



10

METHODS OF ISOLATION OF BACTERIA

10.1 INTRODUCTION

We have learned in earlier chapters that there exist so many bacteria that cause human disease. So now our task is to isolate these bacteria and identify them. The identification is required so as to cure the illness or the infection caused due to these bacteria, using appropriate antibiotics. Identification also holds significance for epidemiological purposes.

This chapter would focus on various methods used for isolation of bacteria. While in subsequent chapters we would learn about identification of bacteria and the ways to contain the infections caused by them.



OBJECTIVES

After reading this chapter, you will be able to :

- Explain the steps involved in the isolation of bacteria.
- describe the significance of Specimen collection.
- describe the significance of Preservation and transportation of specimen.
- explain the role of microscopy in isolation of bacteria.
- explain various methods for isolation of bacteria.

10.2 ISOLATION OF BACTERIA

Isolation of bacteria forms a very significant step in the diagnosis and management of the illness. Isolation of bacteria involves various steps –

- Specimen collection
- Preservation and transportation of specimen



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- Microscopic examination of sample
- Various methods used for isolation of bacteria

Specimen collection

Many different specimens are sent for microbiological examination from patients with suspected bacterial infection. Common specimens include urine, faeces, wound swabs, throat swabs, vaginal swabs, sputum, and blood. Less common, but important specimens include cerebrospinal fluid, pleural fluid, joint aspirates, tissue, bone and prosthetic material (e.g. line tips).

Some types of specimen are normally sterile e.g. blood, CSF. These samples are usually obtained via a percutaneous route with needle and syringe, using appropriate skin disinfection and an aseptic technique. The culture of bacteria from such specimens is usually indicative of definite infection except if they are skin contaminants (bacteria inhabitants of normal skin).



Fig. 10.1: Universal container.

In contrast, many microbiological specimens are obtained from non-sterile sites e.g. vaginal or throat swabs, urine sample, stool sample. Such samples often contain bacteria of no clinical relevance in addition to possible pathogens, making the interpretation of culture results more difficult. In general it is preferable to send samples from sterile sites if available.

It is preferred to obtain the samples for bacteriological culture before antibiotic therapy is started. This maximizes the sensitivity of the investigations and reduces false-negative results. Similarly, samples of tissue or pus are preferred over swabs, to maximize the recovery of bacteria in the laboratory.

Specimens must be accurately labelled and accompanied by a properly completed requisition form, indicating the nature of the specimen, the date of sample collection, relevant clinical information, the investigations required, and details of antibiotic therapy, if any.

This allows the laboratory to perform the correct range of tests, and helps in the interpretation of results and reporting. Along with clinical specimens, medical microbiology laboratories also process samples of food, water and other environmental samples (e.g. air sampling from operating theatres) as part of infection control procedures.

High-risk samples

Certain bacterial infections are a particular hazard to laboratory staff, and specimens that might contain these pathogens should be labelled as 'high risk' to allow for additional safety measures if necessary. For example - blood cultures from suspected typhoid (*Salmonella typhi*) or brucellosis (*Brucella* species), and samples from suspected *Mycobacterium tuberculosis*.

Preservation and Transport of specimen

Most specimens are sent to the laboratory in sterile universal containers. Swabs are placed in a suitable transport medium (eg. charcoal medium) otherwise it leads to false negative reporting.

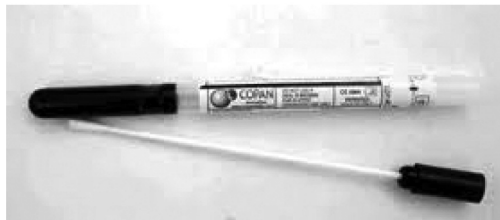


Fig. 10.2: Charcoal laden transport media

Specimens should be transported as soon as possible to the laboratory. In case a delay is anticipated the specimen should be stored at 4° C.

Immediate transport is necessary in order to:

- (i) Preserve the viability of the 'delicate' bacteria, such as *Streptococcus pneumoniae* or *Haemophilus influenzae* (delays in processing can cause false-negative culture results);
- (ii) Minimize the multiplication of bacteria (e.g. coliforms) within specimens before they reach the laboratory. In particular urine and other specimens that utilize a semiquantitative culture technique for their detection, as delays in transport can give rise to falsely high bacterial counts when the specimen is processed.

Microscopy

A Gram stain helps with the visualization of bacteria, and gives an indication of the type of bacteria present, based on the shape of the bacteria and the staining





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properties (Gram positive: purple; Gram negative: pink/red). A Gram stain also helps to identify mixtures of bacteria, helps to determine the appropriate range of agar plates to be used for subsequent culture, and helps with the interpretation of culture results.

