a full-time epidemiologist, at least part time help of an epidemiologist should be available.

Space

Microbiology/Serology laboratory (approx.8 metersx5 meters)	1
Sterilization, disinfection and media preparation laboratory (approx. 6 metersx4 meters)	1
Store-room (approx.3 metersx5 meters)	1
Office (approx. 3 metersx5 meters)	1

Equipment

Binocular microscope	2	Colorimeter	1
Dark-field microscope	1	Refrigerator	1
Inoculating chamber	2	Balances	2
Centrifuge	2	pH meter	1
Autoclave	2	Inspissator	1
Incubator	2	Distil water apparatus	1
Hot air oven	1	Micropipettes	as per workload
Water bath	2	Tips for pipettes	as per workload
VDRL shaker	1		

This manual describes most of the tests that have been suggested to be performed at intermediate-level laboratories.

Further reading

1. Kumari S, Bhatia Rajesh, Heuck CC. Quality Assurance in Bacteriology and Immunology. WHO Regional Publication, South East Asia Series No 28, 1998.
2. Kumari S, Sharma KB et al. Health Laboratory Services in support of Primary Health Care in South-East Asia, WHO Regional Publication, South East Asia Series No 24, 2nd Ed, 1999, New Delhi.

2. Collection and Transportation of Clinical Specimens

The laboratory diagnosis of an infectious disease begins with the collection of a clinical specimen for examination or processing in the laboratory (the *right* one, collected at the *right* time, transported in the *right* way to the *right* laboratory). Proper collection of an appropriate clinical specimen is the first step in obtaining an accurate laboratory diagnosis of an infectious disease. Guidelines for the collection and transportation of specimens should be made available to clinicians in a lucidly written format. The guidelines must emphasize two important aspects:

- ➢ Collection of the specimen before the administration of antimicrobial agents.
- ➢ Prevention of contamination of the specimen with externally present organisms or normal flora of the body.

General rules for collection and transportation of specimens are summarized in Table 1.

Table1: ***Collection and transportation of specimens***

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

Criteria for rejection of specimens

Criteria should be developed by a laboratory on the basis of which the processing of a specimen may not be done by the laboratory. The following are some examples:

- Missing or inadequate identification.
- Insufficient quantity.
- Specimen collected in an inappropriate container.
- Contamination suspected.
- Inappropriate transport or storage.
- Unknown time delay.
- Haemolysed blood sample.

Collection of specimens

The clinical state of the patient will not necessarily be reflected by the result of laboratory investigation despite correct laboratory performance unless the specimen is in optimal condition required for the analysis. Some of the important specimens and their proper collection and transportation methods are described here so as to ensure quality.

Blood

Whole blood is required for bacteriological examination. Serum separated from blood is used for serological techniques. Skin antisepsis is extremely important at the time of collection of the sample. Tincture of iodine (1-2%), povidone iodine (10%) and chlorhexidine (0.5% in 70% alcohol) are ideal agents. However, some individuals may be hypersensitive to iodine present in some of these. While collecting blood for culture,the following points must be remembered:

- Collect blood during the early stages of disease since the number of bacteria in blood is higher in the acute and early stages of disease.
- Collect blood during paroxysm of fever since the number of bacteria is higher at high temperatures in patients with fever.
- In the absence of antibiotic administration, 99% culture positivity can be seen with three blood cultures.
- Small children usually have higher number of bacteria in their blood as compared to adults and hence less quantity of blood needs to be collected from them (Table 2).

Table 2: ***Volume of blood to be collected at different ages***

Age	Volume in 2 bottles
< 2 years	2 ml

2-5 years	8 ml
6-10 years	12 ml
>10 years	20 ml

Cerebrospinal fluid (CSF)

Examination of CSF is an essential step in the diagnosis of any patient with evidence of meningeal irritation or affected cerebrum. Almost 3-10 ml of CSF is collected and part of it is used for biochemical, immunological and microscopic examination and remaining for bacteriological or fungal examination. The following important precautions need to be taken for CSF collection and transportation:

- ➢ Collect CSF before antimicrobial therapy is started.
- ➢ Collect CSF in a screw – capped sterile container and not in an injection vial with cotton plug.
- ➢ Do not delay transport and laboratory investigations.
- ➢ Transport in a transport medium if delay in processing is unavoidable.
- ➢ CSF is a precious specimen, handle it carefully and economically. It may not be possible to get a repeat specimen.
- ➢ Perform physical inspection immediately after collection and indicate findings on laboratory requisition form.
- ➢ Store at 37^{o}C, if delay in processing is inevitable.

The characteristics of the appearance of CSF are outlined in Table 3.

Table 3: ***Appearance and interpretations of CSF***

Clear and colourless	Normal
Clear with Tyndall effect *(sparkling appearance against incident light)*	High protein content
Clear yellowish	Old haemolysis
Clear red	Fresh haemolysis
Turbid blood-stained	Haemorrhage
Turbid white	High cell or protein content
Turbid clot *(after overnight storage)*	Fibrin clots

Sputum

Sputum is processed in the laboratory for aetiological investigation of bacterial and fungal infections of the lower respiratory tract. It is of utmost importance in the diagnosis of pulmonary tuberculosis.

- ➢ Select a good wide-mouthed sputum container, which is preferably disposable, made of clear thin plastic, unbreakable and leak proof material.
- ➢ Give the patient a sputum container with the laboratory serial number written on it. Show the patient how to open and close the container and explain the importance of not rubbing off the number written on the side of the container.
- ➢ 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 (2-3 ml).

Give the patient an additional container with laboratory serial number written on it for an early morning specimen. Explain to the patient to rinse his/her mouth with plain water before bringing up the sputum.

Urine

Under normal circumstances urine is sterile. The lower part of the urethra and the genitalia are normally colonised by bacteria, many of which may also cause urinary tract infection. Since urine is a good growth medium for all sorts of bacteria, proper and aseptic collection assumes greater importance for this specimen.

For microbiological examination urine must be collected as a “clean catch-mid-stream” specimen.

Urine specimens should be transported to the laboratory within one hour for bacteriological examination, because of the continuous growth of bacteria *in vitro* thus altering the actual concentration of organisms.

Stool

Faecal specimens for the aetiological diagnosis of acute infectious diarrhoeas should be collected in the early stage of illness and prior to treatment with antimicrobials. A stool specimen rather than a rectal swab is preferred.

- ➢ The faeces specimen should not be contaminated with urine.
- ➢ Do not collect the specimen from bed pan.
- ➢ Collect the specimen during the early phase of the disease and as far as possible before the administration of antimicrobial agents.
- ➢ 1 to 2 gm quantity is sufficient.
- ➢ If possible, submit more than one specimen on different days.
- ➢ The fresh stool specimen must be received within 1-2 hours of passage.

- Store at 2-8°C.
- Modified Cary and Blair medium (see chapter 5) is recommended as a good transport medium. It is a very stable medium and can be stored for use in screw – capped containers. It is a semi-solid transport medium. At least two swabs should be inoculated. Most pathogens will survive for up to 48 hours at room temperature. Specimens are unacceptable if the medium is held for more than one week or if there is detectable drying of the specimen.

  Alternative transport media are Venkataraman-Ramakrishnan medium (V-R fluid) or alkaline peptone water. VR fluid should be prepared in 30 ml (1 oz) screw capped bottles (MacCartney bottles). It preserves vibrios for more than six weeks and has also proved to be a very convenient medium for transportation as it can be kept at room temperature after collection of the specimen.

Throat swab

- Depress the tongue with a tongue blade.
- Swab the inflammed area of the throat, pharynx or tonsils with a sterile swab taking care to collect the pus or piece of membrane.
- Transport in sterile transport tube.

Bone marrow

Bone marrow is collected by a doctor who is well trained in this procedure

- Decontaminate the skin overlying the site from where specimen is to be collected with 70% alcohol followed by 2% tincture of iodine.
- Aspirate 1 ml or more of bone marrow by sterile percutaneous aspiration.
- Collect in a sterile screw-cap tube.
- Send to laboratory immediately.

Rectal swab

- Insert swab at least 2.5 cm beyond the anal sphincter so that it enters the rectum.
- Rotate it once before withdrawing.
- Transport in Cary and Blair or other transport medium.

Transportation of specimens

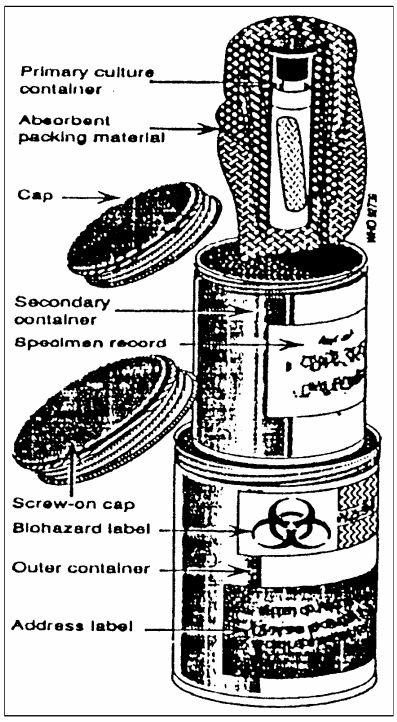
Specimens to be sent to other laboratories require special attention for safe packing of the material. Guidelines are usually issued by national authorities and the same should be strictly followed. For hand-carried transportation

over a short distance, the specimen should be placed upright in appropriate racks. For long distance transportation, it should be placed in three containers, i.e:

- **A primary container** which has the specimen and is leakproof with a screw-cap.
- **A secondary container** which is durable, waterproof and made of metal or plastic with a screw-cap. It should have enough absorptive material to absorb the contents of the primary container should the latter break or leak. On its outside, the details of the specimen should be pasted.
- **A tertiary container** is usually made of wood or cardbox. It should be capable of withstanding the shocks and trauma of transportation. Dry ice can be kept between this and the secondary container along with sufficient absorbents and provision for the escape of carbondioxide to prevent a pressure build-up inside (Fig 1).

In general, most specimens should be processed in the laboratory within 1 to 2 hours after collection. In practice, a 2-to 4-hour time limit is probably more practical during a normal working day. The laboratory must be organized to permit processing of the specimens as soon as they arrive, and the collection of most specimens should be limited to the working hours of the laboratory. However, some arrangements must be made to allow for the initial handling of the few specimens that have to be collected outside of the laboratory's working hours.

Figure 1: *Transportation container*



A continuous effort must be made in order to ensure proper collection and transportation of clinical specimens. Full cooperation of nursing staff and others concerned with specimen collection is required and can be achieved once they are made aware of the principles involved and the significance of what they are being asked to do.

Further reading

1. Lennette HE, Balows A, Hauser WJ et al. Collection, Handling and Processing of Specimen. In Manual of Clinical Microbiology, 4th Ed, ASM, Washington, DC, 73-98, 1985.
2. El-Nageh, Heuck CC, Appel W, Vandepitte et al. Basics of quality assurance in peripheral laboratories. WHO EMRO Series No 2, Alexandria, 1992.

3. Sterilization

Sterilization is defined as the destruction or removal (by filtration) of all microorganisms and their spores, whereas **disinfection** is the destruction of many microorganisms but not usually the bacterial spores. Sterilization is usually achieved with the help of heat whereas chemical agents are employed to effect disinfection.

Sterilization and disinfection are part of the daily routine of microbiological laboratories and constitute a vital activity which ensures that cultures, containers, media and equipment are treated in such a way that only the inoculated organisms will grow while all others will be eliminated.

Sterilization by heat

This can be achieved by autoclaving, by exposing articles to dry heat in hot air ovens or boiling.

Autoclaving

Autoclaves can sterilize anything that can withstand a temperature of 121^{o}C for 30 minutes. A pressure cooker used in homes for cooking purposes can also be used as a makeshift autoclave.

The containers having clinical material are subjected to heat treatment in the autoclave after which these are emptied and washed and put back into service.

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

- ➢ Pressure cooker type.
- ➢ Gravity displacement models with automatic air and condensate discharge.

Pressure-cooker type laboratory autoclaves

The most common type is a device for boiling water under pressure. It has a vertical metal chamber with a strong metal lid which can be fastened down and sealed with a rubber gasket. An air and steam discharge tap, pressure

gauge and a safety valve are fitted in the lid. Water in the bottom of the autoclave is heated by external gas burners, an electric immersion heater or a steam coil.

