Clinical microbiology

Specimen collection

Sample for microbiologic evaluation can be collected quickly and most do not require specialized material or equipment for proper evaluation. Specimens are collected by various methods, including aspiration, swabbing, and scraping. The specific techniques used depend on the type of lesion and its location on the animals body. Careful attention to aseptic technique.

The specific choice of collection method depends on the location of the lesion on the animals body, as well as the specific type of testing desired. Sample that are to be immediately processed can usually be collected by using sterile cotton swabs. However this is the least suitable method of collection because contamination risk is high and cotton can inhibit microbial growth. Oxygen can also trapped in fiber making recovery of anaerobic bacteria less likely.

If delays in processing the sample are expected a rayon swab in transport media must be used to preserve the quality of the sample.

Aspired samples can be collected by fine needle biopsy.

The following guidelines apply to proper specimen collection :

1- The specimen must be collected aseptically. Specimen contamination is the most common cause of diagnosis failure.

2- Samples should be collected as soon as possible after the onset of clinical signs.

3- Multiple specimens must be kept separate to avoid cross contamination.

4- The specimen container is labeled, especially if a zoonotic condition is suspected, such as anthrax, rabies, leptospirosis, brucellosis. Tissues in suspected zoonosis should be submitted in a sealed, leak proof, unbreakable container.

5- Adequate time should be taken. Obtaining results quickly at the expense of accuracy is counterproductive.

Stained Smears from Pathological Specimens

Stained smears made from lesions can yield a considerable amount of information inexpensively and quickly. In a small laboratory, staining techniques can be used alone or in conjunction with cultural methods. However, if only stained smears are used and the pathogen is recognised as one that could be resistant to antimicrobial agents, the

material should be sent to a diagnostic laboratory for culture and antibiotic susceptibility testing.

Preparing bacterial smears

Microscope slides are not always clean enough to use directly from the supplier. Rubbing with a clean, soft cloth and a flick through the Bunsen flame may be sufficient to remove a greasy film. When making a smear from tissue lesions, the specimen is held firmly with the forceps and the scalpel is used to scrape deep into the material. A small amount of the scrapings is placed on the cleaned microscope slide. Another clean slide is used with a scissor action to prepare a thin smear. With liquid or semi-liquid specimens, a little of the sample is placed on the slide with a sterile swab. The contents of the swab are smeared over the surface of the slide, with the aim of having thick and thin areas o f specimen present. The smears are allowed to dry thoroughly before proceeding further.

Fixing the smears

The reasons for fixing the smears include killing the vegetative bacteria, rendering them permeable to the stain and ensuring that the material is firmly fixed to the slide. Fixed and stained smears should be handled carefully as not all bacteria, especially endospores, may have been killed. After use, the stained smears should be autoclaved or soaked in a reliable disinfectant (24 - 48 hours) before discarding. For routine staining the smears are fixed by passing the slide, smear side up, quickly through the Bunsen flame two or three times, taking care not to overheat the smear. This can be tested on the back of the hand; the slide should feel warm but not hot enough to burn. Dried smears to be stained by the Giemsa stain are first fixed in absolute methyl alcohol for 3 minutes and then dried.

Staining the smears: staining techniques

Gram stain

Crystal violet 60 seconds

Gram 's iodine 60 seconds

Gram's decolorize 15 seconds

Counter stain (dilute carbol fuchsin or safronin) 60 seconds

Gram-positive bacteria retain the crystal violet-iodine complex and stain purple-blue. Gram·negative bacteria are decolourised and are stained red by the counter-stain.

Dilute carbol fuchsin (DCF) :

A simple staining technique

Dilute carbol fuchsin 4 minutes

Wash and air dry

The stain is used for some Gram-negative bacteria such as Campylobacter fetus, and Fusobacterium necrophorum.

Modified ziehl--neelsen (MZN)

Dilute carbol fuchsin 15 minutes

Acetic acid (0.5 percent) 15 seconds

Methylene blue 2 minutes

Wash and air dry

MZN-positive bacteria such as Nocardia asteroids, Brucella spp. and Chlamydia psittaci stain bright red with the background and other bacteria staining blue.

Ziehl-neelsen (ZN) or acid-fast stain

Strong carbol fuchsin 10 minutes with heat

Acid-alcohol decolouriser 15 minutes with several changes till the smear becomes colourless.

