VOLUME - VI
ISSUE - XLII
NOV / DEC 2010

The Crux

BIMONTHLY FORUM FOR THE LABORATARIANS

CONTENTS

Editorial

This year is now closing and is at its fag end, however, the complicity of disease diagnosis process with its imperative treatments is an ongoing process. Of all the harmful organisms, the most widely omnipresent are the unicellular organisms – namely bacteria. Fortunately, they are easy to grow, easy to identify and also it is equally easy to establish a cure against them (provided correct methods and protocols are used). There is a sea of antimicrobial agents available to us to choose from. But which will hit the bull's eye and will eventually free the patient from the pathogen remains a big question --that fortunately has a simple answer. Yes, we are talking about bacterial cultures and antibiograms. This whole issue is dedicated to this simple yet perplexing issue of bacterial infections, their diagnosis and subsequent therapy institution. The DISEASE DIAGNOSIS section considers bacterial infections as a whole. What specimen types are usually available and how to handle them. Detailed bacteriology and diagnosis of individual bacteria is not possible to be given on a platform like this, so, important aspects in general as related to cultures are presented. Disease diagnosis also goes on to describe the antimicrobial susceptibility regimens. The broader aspects, methods and principles involved are submitted as a refresher to you.

A simple technique known as the Kirby Bauer's disc diffusion method is available to us to easily and scientifically establish the efficacy of any antibiotic. However, the zone size interpretation is different for different antibiotics and sometimes organisms also. So, presented in a solid single capsule form is provided the Zone Size Interpretative Chart for clinical use in microbiological laboratories. LOOK NO FURTHER, YOU WILL FIND A SEPARATE PRODUCT INSERT FLYER IN THE ENVELOPE OF THIS ISSUE ITSELF.

This flier can be retained for a lifetime and stuck on the wall/ board wherever you judge the inhibitory zones as a ready reckoner to assign resistant/ partially sensitive/ sensitive labels of all antibiotics. All currently available antimicrobials with their zone size interpretations are provided in an easy to comprehend format for various genera/ species of bacteria. INTERPRETATION portion carries forward the overflow from the last issue; it discusses the use of CD4 counts in relation to Highly Active Anti Retroviral Therapy.

Whenever, a process or an SOP is established, it always comes with attendant problems and difficulties. Standards are to be maintained at all costs and times. There are international norms regarding Quality Assurance Systems as related to Antibacterial Susceptibility testing protocols. TROUBLE SHOOTING segment of this issue will iron out all creases that you may come across as you practice the science and art of "ANTIBIOGRAMS"

BOUQUET'S brain- teaser smells of bacteria, beware, though no bacteria would not come out of the pages that follow. Would you believe that you could get infected from the MONEY notes that you handle in everyday life? It's true, BELIEVE IT OR NOT! Wisdom whispers has an assortment of anecdotes, a few funny lines are also strewn along with.

DISEASE DIAGNOSIS

BACTERIAL INFECTIONS
CULTURE & SENSITIVITY (ANTIBIOGRAM)
INTRODUCTION

Infections are by and large the commonest diseases found on our planet amongst the human kind or the animal kingdom. Any part of the body can be infected primarily or secondarily foci can be seen as distant metastatic lesions or diffuse blood borne spread (bacteremia/septicemia) can be observed. Secondary infections can occur because of direct contiguous spread or blood borne spread. Spread occurs faster through body cavities/ spaces. Sample for diagnosis can be drawn from anywhere - they can be liquids, thick pus aspirates or biopsy samples even. Often blood is submitted for cultures.

SPECIMEN COLLECTION

As far as possible obtain specimens before the commencement of therapy. This is important especially for Cerebrospinal Fluid (CSF) cultures. Often a purulent CSF will reveal no bacterial pathogens on smear or culture when an antibiotic has been given within the previous 24 hours. A patient with enteric fever may show a negative stool culture if the specimen has been collected while the patient was receiving suppressive antibiotics. Another important factor for the successful isolation of organisms is the stage of the disease at which the specimen is collected for culture, enteric pathogens are present in much greater numbers during the acute or diarrheal stage of intestinal infections and they are more likely to be isolated at that time. Specimens should be inoculated as soon as possible. If it is not possible then refrigerate the specimens at 4-6°C. Swabs from wounds, urogenital tract, throat, rectum and samples of feces or sputum can be refrigerated for 2-3 hours after procuring them without appreciable loss of pathogens. Urine specimens may be refrigerated for 12 hours without affecting the bacterial flora. On the other hand, cloudy CSF from a patient with purulent meningitis should be examined immediately. Gastric washing, for culture of *Mycobacterium tuberculosis* should be processed soon after delivery as the Mycobacteria die quickly in gastric washing. Specimens submitted for isolation of viruses should be frozen immediately. Specimens of hair scrapings may be submitted for isolation of fungi may be kept at room temperature before inoculation. Sputum, bronchial secretions, bone marrow and purulent material from patients suspected of having a systemic fungal infection should be inoculated to appropriate media as soon as possible. All receptacles (containers) for collection of specimens must be sterile otherwise contaminants from the container will also be grown.