Operating instructions

- ➢ Ensure that there is sufficient water inside the chamber.
- ➢ Load the autoclave and fasten the lid keeping the discharge tap open.
- ➢ Adjust the safety valve to the required temperature and turn the heat on.
- ➢ Allow the mixture of air and steam to pass out freely till all air has been discharged.
- ➢ Close the air discharge tap and let the steam pressure rise within the chamber till it attains a temperature of 121^{o}C (1.5 kg/cm^{2}).
- ➢ Hold on the pressure for 15 minutes.
- ➢ Turn off the heat and let the autoclave cool.
- ➢ Slowly open the air and steam discharge taps after the pressure gauge has reached zero.
- ➢ Allow the material to cool before these are handled (usually agar bottles take hours before these become safe to handle).

Autoclave with air discharge by gravity displacement

These are usually rectangular in shape and arranged horizontally. These autoclaves have a jacket around the chamber.

Operating instructions

- ➢ Bring the jacket of the autoclave to operating temperature.
- ➢ Load the chamber, close the door and open the steam valve so that steam can freely enter the top of the chamber. Air and condensate shall automatically flow out through the drain at the bottom.
- ➢ When the drain thermometer reaches the required temperature, allow further period for the load to reach that temperature (this has to be determined initially and periodically for each autoclave).
- ➢ Continue the autoclave cycle for the holding period.
- ➢ Close the steam valve and let the autoclave cool till a temperature of 80^{o}C is reached.

- ➢ Gradually and softly open the autoclave enabling the steam to escape and allow the load to cool further.

Hot air oven

A hot air oven is electrically operated and should be equipped with a fan to ensure uniform temperature inside. The required temperature for sterilization is generally 160^{o}C for one hour.

Operating instructions

- ➢ Arrange the material to be sterilized loosely and evenly on the racks of the oven allowing free circulation of air and thereby even heating of the load.
- ➢ Do not pack the load tightly since air is a poor conductor of heat.
- ➢ Switch on the power supply and control the temperature of the oven by adjusting the thermostat.
- ➢ Note the time when the desired temperature is reached (heating-up time).
- ➢ Hold the load in the oven at this temperature for a definite period of time (holding period). This is usually 60 minutes at 160^{o}C.
- ➢ Do not overheat since it would char the cotton plugs and paper wrappings.

Autoclaves and hot air ovens can be used for disinfection of infectious waste before it is discarded. In addition, waste can be disposed of by boiling in detergent or by burial.

Boiling in detergent

In the absence of an autoclave, most specimen containers can be boiled in water having detergents to decontaminate. This process kills the vegetative bacteria but fails to destroy the spores and certain viruses. The easiest way to get best results is to add washing powder or sodium carbonate crystals, 60 grams to one litre of water in a big container and boil specimen containers in it for a minimum of 30 minutes.

Disinfection

Disinfection can be undertaken either chemically or by boiling. Boiling is an effective method to disinfect equipment e.g. needles and syringes, if autoclaving facilities are not available. Equipment which has already been cleaned should be boiled for 20 minutes. Chemical disinfection is used for heat-sensitive equipment that is damaged at high temperatures. Commonly-

used chemical disinfectants include chlorine releasing compounds; ethyl and isopropyl alcohol, quaternary ammonium compounds and gluteraldehyde.

The synopsis of a few commonly-used disinfectants is given in Table 1.

Preferred methods of sterilization for common articles are given in Table 2.

Decontamination of some of the commonly reusable equipment has been briefly presented in Table 3.

Table 1: ***Disinfectants and their mode of application****

Target	Disinfectant	Strength to use (disinfectant/ material V/V)	Application	Time of exposure
Skin	Ethanol Iodine Povidone iodine Quaternary ammonium comp	70% 1% 1%	Direct Direct Direct Direct	2 minutes 2 minutes 2 minutes 2 minutes
Blood	Cresol (pH 9) Ca hypochlorite	5% 1%	2:1 2:1	6 hours 6 hours
Urine	Cresol (pH 9)	5%	1:1	4 hours
Sputum	Cresol (pH 9)	5%	1:1	4 hours
Faeces	Cresol (pH 9) Hypochlorite (Na/Ca) Ca hydroxide	5% 1% 20%	2:1 3:1 2:1	6 hours 6 hours 6 hours
Work benches	Lysol Cresol Hypochlorite Chloramine-T	5% 1% 5%	Direct Direct Direct Direct	4 hours 4 hours 4 hours 4 hours
Glassware	Hypochlorite	1%	Direct	4 hours
Lab instruments	Hypochlorite Isopropanol	0.1% 70%	Direct Direct	4 hours 4 hours

* Based upon: Basics of quality assurance: WHO/EMRO, 1992, page 162

Table 2: ***Preferred methods of sterilization for common-use articles***

Autoclaving Animal cages Sugar tubes Lab. coats Cotton Filters Instruments Culture media	***Hot air oven*** Glass ware Beakers Flasks Petridish Pipette Slides Glass syringes Test tubes Powders
Rubber Gloves, stopper, tubing	***Wood*** Tongue depressor, applicator

Glass Slides, syringes, test tubes Enamel metal trays Wire baskets	

Table 3: ***Disinfection of specific equipment***

Container/material	Method of choice for decontamination	Alternative method of decontamination
Reusable stool container	Autoclaving 121°C for 30 minutes	Fill the jar having stool with 5% solution of phenol and keep for 24hours
	Empty into lavatory*	Empty into lavatory*
Reusable containers of CSF, pus, sputum	Autoclaving	Boiling in detergent
Urine bottles (after emptying in lavatory*)	Autoclaving	Fill with 2% phenol or 1% bleach, leave for 4 hours, clean with detergent
Blood containers	Autoclaving	Soak overnight in strong disinfectant(5% cresol; 1% Ca hypochlorite, 1:2 V/V)
Glass microscope slides**	Autoclaving	Soak overnight in 5% phenol

* If the lavatory is connected to a septic tank, phenol or other antiseptics should not be put into the lavatory.

** Glass microscope slides which have been used for the diagnosis of tuberculosis should be discarded after keeping them soaked in detergent overnight.

Biohazard waste management

Waste is defined as any solid, liquid or gaseous material that is no longer used and will either be recycled, disposed of or stored in anticipation of treatment and/or disposal.

Storage

Prior to disposal, all biohazardous waste should be maintained and stored separately from the general waste stream and from other hazardous wastes. The containers used to store biohazardous waste should be leak-proof, clearly labelled with a red or orange universal biohazard symbol and sealed tightly when transported. In certain cases, it may be necessary to double-bag the waste to prevent leakage. Any biohazardous sharps, such as infectious needles and scalpels, must be placed in containers that are puncture-resistant, leak-proof on all sides and the bottom, and close-able. These containers can then be placed in a standard biohazard bag.

Disposal options

There are three main disposal options:

- render the waste noninfectious by autoclaving and dispose it in the general waste stream. If autoclaving is not possible, decontaminate with chemical disinfectants or by boiling for 20 minutes before disposal.
- on-site incineration, if possible.
- transportation of locally-generated waste to a distant appropriate facility.

Incineration is the preferred disposal option. Not only does this method render the waste noninfectious but it also changes the form and shape of the waste. Sterilization is an effective method for decontaminating waste, but it does not alter the appearance of the waste. Steam sterilization in an autoclave at a temperature of 121^{o}C for at least 15 minutes destroys all forms of microbial life, including high numbers of bacterial spores. This type of complete sterilization can also be accomplished using dry heat which requires a temperature of 160-170^{o}C for 2-4 hours. However, it must be ensured that heat comes in contact with the material to be rendered sterile. Therefore, bottles containing liquid material should have loosened caps or cotton plug caps to allow for steam and heat exchange within the bottle. Biohazard bags containing waste should be tied loosely. Once sterilized, biohazardous waste should be sealed in appropriate containers, labelled as disinfected waste and disposed of in an approved facility.

Biological waste should be clearly labelled prior to disposal and complete records should be maintained.

Burial

It is not a decontaminating process *per se*. However, it does prevent the infectious material from becoming a reservoir of infection if properly buried. It requires digging a pit of almost 5 meters depth and 2 meters width and having a tightly fitted heavy lid on top. Disposable containers with clinical material are thrown daily into it and the lid is replaced immediately after throwing the specimens. Once a week, the refuse is covered with a layer of quicklime. If quicklime is not available, the refuse is covered with almost 10 cm thick layer of dried leaves once a week.

Further reading

El-Nageh MM et al. Basics of Quality Assurance for Intermediate and Peripheral Laboratories. WHO Regional Publication, Eastern Mediterranean Series No 2, 156-166, 1992.

4. Staining Techniques

Staining of the clinical material or the bacteria from colonies on laboratory media provide a direct visualization of the morphology of the organisms as well as their reactions to the chemicals present in stains. This is an invaluable and easy-to-use tool for establishing the identity of various microorganisms. Some of the commonly-used staining techniques are:

- ➢ Methylene blue staining
- ➢ Gram staining
- ➢ Albert staining
- ➢ Ziehl Neelsen staining (Acid fast staining)
- ➢ India ink staining
- ➢ Iodine staining for ova and cysts in faeces

Methylene blue staining

Ingredients and preparation

Methylene blue	0.3 gm
Distilled water	100 ml

Dissolve the dye in water. Filter through a filter paper.

Staining procedure

- ➢ Make a smear on a glass slide, dry in air and fix by passing it over the flame of a burner 3-4 times.
- ➢ Stain for one minute by pouring methylene blue solution over the smear.
- ➢ Wash with water, blot dry and examine under the oil immersion of light microscope.

Uses

The stain is used to make out clearly the morphology of the organisms e.g. *Yersinia pestis* in exudate, *Haemophilus influenzae* in CSF and gonococci in urethral pus.

Gram staining

This is the most extensively used differential stain that divides bacteria into two major groups. Those which retain crystal violet dye after treatment with iodine and alcohol appear purple or bluish purple and are designated as Gram positive. Those bacteria which lose the crystal violet show the colour of the counter stain employed. The commonly-used counter stain is saffranin which gives a pink/red colour to bacteria and these organisms are labelled as Gram negative.

Ingredients and preparation

Crystal violet

Solution A

Crystal violet	2.0 gm
Ethanol, 95%	20 ml

Solution B

Ammonium oxalate	0.8 gm
Distilled water	80 ml

Mix solutions A and B. Store for 24 hours before use.

Gram iodine

Iodine crystals	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300 ml

Grind the dry iodine and potassium iodide in a mortar. Add water, a few ml at a time, and grind thoroughly after each addition until the iodine and iodide dissolve. Rinse the solution into an amber glass bottle with the remainder of the distilled water.

Saffranin solution

Stock solution

Saffranin O	2.5 gm
Ethanol, 95%	100 ml

Working solution

Stock solution	10 ml
Distilled water	90 ml

Staining procedure

- ➢ Make a thin smear on a clean glass slide, dry it in air and fix by passing through flame of a burner.
- ➢ Cover the smear with crystal violet, keep for one minute.
- ➢ Wash the slide with water, then cover with Gram iodine and let it stand for one minute.
- ➢ Wash the slide with water.
- ➢ Decolour with acetone/alcohol, rocking the slide gently for 10-15 seconds till the violet colour comes off the slide.
- ➢ Wash with water immediately.
- ➢ Counterstain with saffranin. Let the counterstain stand for 30 seconds.
- ➢ Wash with water, blot dry and examine under the oil immersion lens of a microscope.

Uses

Widely used in diagnostic bacteriology mainly to differentiate organisms on the basis of morphology and Gram reaction.

Albert staining

Ingredients and preparations

Albert stain I

Toluidine blue	0.15 gm
Malachite green	0.20 gm
Glacial acetic acid	1.0 ml
Alcohol(95%)	2.0 ml
Distilled water	100 ml

Grind and dissolve the dyes in alcohol, add water and then add acetic acid. Let the mixture stand for 24 hours and then filter.

Albert stain II

Iodine	2.0 gm
Potassium iodide	3.0 gm
Distilled water	300 ml

Dissolve iodine and potassium iodide in water by grinding in a mortar with a pestle. Filter through a filter paper.

Staining procedure

- ➢ Cover the heat-fixed smear with Albert stain I. Let it stand for two minutes.
- ➢ Wash with water.
- ➢ Cover the smear with Albert stain II. Let it stand for two minutes.
- ➢ Wash with water, blot dry and examine.