Methylene blue 20 seconds

Wash and air dry

ZN-positive or acid - fast bacteria. such as the pathogenic Mycobacterium sp., stain bright red with the background and other bacteria counter-stained blue.

Giemsa stain

The dried smear is first fixed in absolute methyl alcohol for 3 minutes.

I part Giemsa stain + 9 parts buffer: 60 minutes

Wash with the buffer

Drain and air dry

The Giemsa slain is used to stain spirochaetes, to demonstrate the capsule of Bacillus anthracis, to stain rickettsial organisms , and to demonstrate the morphology of Dermatophilus congolensis .

Polychrome methylene blue stain

A thin blood or exudate smear taken from a suspect case of anthrax is air dried, flame-fixed and flooded with the stain for 2-3 minutes. The stained smear is washed and dried. The rods of B. anthracis stain blue and the capsular material a pale pink colour. Any suspect anthrax material should be handled with care and the stained slides autoclaved after use. Viable spores may be present on the slide after staining.

Bacteriological Media

Diagnostic bacteriological media can be divided into the following categories :

1. Basic nutritive media: these are capable of sustaining growth of the less fastidious bacteria. Nutrient agar is an example.

2. Enriched media: agar media, such as blood agar, for the growth of fastidious bacteria. The media are usually enriched with blood, serum or egg yolk.

3. Enrichment broths: liquid media that are selective for a particular bacterium, such as selenite broth for the selection of salmonellae.

4. Selective media: these agar media have been made selective for the growth of a particular bacterium or group of bacteria and are used extensively in diagnostic bacteriology. They contain inhibitory substances that prevent the growth of unwanted bacterial species. Many selective media, such as brilliant green and MacConkey agars, can also be described as indicator media.

5. Indicator media: medium as the colonies of bacteria that ferment lactose take a pink color due to production of acid from lactose. Acid turns the indicator neutral red to pink. These bacteria are called 'lactose fermenter', e.g. Escherichia coli. Colorless colony indicates that lactose is not fermented and the lactose attack the peptone in MacConkey agar producing an alkaline reaction and colorless colonies, i.e. the bacterium is non-lactose fermenter, e.g. Salmonella. Shigella, Vibrio.

Preparation of Culture Media

1- Use a clean glass ware "flask" that has been rinsed free from detergents and other chemicals .

2- Weigh appropriate amount of dehydrated medium, place it in a flask, and then add distilled water to it .

3- Close the flask orifice by the appropriate piece of cotton and aluminum foil .

4- Dehydrated media containing agar is best dissolved by bringing to the boil with continuous stirring using a glass rode or a hot plate that incorporates a magnetic stirrer system, while media not containing agar can usually be dissolved with gentle agitation .

5- The media then sterilized in an autoclave at121 ˚c for 15 minutes.

6- After autoclaving, broth or media should be cooled in a water bath at 50˚c .

7- Pour the broth into a screw-capped bottle ,while the media poured into the Petri dish ,not more than 15 ml per one Petri dish, then allow to dry thoroughly at room temperature or for a few hours in the incubator at 37˚c .

8- Petri dishes stored agar-side upward in a refrigerator at 4˚c .

Preparation of blood agar plates

1 The blood agar base i s prepared from dehydrated powder. sterilized and cooled to 50°C in the usual manner.
2 Sterile blood at the rate of 5-10 percent vol/vol is added to the cooled agar base and mixed well before the plates are poured.
3 If bubbles form on the surface of the poured plates, a low Bunsen flame is quickly passed across the surface of the agar before the agar sets.
4 If the sterile blood has been stored in the refrigerator, it should be warmed to 37°C before being added to the agar medium to avoid thermal shock to the red cells.

Choice of culture media

For routine isolat ion of bacteria. blood agar and MacConkey agar are used. Blood agar will support the growth of most of the pathogenic bacteria and many of the Gram-negative bacteria will grow on the MacConkey

Advantages of culture media

1- This is useful method to detect bacteria when they are present only in very small numbers .

2- Estimate the concentration of bacteria because each colony that grows represent one bacterium from sample.

3- Solid culture media are used to differentiation organism having the same appearance (shape and staining) in stained smear .

4- Differentiation is possibly by observing the shape, size and growth rate of their colony.