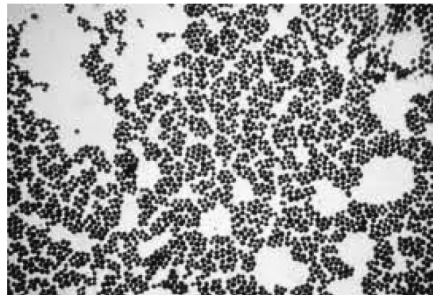


Fig. 10.3: Gram positive cocci

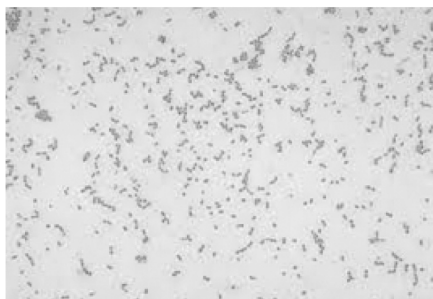


Fig. 10.4: Gram negative bacilli

For liquid specimens e.g. CSF, the sample is first centrifuged to concentrate any bacterial cells in the deposit, and Gram stain and culture is performed from the deposit after the supernatant is decanted. This helps increase the sensitivity of both microscopy and culture.

Ziehl-Neelsen (ZN) stain is used to demonstrate the presence of Mycobacteria. Mycobacteria can also be visualized using the fluorescent dye auramine and a fluorescence microscope. Direct immunofluorescence is employed to detect certain pathogens (e.g. *Legionella*, *Pneumocystis*) using specific antibodies conjugated to a fluorescent dye.

Another microscopic technique is dark ground microscopy. This is mainly used to detect the thin spirochaetal cells of *Treponema pallidum* (syphilis bacteria).



INTEXT QUESTIONS 10.1

1. Specimens that contain pathogens which are hazardous to laboratory staff should be labeled as
2. Swabs are sent to laboratory in medium

Methods of Isolation of Bacteria

3. If delay is anticipated in transporting the specimen, it should be stored at temperature
4. gives an indication of bacteria present in the sample
5. stain is used in demonstration of mycobacteria
6. microscopy is used to detect syphilis organism

10.3 METHODS OF ISOLATION OF BACTERIA

Methods of isolation of bacteria can be broadly classified into two

- Culture methods
 - On Solid media
 - On Liquid media
 - Automated systems
- Non-culture methods

Culture methods

The specimens received in the laboratory are plated on the culture media. The appropriate culture media is selected depending upon the bacteria suspected. The following precautions need to be taken into consideration when the culture methods are processed

- Optimal atmospheric conditions
- Optimal temperature
- Growth requirement of the bacteria

Atmospheric conditions:

Colonies of bacteria are usually large enough to identify after 18–24 hours of incubation (usually at 37°C), but for some bacteria longer incubation times are required (from 2 days to several weeks). Culture plates are incubated (1) in air, (2) in air with added carbon dioxide (5%), (3) anaerobically (without oxygen) or (4) micro-aerophilically (a trace of oxygen) according to the requirements of the different types of bacteria that may be present in specimens.

In case of Mycobacteria especially the scotochromogen the culture bottles are placed in dark or the bottles are covered with black paper and kept for incubation at 37°C.

Temperature:

Most of the bacteria requires a temperature of 37°C for optimal growth. This temperature is provided placing the inoculated culture plates in the incubator set at 37°C temperature.

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Microbiology



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Fig. 10.5: Incubator

Growth requirement of the bacteria

Different bacteria have different growth requirements. For eg Streptococcus pneumoniae requires factor V and factor X for its growth, which are found in chocolate agar. Thus for sample suspected of S. pneumoniae the samples are plated on chocolate agar. Similarly depending upon the growth requirements the appropriate culture media are used.



INTEXT QUESTIONS 10.2

1. & methods are commonly used methods for bacterial isolation
2. Colonies of bacteria can be identified after hours of incubation
3. The optimum temperature most bacteria require to grow are
4. Chocolate agar has & which is used in the diagnosis of streptococci Pneumonia

10.4 CULTURE ON SOLID MEDIA

The principal method for the detection of bacteria from clinical specimens is by culture on solid culture media. Bacteria grow on the surface of culture media to produce distinct colonies.

Different bacteria produce different but characteristic colonies, allowing for early presumptive identification and easy identification of mixed cultures. There are many different types of culture media. Agar is used as the gelling agent to which is added a variety of nutrients (e.g. blood, peptone and sugars) and other factors (e.g. buffers, salts and indicators).