Uses

To demonstrate metachromatic granules in *C.diphtheriae*. These granules appear bluish black whereas the body of bacilli appear green or bluish green.

India ink staining

Staining procedure

- ➢ Place a loopful of India ink on the side of a clean slide.
- ➢ A small portion of the solid culture is suspended in saline on the slide near the ink and then emulsified in the drop of ink, or else, mix a loopful of liquid culture of specimens like CSF with the ink.
- ➢ Place a clean cover slip over the preparation avoiding air bubbles.
- ➢ Press down, or blot gently with a filter paper strip to get a thin, even film.
- ➢ Examine under dry objectives followed by oil immersion.

Use

To demonstrate the capsule which is seen as an unstained halo around the organisms distributed in a black background. This is employed for fungal diagnostics especially for *Cryptococcus neoformans*.

Ziehl Neelsen staining

Ingredients and preparations

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

- ➢ 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 a bamboo stick. A good smear is spread evenly, 2 cms x 3 cms 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 readable through the smear.
- ➢ Let the smear air-dry for 15-30 minutes.
- ➢ 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.
- ➢ 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.
- ➢ Decolor the stained slide by pouring 25% sulphuric acid on the slide and leaving the acid for 2-4 minutes.
- ➢ 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 minutes and rinse gently with tap water.
- ➢ Counter stain the slide by pouring 0.1% methylene blue solution onto the slide and let it stand for one minute.
- ➢ Gently rinse the slide with 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 a microscope using 40 x lens to select the suitable area of the slide and examine under 100 x lens using a drop of immersion oil.

Uses

Distinguishes acid fast bacilli such as *Mycobacterium tuberculosis* and *M.leprae* from other non-acid fast bacilli.

Iodine staining for ova and cysts

➢ On a clean glass slide place one drop of normal saline and one drop of 2% iodine solution at two different sites.

➢ Mix a portion of stool first with normal saline and then with iodine solution with the help of a wire loop or applicator.

➢ Place coverslips on both the emulsions.

➢ Examine the preparations under 10x and 40x of the microscope for various ova and cysts.

Quality control of stains

Test all stains at appropriate intervals for their ability to distinguish positive and negative organisms and document the results. The performance standards for Ziehl-Neelsen and Gram staining are as given below:

Stain	Control organism/ material	ATCC No*	Expected result
Ziehl-Neelsen	*Mycobacterium spp.* *E. coli*	25177 25922	Pink red bacilli Blue bacilli
Gram	*E. coli* *S. aureus*	25922 25923	Gram -ve bacilli Gram +ve cocci
Iodine solution	Formalin treated stool specimen with cysts		Visible cyst nuclei

* If no standard strains are available, known laboratory strains should be used as controls.

The quality control procedure for stains needs to be performed on a weekly basis and also as and when a new lot of reagents for staining is procured/prepared.

Further reading

1. Manual of basic techniques for a health laboratory, WHO, 1980.

2. Bailey & Scott's Diagnostic Microbiology by Baron, Peterson and Finegold, 9th Ed, Mosby, 1994.

5. Bacteriological Media

The role of suitable quality culture media for cultivation of microorganisms cannot be over emphasised. On it depends the very success of isolation of aetiological agents. Only in exceptional cases, can an organism be identified on the basis of its morphological characteristics alone.

Types of media

Bacteriological media can be broadly sub-divided into four categories.

(1) Ordinary culture media

These are routinely employed in a laboratory e.g. nutrient broth, nutrient agar, infusion broth and lysate media.

(2) Enriched media

Certain organisms do not grow on ordinary nutrient media. They require growth- promoting ingredients such as blood, glucose, serum, egg, etc. The media containing ingredients which enhance their growth-promoting qualities are enriched media e.g. blood agar, chocolate agar and Loeffler medium.

(3) Enrichment media

Enrichment media are liquid media containing chemical constituents which inhibit some normal flora and allow pathogens which may be present in very small number in the specimen, to grow unhampered and thus enriching them. Isolated colonies of these organisms may be obtained by subculturing onto solid media. An example of enrichment media is selenite broth used for primary isolation of enteric bacteria.

(4) Differential and selective media

Differential media have got some chemical constituents which characterize different bacteria by their special colonial appearances in the culture e.g. MacConkey agar contains lactose as a substrate and neutral red as an indicator. Bacteria fermenting lactose produce acid and this will change the colour of the indicator and thus the colonies will turn red. The red lactose

fermenting colonies can be differentiated from the pale non-lactose fermenting colonies.

Selective media will selectively permit the growth of pathogens and inhibit the commensals. In addition, it may differentiate the pathogen from commensals that grow by the colour and opacity of the colonies e.g. blood tellurite medium for *C.diphtheriae*.

In addition, transport media are also frequently used to sustain the viability of organisms when a clinical specimen is to be transported from the periphery to laboratory. The transport medium prevents the outgrowth of contaminants during transit and sustains the pathogen. Cary and Blair and Stuart media are two examples of this group of media.

Preparation of media and checking of pH

Presently, a wide range of culture media are available commercially in the form of dehydrated media. These media are simply reconstituted by weighing the required quantities and by adding distilled water, as per the manufacturer's instructions.

The pH determination can be conveniently done with the use of Lovibond comparator with phenol red indicator disc.

- ➢ Take two clean test tubes and add 5 ml of the medium to each of the tubes. One serves as a blank while phenol red indicator is added to the other tube.
- ➢ Compare the colour of the medium with the phenol red indicator at the appropriate pH marking.
- ➢ Add N/10 NaOH or N/10 HCl, drop by drop till the colour of the medium matches the colour of the disc at the required pH reading.
- ➢ Calculate the volume of the NaOH or HCL of 1/10 strength for 5 ml of the medium to get the required pH.
- ➢ Based on the calculation, the volume of 1N NaOH or IN HCl required for the total volume of medium can be calculated and added.
- ➢ Check the pH of the medium once again before use.

The quantity of agar given in the formulae of media may have to be changed depending upon the quality of agar used. The concentration varies from batch to batch and should be such that will produce a sufficiently firm surface on solidification. This can be tested by streaking with inoculating wire.

In some laboratories media are prepared by individual measurement of ingredients and then mixing the same. Hence the method of preparation is given likewise:

Nutrient broth

Meat extract	10.0 gm
Peptone	10.0 gm
Sodium chloride	5.0 gm
Distilled water	1000 ml

Mix the ingredients and dissolve them by heating in a steamer. When cool, adjust the pH to 7.5-7.6.

Nutrient agar

To the ingredients as in nutrient broth, add 15 gm agar per litre. Dissolve the agar in nutrient broth and sterilize by autoclaving at 121°C for 15 minutes. Prepare plates and slopes as required.

Glucose broth

Nutrient broth	900 ml
Glucose (10% solution)	100 ml

- ➢ Dissolve 9 gm glucose in distilled water and sterilize by tyndallisation.
- ➢ Add l00 ml of the glucose solution to 900 ml of sterile nutrient broth.
- ➢ Dispense 60 ml each in 100 ml pre-sterilized culture bottles.
- ➢ Sterilize by open steaming at l00°C for one hour.

Blood agar

Nutrient agar	100 ml
Sheep blood (defibrinated)	10 ml

- ➢ Melt the sterile nutrient agar by steaming, cool to 45°C.
- ➢ Add required amount of sheep blood aseptically with constant shaking.
- ➢ Mix the blood with molten nutrient agar thoroughly but gently, avoiding froth formation.
- ➢ Immediately pour into petri dishes or test tubes and allow to set.

Chocolate agar

The ingredients are essentially the same as in blood agar.

- ➢ Melt the sterile nutrient agar by steaming and cool to about 75°C.
- ➢ Add blood to the molten nutrient agar and allow to remain at 75°C after gently mixing till it is chocolate brown in colour.
- ➢ Pour in petri dishes or test tubes for slopes as desired.

XLD agar

Xylose	3.5 gm
1 – lysine	5.0 gm
Lactose	7.5 gm
Sucrose	7.5 gm
Sodium chloride	5.0 gm
Yeast extract	3.0 gm
Sodium desoxycholate	2.5 gm
Sodium thiosulphate	6.8 gm
Ferric ammonium citrate	0.8 gm
Phenol red	0.08 gm
Agar agar	15.0 gm
Water	1000 ml

Weigh the ingredients into a flask and add distilled water. Mix the contents well and steam it for 15 minutes (do not autoclave). Cool to 56°C and pour in plates.

Buffered glycerol saline

Glycerol	300 ml
Sodium chloride	4.2 gm
Disodium hydrogen phosphate	10.0 gm
Na_2 H PO_4 Anhydrous	15.0 gm
Phenol red aqueous solution 0.02 per cent	15.0 ml
Water	700 ml

- ➢ Dissolve NaCl in water and add glycerol.
- ➢ Add disodium hydrogen phosphate to dissolve.
- ➢ Add phenol red and adjust pH to 8.4.
- ➢ Distribute 6 ml in universal containers (screw -capped bottles of 30 ml capacity). Autoclave at 115°C for 15 minutes.

Loeffler serum medium

Nutrient broth	100 ml
Serum (sheep or horse or ox)	300 ml
Glucose	1.0 gm

- ➢ Dissolve glucose in nutrient broth and sterilize at 121°C for 15 minutes.
- ➢ Add serum aseptically.
- ➢ Mix thoroughly but gently, avoiding froth formation.
- ➢ Distribute in sterile test tubes or quarter ounce screw-cap bottles.
- ➢ Inspissate the medium in a slanting position in a water inspissator at 82°C for two hours.
- ➢ In the absence of an inspissator, the medium may be coagulated by standing over the top of a steam sterilizer for 6-7 minutes.

Blood tellurite agar

Agar base

Meat extract	5.0 gm
Peptone	10.0 gm
Sodium chloride	5.0 gm
Agar	25.0 gm
Water	1000 ml

Dissolve the ingredients and adjust the pH to 7.6. Distribute in 100 ml quantities in a bottle and autoclave at 121°C for 15 minutes.

Glycerolated blood tellurite mixture

Sterile defibrinated sheep blood	14 ml
Sterile glycerol	6 ml
Sterile potassium tellurite solution (1% in water)	4 ml

Sterilize the glycerol in hot air oven at 160°C for 60 minutes and the tellurite solution by autoclaving at 115°C for 20 minutes. Mix the ingredients in a sterile flask, incubate for 1-2 hrs. at 37°C, then refrigerate. Haemolysis is complete after 24 hrs. The mixture keeps well in a refrigerator. One per cent solution of good quality tellurite is sufficient but 2% of some batches may be required.

Preparation of complete medium

Glycerolated blood tellurite mixture	24 ml
Agar base	100 ml

Melt the agar, cool to 45°C, add blood and tellurite and pour in sterile petri dishes.

Salt broth (10%)

Meat extract	10.0 gm
Peptone	10.0 gm
Sodium chloride	100.0 gm
Distilled water	1000 ml

Prepare as for nutrient broth, distribute and sterilize at 121°C for 15 minutes.

Peptone water

Peptone	10.0 gm
Sodium chloride	5.0 gm
Distilled water	1000 ml

Dissolve peptone and sodium chloride in distilled water by heating. Adjust pH to 7.2. Distribute in test tubes and sterilize by autoclaving at 121°C for 15 minutes.

Alkaline peptone water

Prepare peptone water as described above. Adjust pH of peptone water (7.2 to 9.2). Distribute in test tubes and sterilize at 121°C for 15 minutes.

MacConkey agar

Sodium taurocholate	5.0 gm
Peptone	20.0 gm
Sodium chloride	5.0 gm
Lactose	10.0 gm
Agar	15.0 gm
Distilled water	1000 ml
Neutral red (2% solution in 50% ethanol)	3.5 ml

- ➢ Mix 5 gm sodium taurocholate or bile salts, 20 gm of peptone, 5 gm sodium chloride and 15 gm agar with 1000 ml water.
- ➢ Steam until the solids are dissolved.
- ➢ Cool to about 50°C, and at this temperature adjust reaction to pH 7.5 to 7.8. Autoclave at 121°C for 15 minutes and filter while hot through a good grade of filter paper, or a plug of cotton wrapped in gauze placed in the funnel.
- ➢ Adjust reaction of the filtrate to pH 7.3 at 50°C or pH 7.5 at room temperature. Add 10 gm lactose and 3.5 ml of 2% solution of neutral red in 50% ethanol.

