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Laboratory Practical Work on Colony Count, Motility Test and Antimicrobial Susceptibility Test

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Abstract

In this practical laboratory works, three points were focused. These are: Anti-microbial susceptibility test, colony counting, and motility test. Each points discussed in detail as follows. Anti-microbial susceptibility test performed by using disc diffusion method to determine susceptibility of salmonella .In this test, kanamycin formed 24.26 mm ,ciprofloxacin formed 34.23 mm, and chloramphenicol formed 28.46 mmzone of inhibition, while sulphonamides formed no zone of inhibition. Colony counting was done by plate count method. Two medias were used. On VRB (violet red blue agar) media, with dilution of 10-2 was CFU/ml=3.5*103, with dilution of 10-3, CFU/ml=4.1*104 and with dilution of 10-4, CFU=1.5*105. But in this case also it is reported as “TFTC= too few to count”. While on PCA (plate count agar) media, with dilution of 10-3 colony counted was 100, and CFU/ml=1.00*105,with dilution of 10-4 colony counted was 171, and CFU/ml=1.71*106 , with dilution of 10-5colony counted was 58, and CFU/ml=5.8*106,with dilution of 10-6colony counted was 19, and CFU/ml=1.9*107,but in this case it is reported as “TFTC= to few to count”. On the other hand, motility test was done in SIM (sulphure- indole -motility) and results obtained were: Staphylococcus aureus was non-motile; grow only on the line of stab, Escherichia. Coli spp. were motile, grow and move throughout the medium and Salmonella was both motile and produced gas.

Keywords: Anti-Microbial; Susceptibility; Test; Colony Counting; Motility

Introduction

The competency of microbiology professionals is obtained through the theoretical knowledge gained in the class and the practical skills and applications that lead to the better understanding and interpretations the subject matter.In this practical laboratory works, three points are focused. These are: Anti-microbial susceptibility test, colony counting, and motility test. Each points discussed in detail as follows [1].

Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance which resulted in morbidity and mortality from treatment failures and increased health care costs. The results of in-vitro antibiotic susceptibility testing, guide clinicians in the appropriate selection of initial empiric regimens and, drugs used for individual patients in specific situations. The selection of an antibiotic panel for susceptibility testing is based on the commonly observed susceptibility patterns; it became necessary to perform the antimicrobial susceptibility test as a routine. For this, the antimicrobial contained in a reservoir was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Even now a variety of antimicrobial containing reservoirs are used but the antimicrobial impregnated absorbent paper disc is by far the commonest [2].

There are two methods used to count bacteria. These are:-turbidity method (quantitative method) plate count method. The plate count method is based on viable cell counts. The plate count method is performed by diluting the original sample in serial dilution tubes, followed by the plating of aliquots of the prepared serial dilutions into appropriate plate count agar plates by the pour plate or spread plate technique. The pour plate technique utilizes tempered molten plate count agar poured into the respective plate and mixed with the diluted aliquot sample in the plate, whereas the spread plate technique utilizes the addition and spreading of the diluted aliquot sample on the surface of the solid plate count agar in the respective plate [3].

Finally, motility is demonstrated by methods microscopically by hang method and macroscopically by tube methods. Macroscopic is done by making a straight stab of growth into semisolid medium, incubating for 24 to 48 hr at 35° to 37°C, and then observing whether the organism migrates out from the stab line, causing visible turbidity in the surrounding medium. Various formulations of motility medium are available. Enterobacteriaceae can be tested in a 5% agar medium with a tryptose base. Nonfermentative gram-negative organisms that fail to grow in 5% agar medium can be tested using 3% agar in a base containing casein peptone and yeast extract. Fastidious organisms may demonstrate motility better in a 1% agar base containing heart infusion and tryptose. Shigella species and Klebsiella species are always nonmotile. Pseudomonas aeruginosa and Achromobacter xylosoxidans are both motile. Yersinia enterocolitica is often nonmotile at 35° to 37°C but motile at 28°C. Motility may be difficult to demonstrate in semi-solid medium if the organism grows slowly [4].