Urine: Organisms found in a normal urine are staphylococci (coagulase negative), diphtheroid bacilli and coliform bacteria. The important pathogens are *Escherichia coli, Proteus, Citrobacter, Pseudomonas, Klebsiella. Moraxella, Acinetobacter, Staphylococcus, Streptococcus faecalis, Salmonellae, Mycobacterium tuberculosis,* etc. For non-tuberculosis patients a mid stream fresh urine specimen is good enough. Urine samples are streaked on Blood Agar and MacConkey or CLED agar plates. At the same time microscopic examination of the urine should also be carried out. Catheterization is indicated only when a mid stream specimen (MSS) cannot be obtained, if done—all aseptic precautions must be undertaken. Before collecting the specimen, the area is washed well with soapy water and dried and then the MSS collected. A Gram's stain should be done on the centrifuged sediment. If tuberculous nephritis is suspected, a 24 hours specimen, or preferably 5 consecutive early morning specimens are sent to the laboratory. The specimens are centrifuged and the deposits pooled. A Ziehl-Neelsen stain is done and the deposits concentrated before culturing for tubercle bacilli.

Feces: Most of the organisms which make up the intestinal flora in man belong to the family *Enterobacteriaceae*. These may include the intestinal commensals (the coliform bacilli and *Proteus* species) as well as the enteric pathogens of *Salmonella* and *Shigella*, intestinal *Streptococci, Clostridia* and various yeasts including *Candida albicans* may be present. The *Cholera vibrio* may also be isolated. Samples of feces should be sent to the laboratory in disposable containers, e.g. cartons of waxed cardboard which are incinerated after use. Since feces contain inummerable bacteria, and since selective media for enteric (intestinal) pathogens are almost invariably used, aseptic precautions are rather futile and unnecessary. It should be remembered, however, that where blood and mucus are present in the stool, this part should be especially selected for culture, since the pathogens are most likely to be found there. The feces are inoculated into a solid bile salt medium such as MacConkey agar or desoxycholate-citrate-agar (DCA) and also onto Wilson and Blair's bismuth sulphite medium. Liquid media such as Selenite F which will inhibit the growth of the coliform bacilli or brilliant green broth which will enhance the growth of the pathogens: are also inoculated. These are all incubated at 37°C over-night. A Gram stain of the feces is of little value except in cases of fungi and staphylococci infections.

Sputum: The commonly isolated organisms from sputum are Pneumococci, Beta-Hemolytic Streptococci and *Mycobacterium tuberculosis.* If the culture for organisms other than *M. tuberculosis* is needed, all possible care has to be taken while doing so. Rinse the mouth with an antiseptic or clean water to avoid contamination from the oral cavity. The specimen should be collected in a wide mouthed sterile jar with a screw-cap lid. If the sample has to be concentrated when culturing for *M. tuberculosis*, there is less need for a sterile jar, in fact, disposable waxed cartons are preferable since they and their contents can safely be incinerated. For routine cultures, a loopful of the sputum is inoculated onto one/two blood agar plates. When two are inoculated one is incubated in a 5-10% CO_2 atmosphere. A smear is made from specimen and stained by Gram's stain. If the need be Ziehl-Neelsen staining can also be done.

Throat and Nasal Smears: Organisms commonly isolated from a normal throat are Alpha-hemolytic *Streptococci*, *Neisseria catarrhalis*, *Staphylococci*, Non-hemolytic *Streptococci, Pneumococci* and *Coliform bacilli.* The pathogens usually encountered are Beta hemolytic *Streptococci*, *Corynebacterium diphtheriae, Bordetella pertussis, Meningococci, Staphylococcus aureus, Hemophilus influenzae* and *Candida albicans*. The sterile swabs should be first moistened with normal saline (sterile) and then rubbed over the infected area. A blood agar plate is inoculated. A Gram's stain is rarely necessary except in cases where Diphtheria or Vincents angina are suspected. If Diphtheria is suspected the smears should by stained by Albert's, Ponder's or Neisser's techniques.