- ➢ Mix thoroughly, distribute in flasks and sterilize in the autoclave at 121°C for 15 minutes.
- ➢ For use, melt in the steamer, pour into sterile petri dishes and allow to set.

Bile salt agar

Peptone	10.0 gm
Meat extract	5.0 gm
Sodium taurocholate	5.0 gm
Sodium chloride	5.0 gm
Agar	15.0 gm
Distilled water	1000 ml

- ➢ Dissolve by steaming in 1000 ml of water, 10 gm peptone, 5 gm meat extract, 5 gm sodium chloride, 15 gm agar and 5 gm sodium taurocholate.
- ➢ Adjust pH to 8.5 with sodium hydroxide solution.
- ➢ Cool and filter through a good grade filter paper or absorbent cotton wool previously wetted with water.
- ➢ Distribute into sterile flasks in convenient amounts and sterilize by autoclaving at 121°C for 15 minutes.
- ➢ Plates are made by melting the stock medium and pouring into sterile petri dishes.

Agar base

Meat extract	20.0 gm
Peptone (proteose)	20.0 gm
Agar	90.0 gm
Lactose	40.0 gm
Neutral red (2% solution in 50% ethanol)	5.0 gm
Distilled water	4.0 litre

Solution A

Sodium citrate, $2H_2O$	17.0 gm
Sodium thiosulphate	17.0 gm
Ferric ammonium citrate	2.0 gm
Distilled water	100.0 ml

Solution B

Sodium desoxycholate	10.0 gm
Distilled water	100 ml

Agar base

- ➢ Dissolve by heating 20 gm meat extract in 200 ml water and make the solution just alkaline with 50 per cent sodium hydroxide solution.
- ➢ Boil and filter through filter paper.
- ➢ Adjust to pH 7.3, make upto 200 ml and add 20 gm proteose peptone.
- ➢ In another vessel, dissolve 90 gm agar in 3700 ml water by steaming.
- ➢ Filter the agar solution, add the meat extract peptone solution to it and mix.
- ➢ Add 5 ml neutral red solution in 50% ethanol and 40 gm lactose.
- ➢ Make up to 4 litres with water.
- ➢ Mix, bottle accurately in lots of 100 ml, and sterilize in an autoclave by free steaming for one hour and then at 121^{o}C for 10 minutes.

Solution A

Dissolve 17 gm sodium citrate, 17 gm sodium thiosulphate and 2 gm ferric ammonium citrate (green scales) in 100 ml of sterile distilled water with heating.

Solution B

Dissolve 10 gm sodium desoxycholate in 100 ml of sterile distilled water.

- ➢ Sterilize solution A and B at 60^{o}C in water bath for one hour.
- ➢ For preparing desoxycholate citrate agar medium, melt 100 ml of the agar base in a water bath and add 5 ml each of the solutions A and B in the order given, using separate pipettes.
- ➢ Mix well after each addition.
- ➢ Cool the tube at 50 to 55^{o}C.
- ➢ Pour into sterilized petri dishes and allow to set.
- ➢ Dry the surface of the medium in the incubator before use.

Selenite F broth

Sodium hydrogen selenite	4.0 gm
Peptone	5.0 gm
Lactose	4.0 gm
Disodium hydrogen phosphate (Na_2HPO_4,12H_2O)	9.5 gm
Sodium dihydrogen phosphate	0.5 gm
Distilled water	1000 ml

- ➢ Dissolve 4 gm sodium hydrogen selenite, 5 gm peptone, 4 gm lactose, 9.5 gm disodium hydrogen phosphate and 0.5 gm sodium dihydrogen phosphate in 1000 ml of sterile water (water autoclaved at 121°C for 30 minutes) with sterile precautions and distribute the yellowish solution in 10 ml amounts in sterile screw-capped bottles.
- ➢ Steam at 100°C for 30 minutes. The medium should not be autoclaved.
- ➢ There may be a slight red precipitate in the medium, but this does not interfere with the action of the medium.
- ➢ The pH of the medium as prepared should be 7.1 and the quantity of phosphate added may be varied slightly to achieve this.

Media for carbohydrate fermentation

(a)	Peptone	10.0 gm
	Sodium chloride	5.0 gm
	Distilled water	900 ml
(b)	Carbohydrate base	10.0 gm
	Distilled water	90 ml
(c)	Andrade's indicator	10 ml

- ➢ Dissolve 10 gm peptone and 5 gm sodium chloride in 900 ml water.
- ➢ Adjust pH to 7.0 to 7.3 so that after addition of 10 ml of Andrade's indicator the pH should be 7.5.
- ➢ Sterilize at 121°C for 15 minutes.
- ➢ Dissolve 10 gm of the requisite sugar in 90 ml water and steam for 30 minutes or sterilize by filtration.
- ➢ With sterile precautions, add 90 ml of this sugar solution and 10 ml of Andrade's indicator solution to 900 ml of the sterile peptone water solution. Distribute into sterile test tubes containing inverted Durham fermentation tubes. Steam for 30 minutes.
- ➢ Prepare Andrade's indicator solution by adding 1 N sodium hydroxide solution to 0.5% aqueous solution of acid fuchsin until the colour of the indicator solution is just yellow.

Lowenstein-Jensen medium

Salt solution

Potassium dihydrogen phosphate (KH_2PO4)	2.40 gm
Magnesium sulphate ($MgSO_4.7H_2O$)	0.24 gm
Magnesium citrate	0.60 gm
Asparagine L(Pure)	3.60 gm
Glycerol	12.0 ml

Distilled water	600 ml
Whole egg	1000 ml
Malachite green 2%	20 ml

Dissolve the salts and glycerol in water over a water bath. Autoclave for 15 minutes at 121°C, cool to room temperature.

Beaten egg

- Use only fresh eggs preferably not more than three days old.
- Using a soft brush and soap and with soda solution clean the outside of the egg's wall. Leave the eggs in 5% soap and soda solution for 30 minutes.
- Place in running tap water till the water is perfectly clear. Drain and dry the eggs.
- Just before breaking, clean the outside of the eggs with a piece of sterile gauze dipped in alcohol.
- Break the eggs into a sterile flask aseptically.
- Close with a rubber stopper and shake vigorously until well homogenized. Filter through two or three layers of sterile gauze stretched over a sterile funnel and collect the filtrate in a sterile container.

Malachite green solution

- Dissolve l gm malachite green in 50 ml distilled water in a screw capped bottle and sterilise at 121°C for 15 minutes.
- Add the eggs to the salt solution aseptically and mix well.
- Add malachite green and mix.
- Pour into a sterile aspirator jar and let it stand for 30 minutes for the air bubbles to escape.
- Fill into tubes or bottles.
- Inspissate for 40 minutes at 82°C to 85°C.
- While inspissating, the bottle should be kept flat but the tubes have to be sloped.

Cary and Blair transport medium

Sodium thioglycollate	1.5 gm
Disodium phosphate	1.1 gm
Sodium chloride	5.0 gm
Agar	5.0 gm

Calcium chloride, (1% solution)	9.0 ml
Distilled water	1000 ml

- ➢ Dissolve by heating 1.5 gm sodium thioglycollate, 1.1 gm disodium phosphate, 5 gm sodium chloride and 5 gm agar in 990 ml distilled water.
- ➢ Cool to 50°C and add 9 ml of one per cent aqueous solution of calcium chloride. Adjust the pH to 8.4.
- ➢ Dispense in 7 to 10 ml amounts in wide mouth screw capped bottles.
- ➢ Steam for 15 minutes, cool and tighten the caps.

Stuart transport medium

Thioglycolic acid	2 ml
Sodium hydroxide, (IN NaOH)	12-15 ml
Sodium glycerophosphate, 20% aqueous	100 ml
Calcium chloride, $CaCl_2$, 1% aqueous	20 ml
Distilled water	900 ml

Mix the ingredients, adjust pH to 7.2 with IN sodium hydroxide solution.

Agar solution

Agar	6.0 gm
Distilled water	1000 ml
Dissolve by steaming	

Preparation of complete medium

Anaerobic salt solution	900 ml
Agar solution	11 ml
Methylene blue, 0.1% aqueous	4 ml

- ➢ Melt the agar and add the salt solution.
- ➢ Adjust the pH to 7.3-7.4.
- ➢ Add the methylene blue and distribute in small bottles filling nearly to capacity. Autoclave at 121°C for 15 min. and immediately tighten caps.
- ➢ When cool, the medium should be colourless.

Venkataraman-Ramakrishnan (VR) holding medium

- ➢ Dissolve 12.4 gm. boric acid and 14.9 gm potassium chloride in 800 ml hot distilled water, cool and make up to 1000 ml with distilled water.
- ➢ To 250 ml of this stock solution, add 133.5 ml N/5 sodium hydroxide solution. Make up to 1000 ml with distilled water and add 20 gm common salt. Shake and dissolve.
- ➢ Filter through filter paper and dispense in 10 to 15 ml amounts in wide- mouthed screw capped bottles.

A simple modification is as follows:

- ➢ Dissolve 20 gm of crude sea salt and 5 gm of peptone in distilled water to make one litre.
- ➢ Adjust the pH to 8.6 to 8.8.
- ➢ Dispense 10-15 ml amounts in wide-mouthed bottles with screw-caps.
- ➢ About 1-3 ml of stool specimen should be inoculated into the medium.

MacConkey broth for bacteriological examination of water

This medium is used for detecting the presence of coliform organisms in water.

Single strength

Sodium taurocholate (commercial)	5 gm
Peptone (any good make)	20 gm
Sodium chloride (NaCl)	5 gm
Lactose	10 gm
Bromocresol purple, 1%, solution in ethanol	5 ml
or Neutral red, 1% aqueous solution	5 ml
Water	1000 ml

- ➢ Dissolve the bile salt, peptone and sodium chloride.
- ➢ Steam for two hours, cool and transfer to the refrigerator overnight.
- ➢ Add lactose and when dissolved, filter cold through filter paper.
- ➢ Adjust the reaction to pH 7.4.
- ➢ Add indicator.

- ➢ Distribute in 5 ml amounts in 1-oz. bottles or 15 cm x 1.5 cm test tubes with Durham tubes.
- ➢ Autoclave at 121°C for 15 minutes.

Double strength

- ➢ Prepare as above, but with half the amount of water.
- ➢ Distribute in 50 ml amounts in 5-oz bottles using 3x3/8 inch test tubes with Durham tubes, and in 10 ml amounts in 1-oz bottles using 2X0.25 inch Durham tubes.

Sabouraud Dextrose Agar (SDA)

Ingredients

	Original SDA	Emmon's modification
Dextrose Peptone	40 gm	20 gm
Neopeptone	10 gm	10 gm
Agar	20 gm	20 gm
Distilled water	1000 ml	1000 ml
Adjust final pH	5.6	6.8 to 7.0

Preparation

Mix the ingredients in water by heating. Adjust the pH. Sterilize in the autoclave at 121°C for 10 minutes.

10% Potassium Hydroxide

Ingredients and preparation

Potassium hydroxide	10 gm
Distilled water	100 ml

Dissolve 10 grams of potassium hydroxide in 100 ml of distilled water.

Lactophenol cotton blue mounting medium

Ingredients and preparation

Phenol crystals	20 gm
Lactic acid	20 gm
Glycerol	40 gm
Cotton blue (poirrier's blue)	0.05 gm
Distilled water	20 ml

Melt the phenol crystals in water bath. Mix water and phenol crystals. Add 0.05 gram of cotton blue into a mortar and grind it with a pestle. Add water and phenol little by little and make it a nice paste. Add the remaining amount of water and phenol, mix well. Then add lactic acid and glycerol and mix well. Filter through a filter paper.

Desoxycholate Citrate Agar (DCA)

Ingredients

Proteose peptone, No.3	10.0 gm
Lactose	10.0 gm
Sodium citrate	20.0 gm
Ferric ammonium citrate	2.0 gm
Sodium desoxycholate	5.0 gm
Bacto agar	15.0 gm
Neutral red (1% solution)	2.3 ml
Beef infusion broth	1000 ml
pH 7.4	

Preparation

Add Proteose peptone, sodium citrate, ferric ammonium citrate and bacto agar to the beef Infusion broth, pH of which has already been adjusted. Recheck pH. Keep it in a water bath for 30 minutes for dissolving. Then add lactose, sodium desoxycholate and neutral red solution and mix. Then pour in plates.