Antimicrobial Susceptibility Testing (AST)

The purpose of performing antimicrobial susceptibility testing (AST) is to provide in vitro data to help ensure that appropriate and adequate antimicrobial therapy is used to optimize treatment outcomes. In addition, the AST data generated daily can be

statistically analyzed on an annual basis to generate an antibiogram that reflects the antimicrobial susceptibility and resistance patterns of important pathogens that prevail in a particular hospital. These hospital-specific AST epidemiologic data provide valuable guidance to the clinicians for the appropriate selection of empiricthерapy, prior to the availability of culture and susceptibility results that often takes 2 to 3 days.The purpose of the AST of the culture pathogen is to help clinicians correct and/or modify empiric therapy as soon as the results become available.Two basic methods of AST are available to laboratories: (1) qualitative and (2) quantitative. Qualitative methods, such as disk diffusion and abbreviated breakpoint dilution systems, are acceptable options for the testing of isolates from "healthy" patients with intact immune defenses and for less serious infections such as uncomplicated urinary tract infections. Both disk diffusion and breakpoint agar or broth dilution systems are considered satisfactory for predicting treatment outcome in these cases [5].

Quantitative systems that provide a minimum inhibitory concentration(MIC) value are more important in the treatment of serious infections such as endocarditis or osteomyelitis, and for infections in high-risk patient groups such as immunocompromised patients (forexample, transplant patients) and those who are critically ill. Depending on the size of the workloads, personnel resources available, and issues of convenience, many clinical laboratories prefer to use one or more automated systems for AST regardless of whether results obtained are qualitative or quantitative. These systems handle the majority of the higher volume specimens such as urines, and are usually supplemented with manual methods such as disk diffusion and other MIC tests to handle the limitations of automation as well as provide MIC results for different clinical situations. Clinical laboratories should continue to develop and improve their AST algorithms to include different types of test systems in order to provide meaningful and accurate data depending on the type of patient, source of specimen, organism species and anticipated problems in detecting various types of resistance mechanisms.

Disk Diffusion

The principle behind the disk diffusion method, or "Kirby Bauer" test, is the use of a paper disk with a defined amount of antibiotic to generate a dynamically changing gradient of antibiotic concentrations in the agar in the vicinity of the disk. The disk is applied to the surface of an agar plate inoculated with the test organism; and while the antibiotic diffuses out of the disk to form the gradient, the test organism starts to divide and grow and progresses toward a critical mass of cells. The so-called inhibition zone edge is formed at the critical time where a particular concentration of the antibiotic is just able to inhibit the organism before it reaches an overwhelming cell mass or critical mass. At the zone demarcation point, the density of cells on the growth side is sufficiently large to absorb antibiotic in the immediate vicinity, thus maintaining the concentration at a sub-inhibitory level and enabling the test organism to grow. The critical times for the demarcation of the inhibition zone edge for most rapidly growing aerobic and facultative anaerobic bacteria vary between 3 and 6 hr, while critical times of fastidious organisms and anaerobic bacteria can vary from 6 to 12 hr or longer.

A density of approximately 108 CFU mL−1 (CFU = colony-forming unit) of the test organism in the inoculums suspension is used to obtain semi-confluent growth on the agar. The inoculum is prepared by suspending enough well-isolated colonies from an 18- to 24-hr agar plate in broth or physiologic saline (0.85%) to achieve a turbidity matching a 0.5 McFarland Standard. The Prompt™ (BD Diagnostic Systems, Sparks, MD) inoculums preparation system, which consists of a sampling wand and inoculums solution in a plastic bottle, can also be used to optimize workflow because the organisms are maintained at the same density for up to 6 hr. Because the disk diffusion is based on the use of a dynamically changing gradient, inoculum density variations can directly affect the critical times and influence the inhibition zone sizes,regardless of the susceptibility of the test organism. A heavy inoculum will shorten the critical time and lead to falsely smaller inhibition zone sizes, resulting in potentially false resistant results, while a light inoculum will cause the reverse effect and generate potentially false susceptible results.The inoculum suspension should be used within 15 min of preparation. This is particularly important for fastidious organisms that lose their viability rapidly. A sterile cotton swab is dipped into the suspension and pressed firmly on the inside of the tube to remove excess liquid. The dried surface of the appropriate agar plate is inoculated by streaking the entire surface and then repeating this twice, rotating the plate 60° each time. This will result in an even distribution of the inoculum. The inoculated plate is then allowed to dry with the lid left ajar for no more than 15 min. Once the agar plate is completely dry, the different antibiotic disks are applied either manually or with a dispensing apparatus.