5- To observe specific biochemical reactions

a. Colony may become colored either because of their pigment production (*Staphylococci*) or because of the media on which they are grown (lactose fermenter) such as *E. coli* produce pink colony on MacConky agar, *Pseudomonas aerogenus* produce green pigment which diffuse in to the medium .

b. Other factors such as swarming of proteus species in waves of growth across the surface of blood agar.

c. Hemolysis of cells in blood agar example by certain *streptococci* .

6- For pure culture isolation.

7- For storage of culture stocks.

8- As transport media to preserve bacteria during transportation to the laboratory.

Primary Identification of Bacteria

Once a pure culture is obtained, the results from a few comparatively simple tests can often identify the bacterium to a generic level：

1 - A Gram-stained smear from the culture will establish：

a) The Gram reaction (Gram-positive or Gram-negative)
b) The cellular morphology (coccus or rod).

2 - Growth or absence of growth on MacConkey agar .

3 - Catalase and oxidase tests.

4 Motility tests.

5 Oxidation-fermentation test.

Cellular morphology (shape)

Bacteria may be classified by their shape :-

1- Coccus (Cocci) : spherical cell such as *Staphylococcus aureus*, the causative agent of mastitis in animals .

2- Bacillus (bacilli) : shaped like rods or cylinder such as *Bacillus anthracis*, the causative agent of anthrax in animals .

3- Spiral : usually occur singly such as *Leptospira Pomona* which causes red water disease in cattle .

4- Pleomorphic : shape ranging from cocci to rods .

Bacteria are found in a variety of arrangements some grow as single cell and others remain attached after dividing and form chain or clusters. Many exhibit patterns of arrangement such as the following that are important for their identification :

1- Single : some bacteria occur singly, such as spirilla and most bacilli .

2- Pairs : some bacteria occur in pairs, such as *Streptococcus pneumonia* .

3- Clusters : some bacteria occur in clusters, bunches, or groups. For example *staphylococcus aureus* .

4- Chains : some organisms grow in short or long chains, such as the *Streptococcus* species .

6- Palisades : some organisms can be arranged in a palisade or Chinese letter pattern such as *Corynebacterium* species .

Bacterial Cell Counting Techniques

Sometimes it is necessary to enumerate bacterial cells in fluids such as autogenous vaccines, water, milk or urine samples. Both viable and total counts can be carried out .

I- Viable Count

These techniques are more commonly used in diagnostic and food hygiene procedures . Preparation of ten-fold dilutions of bacterial suspension before conducting a viable count to find the number of bacteria/ml in the original sample. The sample should be thoroughly mixed before sampling and a separate pipette must be used for every transfer step .We can calculate the live bacteria by plate method:- which have 2 ways

1- Spreading plate methods

Put 0.1 ml from diluted sample (which contain bacteria) into the nutrient agar spreading by glass spreader (se don't use cotton swap because absorbed part of bacterial suspension). Leave nutrient agar for 5 minutes to absorb the bacterial suspension. Put the plate in incubator in 37 C° for 16-24 hours .Calculate all the bacterial colonies which growing
Bacterial count in the sample = total count of bacteria in plate × 10 × reversible dilution

2- Pour plate method

put 0.1 ml from diluted sample (which contain bacteria) into plate add malting agar in 45 °c in plate then make circular movement to the plate Leave nutrient agar to rigid then put the plate in incubator in 37 C° for 16-24 hours Calculate all the bacterial colonies which growing Bacterial count in the sample = total count of bacteria in plate × 10 × reversible Dilution.

II- Total count

Not accurate method like the methods which counting the live bacteria

1- Direct microscopic counting method .

Depend on take label volume from sample and spreads on counting chamber which called Petroff-Hauser counter. Each slide divided into 25 big square and surrounded by 3 lines. each big square consist of 16 small square surrounded by one line. Total count will be
Total count in 25 big square × 50 × reversible dilutions

2- Breed method

Take standard volume (usually on drop equal to 0.025 ml which means each 1 ml equal to 40 drop) Spreading one small spot (1 cm^2) on glass slide then staining by methylene blue stain after drying & fixation the slide

Determine the bacterial colony by passing on each point of square

Total count of bacteria in square =A

Total count of bacteria = A × 40 × reversible dilution