Bismuth Sulfite Agar (BSA) (Wilson & Blair medium)

Ingredients

Polypeptone peptone	10.0 gm
Beef extract	5.0 gm
Dextrose	5.0 gm
Disodium phosphate	4.0 gm
Ferrous sulfate	0.3 gm
Bismuth sulfite indicator	8.0 gm
Brilliant green	0.025 gm
Agar	20.0 gm
Distilled water	1000 ml
pH 7.5 $\pm$ 0.2	

Preparation

Mix the ingredients in distilled water. Allow to stand for five minutes and mix thoroughly. When a uniform suspension has been obtained, heat with frequent agitation and boil for one minute. Cool to about 50^{o}C. Mix well and pour into sterile plates.

Mueller-Hinton Agar (MHA)

Ingredients

Beef extract	2.0 gm
Acidicase Peptone	17.5 gm
Starch	1.5 gm
Agar	17.0 gm
Distilled water	1000 ml
Final pH 7.4 $\pm$ 0.2	

Preparation

Dissolve the ingredients in one liter of distilled water. Mix thoroughly. Heat with frequent agitation and boil for one minute. Dispense and sterilize by autoclaving at 121^{o}C for 15 minutes. Do not overheat. When remelting the sterile medium, heat as briefly as possible.

Saponin lysed blood agar plus VCNT (A) inhibitors

A selective medium for the growth of *N.gonorrhoeae* using GC agar base

GC agar base (Difco)	36 gm
Saponin lysed horse/sheep blood	90 ml (9% final concentration)
Distilled water	1000 ml
VCN inhibitor (10 ml vial)	1 vial
Trimethoprim (100 mg/L stock)	2 ml
(Amphotericin (1000 mg/L stock)	1 ml (optional))

Method

The GC agar base medium is prepared at single strength which is half the strength recommended by the manufacturer, since the addition of equal volume of 2% haemoglobin is replaced by adding lysed blood. Suspend 36 gm GC agar base in one litre of distilled water. Mix well, then steam or boil gently until it dissolves completely. Sterilize by autoclaving at 15 psi (121^{o}C) for 15 minutes. Cool to 52-54^{o}C in a waterbath. When the molten GC agar medium has cooled to this temperature, aseptically add the saponin lysed horse or sheep blood. VCN inhibitor solution, trimethoprim (and amphotericin if used). Mix well and pour 25 ml volumes in 90 mm diameter petri dishes. Allow the agar to set, then store the plates in an inverted position in the laboratory refrigerator (2-8^{o}C) until required.

Performance of plated media

Samples of plates from each batch are selected for performance-testing and are inoculated with the appropriate stock cultures. For each type of medium, at least two or three microorganisms having growth characteristics with ‘positive’ and ‘negative’ results for the medium should be used. The size of inoculum and method of inoculating the test plates must be standardized as closely as possible. In general, control organisms should be selected from an actively growing broth culture and a standard loopful of culture seeded

directly onto the test medium, which is then streaked so as to obtain isolated colonies. After appropriate incubation, the results of the performance test are recorded. The medium is released for use in the clinical laboratory only if the results indicate satisfactory performance. In initiating a quality control programme, one must establish some priorities, such as beginning by testing those media that are most likely to demonstrate deficiencies. Top priority should be given to blood agar, chocolate agar and Thayer Martin agar media. Secondary priority should be accorded to selective enteric media such as MacConkey agar, XLD and bile salt agars.

A quantitative approach may be more useful for testing of performance of selective or inhibitory media such as Thayer Martin agar. *N gonorrhoeae* and *N.meningitidis* usually grow on Thayer Martin agar when the inoculum is heavy, but when a fairly light inoculum is used, the pathogens might be inhibited. Consequently, a somewhat quantitative performance test could detect deficiencies that would be overlooked if one simply inoculated test plates with undiluted stock cultures.

Further reading

Balows A, Hausler WJ, Herrman KL et al. Manual of Clinical Microbiology 5th Ed, American Soceity of Microbiologists, Washington, 1991.

6. Cultivation of Bacteria on Laboratory Media

Inoculation of Culture Media

For microbiological investigations it is essential to learn the skills of inoculating specimens onto culture media and subculturing from one medium to another.

Instrument for seeding media

This is selected according to the nature of the medium and inoculum. Platinum or nichrome wires of different gauges are used. Nichrome has oxidizing properties and hence in some of the tests where this property of bacterium is to be tested (e.g. oxidase test), platinum wire, instead of nichrome should be used. This wire is sterilized by holding it vertically in the flame of the burner so that the whole length of wire becomes red hot. It is allowed to cool down before it touches any material suspected to be having bacteria to avoid the heat killing the organisms. Presterilized disposable loops are now available commercially. The wire can be used as a:

- Straight wire to stab the culture, picking of single colonies as well as for inoculating the liquid media,
- Thick wire which is useful for lifting the viscid material such as sputum, and
- Wire loop which is usually of 2 mm diameter is most useful of all inoculating wires. These are preferred to seed a plate of medium as the straight wire usually cuts the agar.

Seeding a culture plate

There are three commonly employed techniques for seeding culture plates. The most common is shown in Fig. 1.

The inoculum from the clinical material or another plate is first spread out in the form of a primary inoculum (as at A in Fig 1) which is also called as ‘well-inoculum’ or only ‘well’. The successive series of strokes B, C, D and E are made with the loop sterilized between each sequence. At each step the inoculum is derived from the most distal part of the immediately preceding

strokes so as to gradually reduce the number of bacteria. This helps in obtaining isolated colonies.

In an alternative plating procedure one edge of a large loop is used to make a secondary well (see B in Fig 1). The other edge is then used to make succession of strokes across the remaining unseeded area.

When the inoculum is small or the medium is selective it can be more heavily inoculated (Fig 2). Several loop-fulls of the specimen are used to spread the primary inoculum (see A in Fig 2).

After sterilizing the loop, it is recharged by rubbing it over area A and the plate is seeded in parallel strokes.

Figure1: *Seeding a culture plate inoculum*

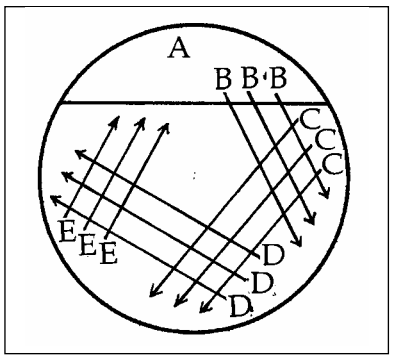
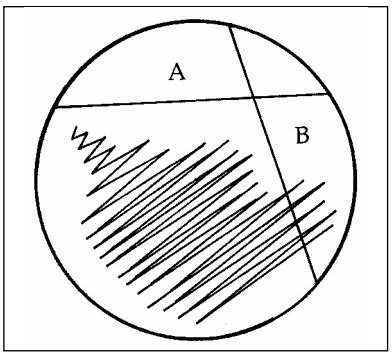


Figure 2: *Seeding with heavy*



Seeding a liquid medium

If the tubes have got cotton plugs, the mouth of the tubes should be heated in flame before and after any handling of tube to prevent contamination from the rims of tubes getting into the medium. It is not required when metal caps and screw-capped tubes are handled. Incline the tube containing the liquid medium to 45^{o} and deposit the inoculum on its wall above the surface of the liquid at its lower end. Return the tube to a vertical position. Now the inoculum shall be below the surface of the liquid.

Subculture from a solid medium to solid medium

Using a sterile wire or loop, a representative colony is touched and subcultured onto appropriate solid medium by touching the wire or loop onto the surface of the medium.

Important points about inoculation of culture media

➢ Aseptic technique is important to avoid contamination.

- ➢ When more than one medium is inoculated, follow a particular order. Inoculate media without inhibitors, followed by indicator and then selective media.
- ➢ While processing fluid specimen inoculate liquid media first to reduce the chances of carry over from contaminated solid media.
- ➢ Prepare smears for staining after all media have been inoculated.
- ➢ Properly label the media to be inoculated to avoid any mix-up of the specimens.
- ➢ Inoculate the media with clinical specimens as soon as possible.
- ➢ Minimize the aerosol production by opening the caps of liquid media slowly, avoiding vigorous shaking of the specimen and avoiding the expulsion of the last drop from the pipette.

Inoculation of carbohydrate fermentation media

These are inoculated as liquid media and incubated at 37^{o}C for 18-24 hours. When the particular sugar is fermented, acid is produced which changes the pH of the medium thus turning phenol red into yellow. In case the fermentation is with the production of gas, a bubble of air is visible in Durham tube.

Seeding solid media in test tubes

Slopes of solid media are inoculated by streaking the surface of the agar with loop in a zig zag manner. Stab cultures are inoculated by plunging the wire into the centre of the medium.

Aerobic Incubation of cultures

For bacteria of medical importance, incubation is uniformally done at 37^{o}C. Depending upon the workload a laboratory may have a tabletop incubator (suitable for peripheral laboratories) or a walk-in incubator. For prolonged incubations, as are required for the growth of *Mycobacterium tuberculosis,* screw-capped bottles should be used instead of petri dishes or tubes to prevent the drying of medium.

Incubation in an atmosphere with added carbon dioxide

Extra carbon dioxide is needed for optimal growth of organisms such as *Brucella abortus,* pneumococci and gonococci. The concentration of additional carbon dioxide needed is 5-10 per cent. The simplest method for having this environment is to put the plates in a container and generate CO_2 inside by lighting a candle in it just before putting on the lid. Pure CO_2 can also be introduced in a container. Carbon dioxide-generating kits are now available and so are incubators which can provide a predetermined and

regulated amount of this gas. Special CO_2 incubators (also called capnoeic incubators) are available commercially.

Aseptic techniques

Aseptic techniques are important to protect the worker from infection from the clinical specimen and also to prevent contamination of the material under process. Aseptic conditions can be achieved by following steps:

- ➢ Open the caps and lids of the containers containing the specimen for the briefest period required.
- ➢ Do not keep the lids on the workbench.
- ➢ Inoculating loops should be put through the flame properly prior to introducing them into the specimen container.
- ➢ While working on the infectious material, keep the specimen away from the face.
- ➢ Loops should not contain fluid or large particles of matter that may splatter when placed in the flame.
- ➢ Avoid vigorous shaking of the specimen prior to opening; open the caps slowly to minimize aerosol production.
- ➢ Perform homogenization and grinding procedures involving tissue or biopsy specimen in safety cabinet.
- ➢ Keep all specimens, tubes and bottles of media in racks to reduce the risk of accidental spillage.
- ➢ Mop up the workbench clean with any disinfectant at the start and close of work.
- ➢ Wash hands with soap and water before and after handling infectious specimens.

Further reading

1. Isenberg HD(Ed) Clinical Microbiology Procedures handbook. American Society for Microbiology, Washington, DC, Vol 1, Section 1.4, 1992.
2. Collins CH, Lyne PM and Grange JM. Microbiological Methods. Butterworths, London, 94-96, 1995.
3. PHLS Standard Operating Procedures – Inoculation of culture media No B.SOP 54 Version:1, 1998.

7. Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing has become a very essential step for the proper treatment of infectious diseases. It is used

- ➢ To guide the clinician in selecting the best antimicrobial agent.
- ➢ To accumulate epidemiological information on the resistance of microorganisms of public health importance.

The choice of drugs used in a routine antibiogram is governed by various considerations since only a few antimicrobial agents can be tested. Table 1 suggests the drugs to be tested in various situations. The drugs in Table 1 are divided into two sets. Set 1 includes drugs that are available in most hospitals and for which routine testing should be carried out for every strain. Tests for drugs in set 2 are to be performed only at the special request of the physician, or when the causative organism is resistant to the first-choice drugs, or when other reasons (allergy to a drug, or its unavailability) make further testing justified.