In general, no more than twelve disks should be placed on a 150-mm agar plate or five disks on a 90-mm plate. Fewer disks are used when anticipating highly susceptible organisms. Optimally, disks should be positioned at a distance of 30 mm apart and no closer than 24 mm apart when measured center to center, to minimize the overlap of inhibition zones. Most dispenser devices are self-tamping (disks are tapped or pressed onto the agar surface). If applied manually, the disk must be pressed down to make immediate and complete contact with the agar surface. Once in contact with the agar, the disk cannot be moved because of instantaneous diffusion of antibiotics from the disk to the agar. Agar plates are incubated in an inverted position (agar side up) under conditions appropriate for the test organism. Plates should not be stacked more than five high to ensure that the plate in the middle reaches incubator temperature within the same time frame as the other plates. After 16 to 18 hr of incubation at 35°C for rapidly growing aerobic bacteria, or longer where appropriate for fastidious organisms or specific resistance detection conditions, the agar plate is examined to determine if a semi-confluent and even lawn of growth has been obtained before reading the plate. If individual colonies are seen, the inoculum is too light and the test should be repeated because zone sizes may be falsely larger. The same holds true for excessively heavy inoculum where zone edges may be very hazy and difficult to read and zone sizes may be falsely small.

If the lawn of growth is satisfactory, the zone diameter is read to the nearest millimeter using a ruler or sliding calipers. For Mueller Hinton agar (without blood supplements), zone diameters are read from the back of the plate. For blood containing agar, zone diameters are read from the surface of the agar. The zone margin, unless otherwise specified, is identified as the area in which no obvious visible growth is seen by the naked eye. Faint growth or micro-colonies detectable only with a magnifying glass or by tilting the plate should be ignored in disk diffusion tests. Reading of zone sizes can be simplified using automated zone readers that use a camera-based image analysis system such as the BIOMIC (Giles Scientific, New York, NY); Aura (Oxoid, Basingstoke, U.K.); Protozone (Microbiology International, Frederick, MD); or SirScan (i2a., France).

The agar plate is placed into the zone reader instrument that is connected to a computer system. The system then reads the different zone sizes directly from the agar plate and converts the result to a susceptibility category interpretation using various zone-MIC interpretive guidelines in the Software.

Although zone sizes are automatically read, the user can intervene and adjust results. The BIOMIC system also converts zone sizes to MIC equivalents using a database of regression analyses based on the assumption of a linear correlation between the zone size and the MIC value .

This may not always be the case, however, particularly for fastidious, slow-growing organisms.

Materials and Methods

- Stock solution of H2SO4 and BaCl2
- Glass bottles
- Vortex
- Pipettes
- Measuring cylinder
- Rack

Procedures of Mc farlan preparation

1.1% barium chloride (BaCl2•2H2O) and 1% sulfuric acid (H2SO4) prepared first as shown below.

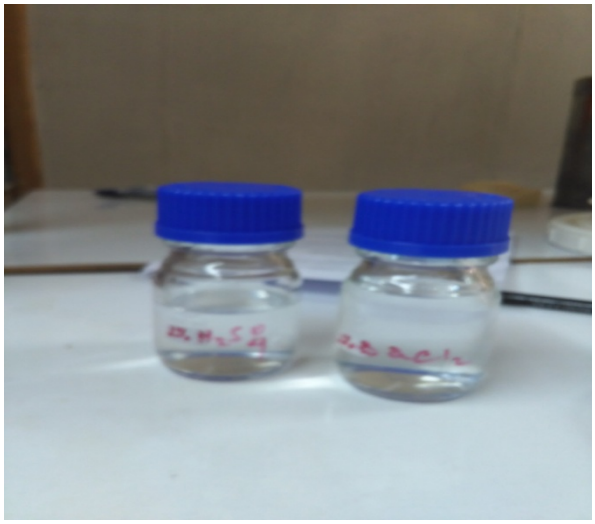

Figure1: Solution of 1%of H2SO4 and 1% BaCl2.