Some culture media are nonselective (e.g. blood agar, nutrient agar) and these will grow a wide variety of bacteria. While some e.g. MacConkey agar are more selective (in this case through the addition of bile salts selecting for the 'bile-tolerant' bacteria found in the large intestine such as *Escherichia coli* and *Enterococcus faecalis*). MacConkey agar also contains lactose and an indicator system that identifies lactose-fermenting coliforms (e.g. *Escherichia coli*, *Klebsiella*) from lactose-non fermenting coliforms (e.g. *Morganella Salmonella*). Media can be made even more selective by the addition of antibiotics or other inhibitory substances, and sophisticated indicator systems can allow for the easy detection of defined bacteria from mixed populations.

Method of inoculating the solid culture media

Method used for inoculating the solid media depends upon the purpose of inoculation- whether to have isolated colonies or to know the bacterial load of the sample (quantitative analysis).

For obtaining the isolated colonies streaking method is used, the most common method of inoculating an agar plate is streaking.

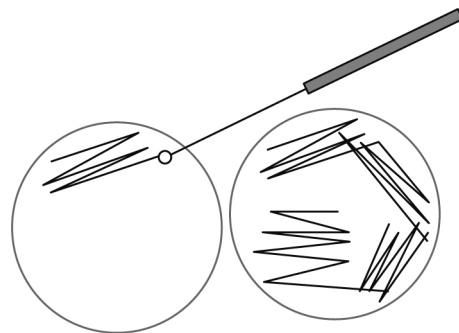


Fig. 10.6: Streaking method



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Streak plates

1. A small amount of sample is placed on the side of the agar plate (either with a swab, or as a drop from an inoculating loop).
2. A sterile loop is then used to spread the bacteria out in one direction from the initial site of inoculation. This is done by moving the loop from side to side, passing through the initial site.
3. The loop is then sterilised (by flaming) again and the first streaks are then spread out themselves.
4. This is repeated 2-3 times, moving around the agar plate as shown in the figure.

In this method single bacterial cells get isolated by the streaking, and when the plate is incubated, forming discrete colonies that will have started from just one bacterium each.

For quantitative analysis or semi quantitative analysis of the sample for example in case of urinary tract infection. In fact *E.coli* is implicated as the causative organism in urinary tract infection only if there are $>10^5$ Colony forming units per millilitre of urine. The method of inoculating the solid culture media is as shown in the figure.

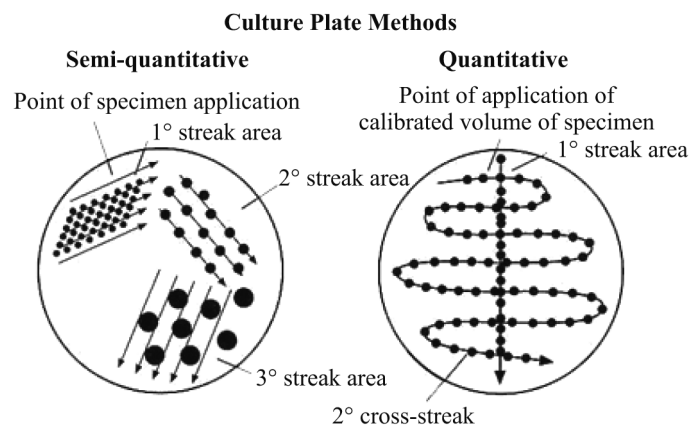


Fig. 10.7: Inoculation methods

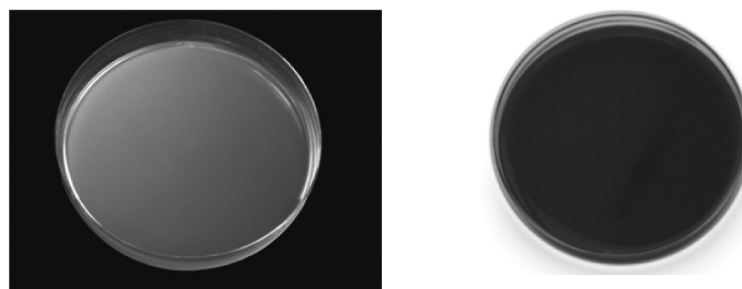


Fig. 10.8: Uninoculated Mac conkey Agar and Blood agar plate



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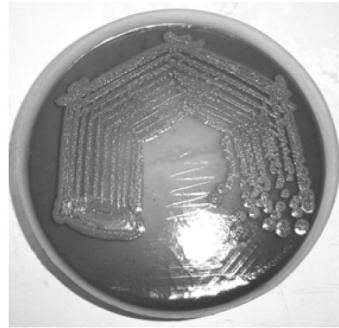


Fig. 10.9: Lactose fermenting (pink coloured) colonies on mac conkey agar

Culture in liquid media

Bacteria can also be grown in liquid media (broth). Like agar plates, broth cultures may be non selective or selective. Bacterial growth is easy to detect as the clear liquid turns turbid, usually within 24–48 hr, but incubation may need to be extended to 14 days or more.