Table 1: ***Basic sets of drugs for routine susceptibility tests***

	Set 1	Set 2
Staphylococcus	Benzyl penicillin Oxacillin Erythromycin Tetracycline Chloramphenicol	Gentamicin Amikacin Co-trimoxazole Clindamycin
Intestinal	Ampicillin Chloramphenicol Co-trimoxazole Nalidixic acid Tetracycline	Norfloxacin
Enterobacteriaceae **Urinary**	Sulfonamide Trimethoprim Co-trimoxazole Ampicillin Nitrofurantoin Nalidixic acid Tetracycline	Norfloxacin Chloramphenicol Gentamicin
Blood and tissues	Ampicillin Chloramphenicol Cotrimoxazole Tetracycline Gentamicin	Cefuroxime Ceftriaxone Ciprofloxacin Piperacillin Amikacin
Pseudomonas aeruginosa	Piperacillin Gentamicin	Amikacin

	Tobramycin	

Antimicrobial susceptibility tests measure the ability of an antibiotic or other antimicrobial agent to inhibit bacterial growth *in vitro*. This ability may be estimated by either the dilution method or the diffusion method. The recommended method for intermediate and peripheral laboratories is the modified Kirby-Bauer method, the methodology of which is given below:

Modified Kirby-Bauer method

Reagents

Mueller-Hinton agar

- Mueller-Hinton agar should be prepared from a dehydrated base according to the manufacturer's recommendations. The medium should be such that with standard strains, zone sizes within the acceptable limits are produced. It is important not to overheat the medium.
- Cool the medium to 45-50^{o}C and pour into plates. Allow to set on a level surface, to a depth of approximately 4 mm. A 9 cm diameter plate requires approximately 25 ml of the medium.
- When the agar has solidified, dry the plates for immediate use for 10-30 minutes at 36^{o}C by placing them in an upright position in the incubator with the lids tilted.
- Any unused plates may be stored in a plastic bag, which should be sealed and placed in a refrigerator. Plates stored in this way can be kept for two weeks.
- In order to ensure that the zone diameters are sufficiently reliable for testing susceptibility to sulfonamides and co-trimoxazole, the Mueller-Hinton agar must have low concentrations of the inhibitors thymidine and thymine. Each new lot of Mueller-Hinton agar should therefore be tested with a control strain of *Enterococcus faecalis* (ATCC 29212 or 33186) and a disc of co-trimoxazole. A satisfactory lot of medium will give a distinct inhibition zone of 20 mm or more that is essentially free of hazy growth or fine colonies.
- For testing the susceptibility of fastidious organisms, 5% blood should be added to the Mueller-Hinton agar base.

Antibiotic discs

Any commercially available discs with the proper diameter and potency can be used. Stocks of antibiotic discs should preferably be kept at -20^{O}C, or the freezer compartment of a home refrigerator is convenient. A small working supply of discs can be kept in the refrigerator for up to one month. On removal from the refrigerator, the containers should be left at room

temperature for about one hour to allow the temperature to equilibrate. This procedure reduces the amount of condensation that occurs when warm air reaches the cold container.

Turbidity standard

Prepare the turbidity standard by pouring 0.6 ml of a 1% (10 gm/L) solution of barium chloride dihydrate into a 100-ml graduated cylinder, and filling to 100 ml with 1% (10 ml/L) sulphuric acid. The turbidity standard solution should be placed in a tube identical to the one used for the broth sample. It can be stored in the dark at room temperature for six months, provided it is sealed to prevent evaporation.

Swabs

A supply of cotton wool swabs on wooden applicator sticks should be prepared. These can be sterilized in tins, culture tubes, or on paper, either in the autoclave or by dry heat.

Procedure

- To prepare the inoculum from the primary culture plate, touch with a loop the tops of each of 3-5 colonies, of similar appearance, of the organism to be tested.
- When the inoculum has to be made from a pure culture, a loopful of confluent growth is similarly suspended in saline. Inoculum from colonies of streptococci cannot be made by emulsification. Hence, with streptococci, after inoculation the culture tubes are incubated for 4-6 hours to get uniform turbidity which should be matched with the turbidity standards.
- Compare the tube with the turbidity standard and adjust the density of the test suspension to that of the standard by adding more bacteria or more sterile saline. Proper adjustment to the turbidity of the inoculum is essential to ensure that the resulting lawn of growth is confluent or almost confluent.
- Inoculate the plates by dipping a sterile swab into the inoculum. Remove excess inoculum by pressing and rotating the swabs firmly against the side of the tube above the level of the liquid.
- Streak the swab all over the surface of the medium three times, rotating the plate through an angle of 60^{o} after each application. Finally, pass the swab round the edge of the agar surface. Leave the inoculum to dry for a few minutes at room temperature with the lid closed. The antibiotic discs may be placed on the inoculated plates using a pair of sterile forceps.

- A sterile needle tip may also be used to place the antibiotic discs on the plate. Alternatively, an antibiotic disc dispenser can be used to apply the discs to the inoculated plate.
- A maximum of seven discs can be placed on a 9-10 cm diameter plate. Six discs may be spaced evenly, approximately 15 mm from the edge of the plate, and one disc placed in the centre of the plate. Each disc should be pressed down gently to ensure even contact with the medium.
- The plates should be placed in an incubator at 35°C within 30 minutes of preparation. Temperatures above 35°C invalidate the results for oxacillin/ methicillin.
- Do not incubate in an atmosphere of carbon dioxide.
- After overnight incubation, the diameter of each zone(including the diameter of the disc) should be measured and recorded in mm. The results should then be interpreted according to the critical diameters by comparing with standard tables (Table 2).
- The measurements can be made with a ruler on the under surface of the plate without opening the lid.
- The end-point of inhibition is judged by the naked eye at the edge where the growth starts, but there are three exceptions:
  - With sulfonamides and co-trimoxazole, slight growth occurs within the inhibition zone; such growth should be ignored.
  - When β-lactamase producing staphylococci are tested against penicillin, zones of inhibition are produced with a heaped-up, clearly defined edge; these are readily recognizable when compared with the sensitive control, and regardless of the size of the zone of inhibition, they should be reported as resistant.
  - Certain *Proteus* spp. may swarm into the area of inhibition around some antibiotics, but the zone of inhibition is usually clearly outlined and the thin layer of swarming growth should be ignored.

Table 2:* ***Zone sizes with different antimicrobial agents***

Antimicrobial agent	When testing against	Disc content	Zone diameter nearest whole mm		
			Resistant	Intermediate	Susceptible
ß-LACTAMS					
Ampicillin	Enterobacteriaceae	10 µg	<13	14-16	>17
	Staphylococci	10 µg	<28		>29
	Enterococci	10 µg	<16		>17
Carbenicillin	Pseudomonas	100 µg	<13	14-16	>16
	Gram negatives	100 µg	<19	20-22	>23
Methicillin	Staphylococci	5 µg	<9	10-13	>14

Antimicrobial agent	When testing against	Disc content	Zone diameter nearest whole mm		
			Resistant	Intermediate	Susceptible
Mezlocillin	Pseudomonas	75 µg	<15		>16
	Other Gram negatives	75 µg	<17	18-20	>21
Nafcillin	Staphylococci	1 µg	<10	11-12	>13
Oxacillin	Staphylococci	1 µg	<10	11-12	>13
Penicillin	Staphylococci	10 units	<28		>29
	Enterococci	10 units	<14		>15
Piperacillin	Pseudomonas	100 µg	<17		>18
	Other Gram negatives	100 µg	<17	18-20	>21
Ticarcillin	Pseudomonas	75 µg	<14		>15
	Other Gram negatives	75 µg	<14	15-19	>20
ß-LACTAM/ß-LACTAMASE INHIBITOR COMBINATIONS					
Amoxycillin/clavulanic acid	Staphylococci	20/10 µg	<19		>20
	Other organisms	20/10 µg	<13	14-17	>18
Ampicillin/sulbactam	Staph & Gram -ve	10/10 µg	<11	12-14	>15
Piperacillin/tazobactam	Pseudomonas	100/10 µg	<17		>18
	Other Gram negatives	100/10 µg	<17	18-20	>21
	Staphylococci	100/10 µg	<17		>18
Ticarcillin/clavulanic acid	Pseudomonas	75/10 µg	<14		>15
	Other Gram negatives	75/10 µg	<14	15-19	>20
	Staphylococci	75/10 µg	<22		>23
CEPHEMS					
Cefamandole		30 µg	<14	15-17	>18
Cefazolin		30 µg	<14	15-17	>18
Cefotaxime		30 µg	<14	15-22	>23
Cefoxitin		30 µg	<14	15-17	>18
Ceftazidime		30 µg	<14	15-17	>18
Ceftizoxime		30 µg	<14	15-19	>20
Ceftriaxone		30 µg	<13	14-20	>21
Cefuroxime oral		30 µg	<14	15-22	>23
Cefuroxime parentral		30 µg	<14	15-17	>18
Cephalothin		30 µg	<14	15-17	>18
CARBAPENEMS					

Antimicrobial agent	When testing against	Disc content	Zone diameter nearest whole mm		
			Resistant	Intermediate	Susceptible
Imipenem		10 µg	<13	14-15	>16
MONOBACTAMS					
Aztreonam		30 µg	<15	16-21	>22
GLYCOPEPTIDES					
Telcoplanin		30 µg	<10	11-13	>14
Vancomycin	Enterococci	30 µg	<14	15-16	>17
	Other Gram positives	30 µg	<9	10-11	>12
AMINOGLYCOSIDES					
Amikacin		30 µg	<14	15-16	>17
Gentamicin	except high resistant enterococci	10 µg	<12	13-14	>15
Kanamycin		30 µg	<13	14-17	>18
Netilmycin		30 µg	<12	13-14	>15
Streptomycin		10 µg	<11	12-14	>15
Tobramycin		10 µg	<12	13-14	>15
MACROLIDES					
Azithromycin		15 µg	<13	14-17	>18
Clarithromycin		15 µg	<13	14-17	>18
Erythromycin		15 µg	<13	14-22	>23
TETRACYCLINES					
Doxycycline		30 µg	<12	13-15	>16
Minocycline		30 µg	<14	15-18	>19
Tetracycline		30 µg	<14	15-18	>19
QUINOLONES					
Ciprofloxacin		5 µg	<15	16-20	>21
Enoxacin		10 µg	<14	15-17	>18
Lomefloxacin		10 µg	<18	19-21	>22
Nalidixic acid		30 µg	<13	14-18	>19
Norfloxacin		10 µg	<12	13-15	>16
Ofloxacin		5 µg	<12	13-15	>16
OTHERS					
Chloramphenicol		30 µg	<12	13-17	>18
Clindamycin		2 µg	<14	15-20	>21
Nitrofurantoin		300 µg	<14	15-16	>17
Rifampin		5 µg	<16	17-19	>20
Sulfonamides		250/300 µg	<12	13-16	>17

Antimicrobial agent	When testing against	Disc content	Zone diameter nearest whole mm		
			Resistant	Intermediate	Susceptible
Trimethoprim		5 µg	<10	11-15	>16
Trimethoprim/ sulfamethoxazole		1.25/ 23.75 µg	<10	11-15	>16

* For *Vibrio cholerae*, the results of disc diffusion tests for ampicillin, tetracycline and trimethoprim/sulfamethoxazole correlate with results obtained by broth microdilution methods.

Results

The result of the susceptibility test, as reported to the clinician, is the classification of the microorganism in one of two or more categories of susceptibility. The simplest system comprises only two categories, susceptible and resistant. This classification, although offering many advantages for statistical and epidemiological purposes, is too inflexible for the clinician to use. Therefore, a three-category classification is often adopted. The Kirby-Bauer method and its modifications recognize three categories of susceptibility and it is important that both the clinicians and the laboratory workers understand the exact definitions and the clinical significance of these categories.

- **Susceptible:** An organism is called "susceptible" to a drug when the infection caused by it is likely to respond to treatment with this drug, at the recommended dosage.
- **Intermediate susceptibility** covers two situations. It is applicable to strains that are "moderately susceptible" to an antibiotic that can be used for treatment at a higher dosage because of its low toxicity or because the antibiotic is concentrated at the focus of infection (e.g. urine). The term also applies to those strains that are susceptible to a more toxic antibiotic that cannot be used at a higher dosage. In this situation the intermediate category serves as a buffer zone between susceptible and resistant.
- **Resistant:** This term implies that the organism is expected not to respond to a given drug, irrespective of the dosage and the location of the infection.

For testing the response of staphylococci to benzylpenicillin, only the categories 'susceptible' and 'resistant' (corresponding to the production of β-lactamase) are recognized. Staphylococci that are resistant to methicillin or oxacillin are also resistant to other penicillins or cephalosporins even though they show a zone of inhibition against these drugs.