2. Then, 9.95ml solution of 1%of H2SO4 and 0.05ml 1% BaCl2 poured in to test tube and mixed with the help of vortex.

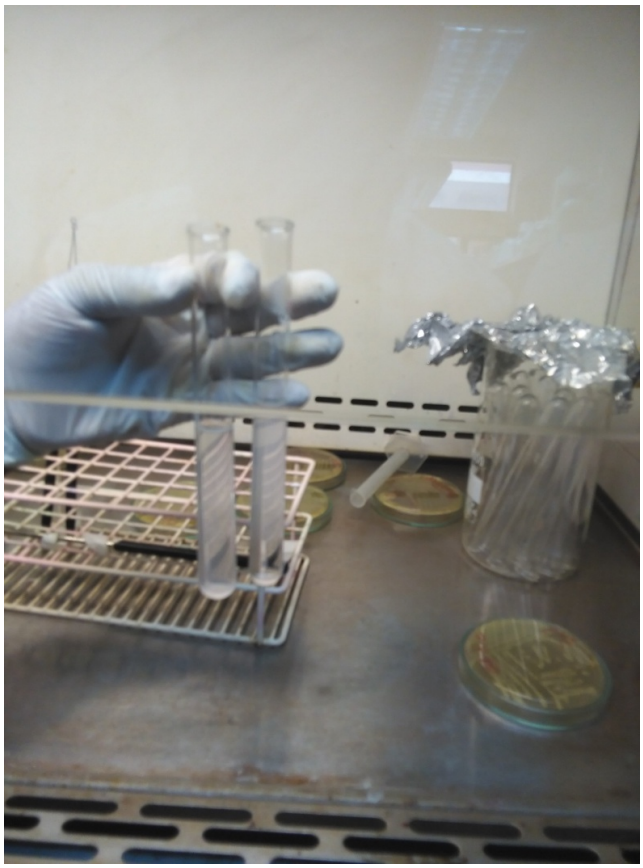

Figure 2: Solution of 1%of H2SO4 and 0.05ml 1% BaCl2.

3. The prepared MC farland turbidity kept at room temperature.

Procedures of Disk Diffusion Test

- Five isolated colonies of salmonella were picked up from an agar plate culture.
- The colonies were added to tube containing saline water and mixed until suspensions formed.
  The suspensions formed are compared with Mc farland turbidity standard as shown in fig.6 below.
- Within 15 minutes ,after adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the adjusted suspension rotated several times and pressed firmly on the inside wall of the tube above the fluid level.
- The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two times, rotating the plate approximately 60 each time to ensure an even distribution of inoculums. As a final step, the rim of the agar is swabbed.
- The plate was allowed for 5 minutes for any excess surface moisture to be absorbed before applying the drug impregnated disks.
- The predetermined battery of antimicrobial discs was dispensed onto the surface of the inoculated agar plate.
- Allowed for 15 minutes to dry and incubated for 24 hours.

Result Anti-Microbial Susceptibility Test

After 24 hours of incubation, Zones are measured, using sliding calipers which is held on the back of the inverted petridish plate, salmonella isolate showed different zone of inhibition for different types of anti-microbial discs.

After incubation, observation and measuring zone of inhibition, the following conclusions were drawn.

- Salmonella was susceptible to ciprofloxacin, gentamycin, and chloramphenicol, because their zone of inhibition measured was within the range of susceptibility (see annex1).
- Salmonella was resistant to sulphonamides, because its zone of inhibition measured was zero.