The advantage of broth culture is that it is significantly more sensitive than direct culture on agar. The disadvantage is that, by itself, it is not easy to determine the type of bacteria present or whether a mixed growth has occurred, and in most cases the broth must be subcultured onto solid agar plates. This causes an additional delay in culture results. Broth cultures are also prone to contamination.

Broth enrichment media are used when high sensitivity is required e.g. for detection of bacteria from CSF, or to detect small numbers of *Salmonella* in a stool sample containing many millions of other bacteria.

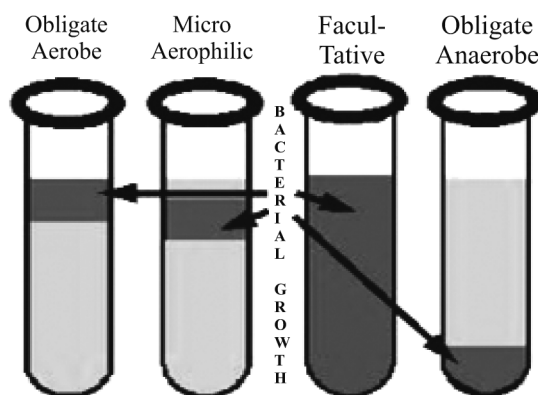


Fig. 10.10: Liquid media

Automated system

Automated blood culture systems eg. BACTEC, BacteAlert utilize liquid culture. Bacterial growth may be detected by a variety of methods (e.g. detection of bacterial CO₂ production).



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Fig. 10.11: Bactec



Fig. 10.12: Bactec

Automated liquid culture systems are also available for the culture of Mycobacteria, and similar technology can be used to automate sensitivity

The advantage of automated system are

Rapidity : they aid in faster growth of bacteria. Thus less time consuming.

The incidence of contamination during the processing of sample are minimised

Real time monitoring of the growth

One of the main limitations is the commercial viability.

Non culture methods

Isolation of bacteria can also be carried out by non-culture methods. In particular the more advanced Amplification techniques like Polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), and nucleic acid sequence based amplification (NASBA) are being used in clinical laboratories for isolation and identification of bacteria.

Methods of Isolation of Bacteria

The following are some of the factors that are considered in interpreting bacteriological culture results:

- type of specimen
- any delays in processing
- types of bacteria recovered
- knowledge of the normal human flora at different sites
- clinical information provided on the request form
- details of recent antibiotic therapy

There must be good liaison between healthcare workers and the microbiology laboratory, in order to ensure that the most appropriate investigations are performed, results are interpreted correctly, and clinically relevant bacteriological reports are produced.



INTEXT QUESTIONS 10.3

1. is used as gelling agar in culture media
2. culture media grow a wide variety of bacteria
3. is an example of selective media
4. For obtaining the isolated colonies method is common method of inoculating
5. is the liquid medium in which bacteria may be grown
6. Examples of Amplification techniques are, &



WHAT YOU HAVE LEARNT

- Isolation of bacteria forms a very significant step in the diagnosis and management of the illness. Isolation of bacteria involves various steps – Specimen collection, Preservation and transportation of specimen, Microscopic examination of sample. Various methods used for isolation of bacteria culture methods which includes culture on solid or liquid media and automated system. Non culture methods include the molecular techniques eg PCR, SDA, NASBA.

MODULE

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**Notes****TERMINAL QUESTIONS**

1. What is the need for isolation of bacteria?
2. Describe in brief various steps involved in the isolation of bacteria.
3. What is difference between blood agar and chocolate agar
4. Explain the term selective and non selective media with proper examples.
5. Draw a labeled diagram of inoculation of solid culture media for isolation of bacteria.
6. Draw a labeled diagram for inoculation of solid media for processing the urine sample of a patient suspected of urinary tract infection.
7. Describe in brief the advantages and the limitation of use of liquid culture media for isolation of bacteria.
8. Mention the advantages and the disadvantages of automated system for isolation of bacteria.
9. Name some non culture methods for isolation of bacteria

**ANSWERS TO INTEXT QUESTIONS****10.1**

1. High-risk
2. Charcoal
3. 4°C
4. Gram stain
5. Ziehl-Neelson
6. Dark Ground

10.2

1. Direct culture & Non-culture
2. 18-24
3. 37°C
4. Factor V & Factor X

10.3

1. Agar
2. Non-selective
3. MacConkey
4. Streaking
5. Broth
6. Polymerase Chain Reaction, Ligase Chain Reaction, Nucleic Acid Sequence Based Amplification