Zone diameters, to the nearest whole mm for various antimicrobial agents with disc content specified for each one for interpretation as susceptible, intermediate and resistant are given in Table 2.

Quality assurance in susceptibility test

The precision and accuracy of the test are controlled by the parallel use of a set of control strains, with known susceptibility to antimicrobial agents. These quality control strains are tested using exactly the same procedure as for the test organisms. The zone sizes shown by the control organisms should fall within the range of diameters given in Table 3. When the results regularly fall outside this range, they should be regarded as evidence that a technical error has been introduced into the test, or that the reagents are at fault. Each reagent and each step in the test should then be investigated until the cause of the error has been found and eliminated.

The quality assurance programme should use standard reference strains of bacteria that are tested in parallel with the clinical culture. They should preferably be run every week, or with every fifth batch of tests, and in addition, every time that a new batch of Mueller-Hinton agar or a new batch of discs is used. The standard strains are:

Staphylococcus aureus (ATCC 25923)
Escherichia coli (ATCC 25922)
Pseudomonas aeruginosa (ATCC 27853)

Culture for day-to-day use should be grown on slants of nutrient agar (tryptic soya agar is convenient) and stored in a refrigerator. These should be subcultured onto fresh slants every two weeks.

Table 3: ***Quality Control – Susceptibility of Control Strains***

Zone diameter of inhibition (mm)

Antibiotic	Disc potency (IU or µg)	*S.aureus* (ATCC 25923)	*E.coli* (ATCC25922)	*P.aeruginosa* (ATCC 27853)
Amikacin	30	20-26	19-26	18-26
Ampicillin	10	27-35	16-22	-
Ceftriaxone	30	22-28	29-35	17-23
Cephalothin	30	29-37	15-21	-
Chloramphenicol	30	19-26	21-27	-
Ciprofloxacin	5	22-30	30-40	25-33
Clindamycin	2	24-30	-	-
Erythromicin	15	22-30	-	-
Gentamicin	10	19-27	19-26	16-21
Nalidixic acid	30	-	22-28	-
Nitrofurantoin	300	18-22	20-25	-
Norfloxacin	10	17-28	28-35	-
Oxacillin	1	18-24	-	-
Penicillin G	10	26-37	-	-

Antibiotic	Disc potency (IU or µg)	*S.aureus* (ATCC 25923)	*E.coli* (ATCC25922)	*P.aeruginosa* (ATCC 27853)
Piperacillin	100	-	24-30	25-33
Tetracycline	30	19-28	18-25	-
Tobramycin	10	19-29	18-26	19-25
Trimethoprim	5	19-26	21-28	-
Trimethoprim-sulfamethoxazole	25	24-32	24-32	-

Biosafety

- ➢ Observe good laboratory practices as outlined in Chapter 8.
- ➢ Antimicrobial susceptibility of organisms such as *Yersinia pestis* and *Bacillus anthracis* should be performed under BSL-3 environment.
- ➢ The culture plates with organisms such as *Bacillus anthracis* be appropriately autoclaved after chemical treatment with hypochlorite solution before disposal.

Referral

- ➢ In the event of disease outbreak the susceptibility results must be verified by the reference laboratory.
- ➢ When unusual susceptibility patterns are encountered.
- ➢ As a part of quality assurance programme.

When intermediate laboratories need not undertake suscpetibility tests?

- ➢ In organisms with predictable susceptibility patters e.g. *Streptococcus haemolyticus, Corynebacterium diphtheriae* and *Treponema pallidum* infections.
- ➢ In cases where susceptibility tests are technically more demanding such as *Mycobacterium tuberculosis* and *Neisseria* spp.

Salient features of quality assurance in antibiotic susceptibility testing

- ➢ Use antibiotic discs of 6 mm diameter.
- ➢ Use correct content of antimicrobial agent per disc.

- Stock the supply of antimicrobial discs at -20^{0}C.
- Use Mueller-Hinton medium for antibiotic sensitivity determination.
- Use appropriate control cultures.
- Use standard methodology for the test.
- Use coded strains from time to time for internal quality control.
- Keep the antibiotic discs at room temperature for one hour before use.
- Incubate the sensitivity plates for 16-18 hours before reporting.
- Incubate the sensitivity plates at 35^{0}C.
- Space the antibiotic discs properly to avoid overlapping of inhibition zone.
- Use inoculum size that produces near confluent growth.
- Ensure an even contact of the antibiotic disc with the inoculated medium.
- Measure the zone sizes precisely.
- Interpret the zone sizes by referring to standard charts.

Further reading

NCCLS: Performance Standards for Antimicrobial Susceptibility Testing: Eighth Informational Supplement.M100-S8, Vol 18 No 1 January 1998, NCCLS, Pennysylvania, USA.

8. Safety in Laboratories

Laboratory safety is a vital component of functioning of any laboratory. Safety procedures and precautions to be followed in the microbiology laboratory should be designed to:

- ➢ Restrict microorganisms present in specimens or cultures to the vessels in which they are contained.
- ➢ Prevent environmental microorganisms (normally present on hand, hair, clothing, laboratory benches or in the air) from entering specimens or cultures and interfering with the results of the studies.

Laboratory biosafety levels

Four biosafety levels have been recommended based on the infectiousness of the agent/s.

Biosafety Level -1 (BSL-1): Adherance to standard microbiological practices. No special requirement as regards containment equipment.

Biosafety Level-2 (BSL-2): In addition to the use of standard microbiological practice, laboratory coats, decontamination of infectious wastes, limited access, protective gloves and display of biohazard sign and partial containment equipment are the requirements for this level.

Most peripheral and intermediate laboratories need BSL-1 or BSL-2 laboratory facilities.

BSL-3: In addition to BSL-2, it has special laboratory clothing, controlled access to laboratory and partial containment equipment.

BSL-4: BSL-3 plus entrance through change room where laboratory clothing is put on, shower on exit, all wastes are decontaminated before exit from the facility. It requires maximum containment equipment.

Laboratory facilities in BSL-2

- ➢ Laboratory should be designed in such a way that it can be easily cleaned.
- ➢ Laboratory contains a sink for washing.
- ➢ Laboratory tops are impervious to water but resistant to acids, alkalies and organic solvents.

- An autoclave to decontaminate infectious material is available.
- Illumination is adequate for all laboratory activities.
- Storage space is adequate.

Preventive measures against laboratory infections

These are aimed to protect workers, patients and cultures. Following steps are suggested:

- Perform adequate sterilization before washing or disposing waste.
- Provide receptacle for contaminated glassware.
- Provide safety hood.
- Ensure that tissues are handled and disposed of properly.
- Promote regular handwashing and cleaning of bench tops.
- Ensure use of gloves.
- Provide mechanical pipetting devices.
- Protect patients from laboratory personnel with skin or upper respiratory tract infections.
- Provide special disposal containers for needles and lancets.

Pipetting

Pipetting and suctioning have been identified as the significant and consistent causes of occupational infections. Various important precautions that must be taken while pipetting are:

- Develop pipetting techniques that reduce the potential for creating aerosols.
- Plug pipettes with cotton.
- Avoid rapid mixing of liquids by alternate suction and expulsion.
- Do not forcibly expel material from a pipette.
- Do not bubble air through liquids with a pipette.
- Prefer pipettes that do not require expulsion of last drop of liquid.
- Drop material having pathogenic organisms as close as possible to the fluid or agar level.
- Place contaminated pipettes in a container having suitable disinfectant for complete immersion.

A variety of pipettes are available. Selection should depend upon the ease of operation and the type of work to be performed.

Hypodermic syringes and needles

Accidents involving the use of syringes and needles while drawing blood from patients or performing experiments on laboratory animals are among the most common causes of occupational infections in laboratories and health care facilities. They account for almost 25% of the laboratory-acquired infections that occur by accidents. The practices which are recommended for hypodermic needle and syringes are:

- ➢ Avoid quick and unnecessary movements of the hand holding the syringe.
- ➢ Examine glass syringes for chips and cracks, and examine needles for barbs and plugs.
- ➢ Use needle locking (Luer Lock type) syringes only and be sure that needle is locked securely.
- ➢ Wear surgical or other gloves.
- ➢ Fill syringes carefully to minimize air bubbles and frothing.
- ➢ Expel excess air, liquid and bubbles vertically into a cotton pledget moistened with suitable disinfectant.
- ➢ Do not use syringe to forcefully expel infectious fluid into an open vial for mixing. Mixing with a syringe is appropriate only if the tip of the needle is held below the surface of the fluid in the tube.
- ➢ Do not bend, shear, recap or remove the needle from syringe by hand.
- ➢ Place used needle-syringe units directly into a puncture-resistant container and decontaminate before disassembly, reuse or disposal.

Opening containers

The opening of vials, flasks, petri dishes, culture tubes, embryonated eggs, and other containers of potentially infectious materials poses potential but subtle risks of creating droplets, aerosols or contamination of the skin or the immediate work area. The most common opening activity in most health care laboratories is the removal of stoppers from containers of clinical materials. It is imperative that specimens should be received and opened only by personnel who are knowledgable about occupational infection risks. Various precautions that can be taken in this regard are:

- ➢ Open containers with clinical specimens in well-lighted and designated areas only.

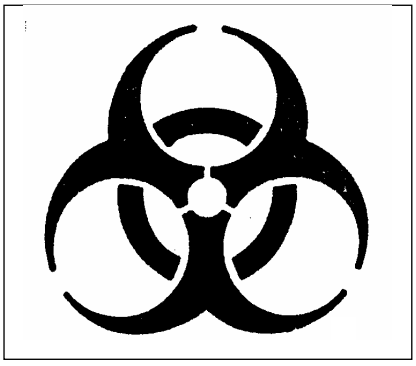
- ➢ Wear a laboratory coat and suitable gloves.
- ➢ If possible, use a plastic-backed absorbent paper towel to:
  - facilitate clean-up
  - reduce generation of aerosols
- ➢ Specimens which are leaking or broken may be opened only in safety cabinets.

Tubes containing bacterial cultures should be handled with care. Vigorous shaking of liquid cultures creates a heavy aerosol. When a sealed ampoule containing a lyophilized or liquid culture is opened, an aerosol may be created. Ampoules should be opened in a safety cabinet.

Laboratory access

- ➢ As far as possible children and pregnant women visitors should not enter the microbiological laboratories.
- ➢ Appropriate signs should be located at points of access to laboratory areas directing all visitors to a receptionist or receiving office for access procedures.
- ➢ The universal biohazard symbol (Fig 1) shall be displayed at specific laboratories in which manipulations of organisms with moderate and heavy risk are being carried out. Only authorized visitors shall enter the laboratory showing universal biohazard sign. Doors displaying biohazard symbol shall not be propped open, but shall remain closed when in use.

Figure 1: *Universal biohazard sign*



Clothing

- ➢ All employees and visitors in microbiological laboratories shall wear laboratory clothing and laboratory shoes or shoe covers.

- ➢ Disposable gloves shall be worn wherever radiological, chemical, carcinogenic materials or virus preparations of moderate to high risk are handled.
- ➢ Laboratory clothings including shoes shall not be worn outside the work area.

Accidents in laboratory

In the microbiological laboratory, bacterial infections pose the most frequent risk. The important diseases/organisms are:

Hepatitis B virus
HIV
Brucella spp.
Leptospires
Mycobacteria spp.
Histoplasma
Shigella spp.
Salmonella spp. including S typhi
Bacillus anthracis
Yersinia pestis

Accidents and spills

The order of priorities is as follows:

- ➢ Protection of personnel
- ➢ Confinement of contamination
- ➢ Decontamination of personnel
- ➢ Decontamination of area involved

Decontamination of skin. The area is washed thoroughly with soap and water. Detergents or abrasive materials must not be used and care must be taken not to damage the skin.

Decontamination of cuts\eyes. These are irrigated with water taking care to prevent the spread of contamination from one area to another.

Decontamination of clothing. Contaminated garments should be removed immediately and placed in a container. They should not be removed from the spill location until contamination has been monitored.

Decontamination of work surfaces

- ➢ Flood the total spillage area including the broken container with disinfectant.
- ➢ Leave undisturbed for 10 minutes.
- ➢ Mop with cotton wool or absorbent paper.