Colony Counting

The plate count method is based on viable cell counts. The plate count method is performed by diluting the original sample in serial dilution tubes, followed by the plating of aliquots of the prepared serial dilutions into appropriate plate count agar plates by the pour plate or spread plate technique (Goldman and Green, 2009).

In microbiological research, it is often necessary to be able to quantify the number of living bacteria in a particular sample. There are two methods used to count bacteria. These are total cell count and viable cell count. One of the major ways to do this is using viable plate counts, in which bacterial cells from a liquid culture are spread onto an agar plate. The plate is incubated, the numbers of colonies that grow on the plate are counted, and the number of original bacterial cells in the culture is determined. In most cases, however, the liquid culture being quantified contains too many cells to be directly plated onto agar plates – there would be so much growth that it would be impossible to count individual colonies! Therefore, the liquid culture needs to be diluted, often 1-million-fold, before it can be plated (sultan, 2014).

When such a large dilution is required, an accurate dilution cannot be made in a single dilution step and it is necessary to make serial dilutions. Serial dilutions are a step-wise set of dilutions which sequentially dilute the bacterial culture. One or more of the dilutions are then plated on the agar plates to determine the number of colonies present in the original culture. Only plates containing between 30 and 300 colonies are counted to ensure statistically significant data. To estimate the number of bacterial in the original culture, the number of colonies on the plate is multiplied by the total dilution plated. For example, suppose 0.1 ml of a 10-6 dilution was plated, and 123 colonies were counted following incubation. The total dilution plated would be 10-7 (since only 0.1 ml was plated), and the number of bacteria/ml of the original culture would be: (123) x 1/10-7 = 1.23 x 109 CFU/ml. Note that the results are expressed as “colony forming units (CFU)” per ml (sultan, 2014).

The plate count method is used primarily in the enumeration of samples with high microorganism numbers or microorganisms that do not grow well in liquid media. Plate count methods are used in the areas of food, pharmaceutical, environmental including drinking water applications, and biofilm testing. Modified or alternate versions of the plate count methods have also been developed to further enhance the use of the standard plate count method approach to estimate bacteria or fungi by utilizing the roll tube method,drop plate method,spiral plate count method, Petrifilm Sim PlateRODAC (replicate organism detection and counting plate) for environmental surface sampling, dipslide or dipstick paddle method, and adhesive sheet method (Gracias and McKillip, 2004).

Result s

After 2days of incubation, visible colonies counted and different numbers scored.

In PCA media

- PCA with dilution of 10-3 colony counted was 100, and CFU/ml=1.00*105.
- PCA with dilution of 10-4 colony counted was 171, and CFU/ml=1.71*106.
- PCA with dilution of 10-5colony counted was 58, and CFU/ml=5.8*106.
- PCA with dilution of 10-6colony counted was 19, and CFU/ml=1.9*107, but in this case it is reported as “TFTC= too few to count”.

In VRB media

- VRB with dilution of 10-2 colony counted was 35, and CFU/ml=3.5*103.
- VRB with dilution of 10-3 colony counted was 41, and CFU/ml=4.1*104.
- VRB with dilution of 10-4colony counted was 15, and CFU=1.5*105. But in this case also it is reported as “TFTC= too few to count”

Conclusion

Colony counting is affected by various factors from early sampling to final colony counting and what concluded were:

- When the sample is highly diluted, chance of obtaining colony forming unit (CFU) decreases.
- Types of media used for colony counting affects colony forming units (CFU), because in this practical work two dilutions (10-3 and 10-4) were inoculated in PCA and VRB and different results obtained.

Motility Test

To determine if the isolated micro-organisms (E.coli, salmonella and s.aureus) are motile or not motile.

Using Motility Test Medium

Principle: Some bacteria have the ability to propel themselves through liquids by means of flagella (flagellum = singular). These long fibers of protein are found on many bacteria, including most supported by this simulation. However, not all bacteria are able to "swim", even if they have flagella. The purpose of this test is to see if the microbe can "swim" by means of flagella.Motility medium is a colorless, semisolid agar medium dispensed in a test tube that is soft enough that motile organisms can spread through the agar as they grow.

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