- ➢ Wear disposable gloves, apron and goggles.
- ➢ If a dustpan and brush or forceps have been used these too require disinfection.
- ➢ For blood or viruses, hypochlorites (10 gm/L) are used.
- ➢ Do not use hypochorite solution in centrifuges.
- ➢ Use activated gluteraldehyde (20 gm/L) on surfaces for viral decontamination.
- ➢ Place all potentially contaminated materials in a separate container and retain until monitored.
- ➢ Restrict the entry to such an area until contamination monitoring has been carried out.

Management of laboratory accidents

An adequately equipped first-aid box should be kept in the laboratory in a place that is known and accessible to all members of staff. The box must be clearly marked and preferably be made of metal or plastic to prevent from damage by pests. A medical officer should be consulted regarding the contents of the box. A first-aid chart giving the immediate treatment of cuts, burns, poisoning, shock and collapse, should be prepared and displayed in the laboratory.

General laboratory directions for safety

The salient general laboratory directions which must be obeyed by all are:

- ➢ Long hair should be bound back neatly away from shoulders.
- ➢ Do not wear any jewellery to laboratory sessions.
- ➢ Keep fingers, pencils, bacteriological loops etc. out of your mouth.
- ➢ Do not smoke in the laboratory.
- ➢ Do not lick labels with tongue (use tap water).
- ➢ Do not drink from laboratory glasswares.
- ➢ Do not wander about the laboratory; uncontrolled activities cause:
  - – accidents
  - – distract others
  - – promote contamination
- ➢ Do not place contaminated pipettes on the bench top.
- ➢ Do not discard contaminated cultures, glasswares, pipettes, tubes or slides in wastepaper basket or garbage can.

- ➢ Avoid dispersal of infectious materials.
- ➢ Operate centrifuges, homogenizer and shakers safely.
- ➢ Immunize the laboratory workers against vaccine-preventable diseases such as hepatitis B, meningococcal meningitis, rabies, etc.

Further reading

1. Guidelines for Preventing HIV, HBV and other Infections in the Health Care Setting, SEARO WHO, Delhi 1996.
2. World Health Organization: Laboratory biosafety manual, 2nd Edition, WHO, 1993.
3. Miller B.M. (et al). Laboratory safety: principles and practices, Washington DC, American Society for Microbiology, 322, 1986.

9. Quality Assurance

Quality Assurance (QA) is a wide ranging concept covering all matters that individually or collectively influence the quality of a product. It denotes a system for continuously improving reliability, efficiency and utilization of products and services. In the context of quality assurance two important definitions need to be clearly understood:

(i) **Internal Quality Control (IQC):** which denotes a set of procedures undertaken by the staff of health facility (medical, paramedical workers as well as laboratorians) for **continuously** and **concurrently** assessing laboratory work so that quality results are produced by the laboratory for supporting quality health care at patient and community levels.

(ii) **External Quality Assessment (EQA):** is a system of objectively assessing the laboratory performance by an outside agency. This assessment is **retrospective** and **periodic** but is aimed at improving the IQC.

IQC and EQA are complementary in ensuring the reliability of the procedures, results and quality of the product.

What is the objective of QA?

QA programmes are required for the following reasons:

- ➢ To improve the quality of health care.
- ➢ To generate reliable, reproducible results.
- ➢ To establish inter-laboratory comparability in laboratory testing.
- ➢ To establish the credibility of the laboratory among doctors and the public at large.
- ➢ Motivating the staff for further improvement.
- ➢ Prevention of legal complications which may follow poor quality results.

Factors affecting the quality

It is commonly believed that the quality of laboratory results solely depends upon the laboratory undertaking this analysis. However, there are many pre-analytical and post-analytical factors which influence the quality of the end results to a very significant extent. The principle of "GIGO" – "Garbage in Gabage Out" very well applies to the laboratory tests also. Some of the important factors influencing quality are listed here:

(i) **Specimen:** This is the single most important factor. Selection of the right sample, collection in a right manner, adequate quantity, proper transportation to the laboratory, and processing of the sample before testing, are crucial factors.

(ii) **Personnel:** The quality of the laboratory results generated is directly proportional to the training, commitment and motivation of the technical staff.

(iii) **Environmental factors:** Inadequate lighting, workspace or ventilation or unsafeworking conditions may influence the laboratory results.

(iv) **Analytical factors:** The quality of reagents, chemicals, glassware, stains, culture media,use of standard procedures and reliable equipment all influence laboratory results. Failure to examine a sufficient number of microscope fields can lead to false negative results.

(v) **Post analytical factors:** Transcription errors, incomplete reports, and improper interpretation can adversely influence the laboratory results.

IQC – the mainstay of QA

The backbone of a good quality assurance programme is a good IQC. Intermediate and peripheral laboratories must put in place various IQC procedures and may participate in any EQAS that is in operation.

Requirements of IQC

- ➢ Comprehensive: Cover all steps from collection of sample to reporting.
- ➢ Regular continuous monitoring.
- ➢ Rational: Focus more on critical factors.
- ➢ Practical: Should not atttempt to evaluate everything.
- ➢ Economical: Should be cost-effective and within the provided budget.

Each laboratory should have Standard Operating Procedure Manuals (SOPMs) which should include the following information about the infrastructure of a laboratory

- ➢ Biosafety precautions
- ➢ Disposal of infectious waste
- ➢ Collection, transport and storage of specimens
- ➢ Criteria of rejection of samples
- ➢ Processing of specimens
- ➢ Maintenance of equipment
- ➢ Recording of results
- ➢ Reporting of results
- ➢ Procedure of quality control
- ➢ Referral

SOPMs should be periodically reviewed and revised and religiously followed in the laboratories.

Most of the above-mentioned factors have been described in various chapters of this document. The remaining have been presented briefly here.

Maintenance of equipment

Good quality equipment is absolutely essential to generate quality results. Care of the equipment purchased is also crucial. The quality control steps for some of the commonly-used equipment at the intermediate/peripheral laboratory level is depicted in Table 1.

Table 1: ***Suggested maintenance of commonly-used equipment***

Equipment	Maintenance Instructions
Autoclave	Clean and change water monthly Adjust water level before each run Record time, temperature and pressure for each run Inspect gasket in the lid weekly Technical inspection every six months
Incubator	Clean inside walls once in a month Record temperature at the start of each working day Technical maintenance every six months
Hot air oven	Clean the inside at least once a month Record time and temperature with every run Technical inspection every six months

Equipment	Maintenance Instructions
Microscope	Wipe lenses with lens paper at the end of each day's work
	Protect the microscope from dust, vibrations and moisture
	Place a shallow plate containing dry blue silica gel in a box to absorb moisture
	Check alignment of the condenser once a month
	Technical inspection once in a year
Balance	Keep the balance and weights clean and dry
	Always use a container or weighing paper, do not put material directly on the pan
	Prevent the balance from drafts of air
Refrigerator	Place at least 10 inches away from the wall
	Clean and defrost at least every two months
	Record temperature daily
	Technical service at least once a year
Water Bath	Check water level daily
	Check temperature before and during use
	Clean monthly
	Technical inspection once in six months
Inspissator	Check temperature daily
	Clean after each batch of culture media prepared
Centrifuge	Wipe inner walls with antiseptic solution weekly
	Check brushes and bearings every six months
Glassware	Discard chipped glassware
	Ensure these are free of detergents
	Do not store sterile glassware for more than three weeks before it is used.

Performance tests on culture media

Culture media may be prepared from the individual ingredients or may be prepared from dehydrated powders available commercially. The important points in QC of media are listed here:

- ➢ Do not over-stock the media. Store the required quantities only which can be used in 6-12 months.

- ➢ Store the media away from moisture by securing the caps of all the containers tightly.
- ➢ Store in a dark, cool and well-ventilated place.
- ➢ Keep a record of the receipt, and opening of the media container.
- ➢ Discard all dehydrated media that are either darkened or caked. Rotate the stock of media, following the principle of "first in, first out".
- ➢ For preparation of media adhere strictly to the manufacturer's instructions.
- ➢ Prepared media should be protected from sunlight and heat.
- ➢ Sterility testing, performance testing and pH test of the prepared media should be done as listed in Table-2.

Table 2: ***Performance tests on commonly-used media***

Medium	Incubation	Control Organism	Expected Result
Blood Agar	24h, CO_2	*S. aureus*	Growth and beta-haemolysis
		S.pneumoniae	Growth and alpha-haemolysis
Chocolate agar	24h, CO_2	*H.influenzae*	Growth
MacConkey agar With crystal violet	24h	*E.coli*	Red colonies
		P.mirabilis	Colourless colonies (no swarming)
		E.faecalis	No growth
Methyl red/Voges-Proskauer	48h	*E.coli*	Positive/negative
		K.pneumoniae	Negative/positive
Mueller-Hinton	24h	*E.coli* ATCC 25922	Acceptable zone sizes
		P.aeruginosa ATCC 27853	Acceptable zone sizes
Peptone water (indole)	24h	*E.coli*	Positive
		K.pneumoniae	Negative
Simmons citrate (incubate with loose screwcap)	48h	*E.coli*	No growth
		K.pneumoniae	Growth, blue colour
Thiosulfate citrate bile salt (TCBS) agar	24h	*Vibrio spp.* (non agglutinable	Yellow colonies
Thayer Martin Agar	24h, CO_2	*N.meningitidis*	Growth
		N.gonorrhoeae	Growth

		Staphylococci	No growth
		E.coli	No growth

Quality control for commonly-used tests

The QC for commonly-used tests at the intermediate/peripheral level is listed in Table 3.

Quality control of immunological tests

Quality control procedures used for the detection of antigen or antibodies by various test methods are listed in Table 4.

QA of antibiotic susceptibility testing

For details refer to chapter 7.

Table 3: ***QC for commonly-used tests***

Procedure/result	Test Control organism		Expected reaction
Catalase	*S. aureus*	+	Bubbling reaction
	Streptococcus spp.	–	No bubbling
Coagulase	*S. aureus*	+	Clot formation in 4 hours
Indole	*E. coli*	+	Red ring at surface
	E. aerogenes	–	Yellow ring at surface
Methyl red	*E. coli*	+	Instant red colour
	E.. aerogenes	–	No colour change
Oxidase	*P.aeruginosa*	+	Purple colour in 20 seconds
	E. coli	–	No colour in 20 seconds
Voges	*E. aerogenes*	+	Red colour
Proskauer	*E. coli*	–	No colour change
Bacitracin disc	*Streptococcus group A*	+	Zone of inhibition
	E. faecalis	–	Zone of inhibition
Optochin disc	*S. pneumoniae*	+	Zone of inhibition
	S. viridans	–	No zone of inhibition
Oxidase disc	*P.aeruginosa*	+	Purple colour in 30 seconds
	E. coli	–	No change in colour

The testing should be done each time a new batch of working solution is prepared.

Table 4: ***QC procedures for immunological tests***

Test	Control procedures required	Expected results
1. Flocculation test (RPR)	Nonreactive serum control	No clumping
	Weakly reactive serum control	Clumping of graded activity
	Reactive serum control	Clumping of graded activity
2. Latex agglutination test (ASO)	Negative control serum	No clumping
	Positive control serum	Clumping
3. Direct agglutination (Widal test)	Antigen control	No clumping
	Negative control serum	No clumping
	Positive control serum	Clumping
4. Capsular Quellung reaction (Omni serum, *H.influenzae* type b)	Pneumococci	Capsular swelling
	Haemolytic streptococci	No reaction
	H.influenzae type b	Capsular swelling
	Acinetobacter anitratum	No reaction

In service training of staff

Periodic updating of the skills and knowledge of the laboratory workers is essential for maintaining quality. Course-curriculum of such trainings should focus on the issues highlighted above.

Participation in external quality assessment

Participation in EQAS reassures about the correctness of the results generated by the laboratory and finds out whether IQC is in place or not. The control or referral laboratories should organise EQAS in some commonly used tests.

Further reading

1. Sudarshan Kumari, Rajesh Bhatia, CC Heuck: Quality Assurance in Bacteriology & Immunology WHO Regional Publication, South-East Asia Series No. 28, 1998.
2. Bhatia Rajesh & lchhpujani R.L.: Quality Assurance in Microbiology CBS Publishers and Distributors, New Delhi, 1995.
3. Howaritz PJ, Kowanitz JH: Laboratory quality assurance (McGraw Hill Book Companry, New York (1987).