Pus Swabs: The infected area is carefully swabbed with spirit before the swab is taken, or the pus aspirated. Carry out a Gram's stain, streak on blood agar, MacConkey agar and then place into nutrient broth or, if anaerobes are suspected, into thioglycollate broth or Robertson's cooked meat medium. In cases of suspected gangrene or tetanus: two blood agar plates should be inoculated, one for aerobic culture and the other for incubation in an anaerobic atmosphere.

Blood Cultures: Bacteremia is an important part of any systemic infection and hence, blood culture acquires similar significance. Blood culture permits the prompt commencement of specific treatment against the offending organism and may prove to be lifesaving. Bacteremia occurs transiently in pneumococcal pneumonia, bacterial meningitis,

urinary tract infections, enteric fever and generalized *Salmonella* infections. A mild transitory bacteremia is a frequent finding in many infectious diseases but a persistent bacteremia points towards a more serious infection. When the classic syndrome of a septicemia is due to pyogenic organism, chills, fever prostration is found, one rarely finds difficulty in isolation of the causative organism. In some diseases the chances of isolating bacteria from blood culture depends on the stage of the disease at which the culture is done. For example, bacteria can be cultured during the early course of the disease, thus the cultivation of the bacteria from the blood is important since, it may be the only reliable means of making an early diagnosis available for the physician. A diagnosis of bacteremia can only be made by growing the pathogenic agents on suitable culture media. Perfect aseptic conditions must be observed while collecting blood for blood culture. Enriched aerobic and anaerobic culture media must be utilized in order to provide optimal conditions for bacterial growth. Commonly isolated pathogens from blood cultures are:

α and β hemolytic *Streptococci*. *Staphylococci,* pathogenic and saprophytic. *Coliform bacilli* and related organisms. *Pneumococci. Hemophilus influenzae. Enterococci. Clostridium perfringens. Pseudomonas* species. *Bacteroides* species. *Neisseria meningitidis. Salmonella* species. *Pasteurella tularensis. Leptospira* species.

Pathogenic yeasts and molds: Keep all the required things—the culture media (nutrient or glucose broth), spirit lamp, pen for labeling, etc.—by the bedside of the patient. Three bottles of broth should be incubated, one for aerobic cultivation, one for anaerobic and other for incubation in 5-10% CO_2. The site selected for venipuncture is well swabbed with cotton moistened in spirit and with tincture of iodine. The needle is fitted onto the syringe without touching the needle or the nozzle of the syringe. Blood is withdrawn—about 15 ml. After withdrawing needle from arm the needle is passed through the flame. Remove protective covering from culture bottle. The top of the screw-capped bottle is flamed and the needle inserted into the bottle through the rubber washer and 5 ml of blood is placed in each bottle. The bottle is again flamed and protective covering replaced on the bottle-top. The same procedure is carried out for each of the three bottles. Once collected the cultures should be incubated at 37°C immediately and left overnight in the appropriate atmospheres. Subcultures are made every two days, either until growth is found or until 2 weeks have elapsed. Only after two weeks have elapsed can a report of 'No growth' be sent. If the patient has already been on penicillin or sulphonamide therapy, penicillinase or para-minobenzoic acid may be added respectively. These substances will counteract the effect of any of the drugs present in the serum. Counteracting agents for other antibiotics are not yet known.

CSF, Pleural Fluid and Other Body Fluids: The aspirated material should be sent to the laboratory immediately in sterile tubes or bottles. They are cultured on blood agar, aerobically, anaerobically and in 5-10% CO_2. Gram and Ziehl-Neelsen stains are done on smears prepared from centrifuged sediments. Broth cultures should also be setup. The plates and subcultures of the broth should be incubated for 48 hours before reporting as negative (i.e. No growth). Organisms isolated from CSF are: *Hemophilus influenzae, Pneumococcus, Neisseria meningitidis, Mycobacterium tuberculosis, Staphylococcus, Streptococcus, Coliform bacilli, Pseudomonas* and Viruses.

Ear Discharge Cultures: Organisms isolated from the ear are: Nonpathogenic: Coagulase negative Staphylococci and Diphtheroids.

Pathogenic: *Pseudomonas, Staphylococcus, Proteus, Pneumococci,* α and β *hemolytic Streptococci* and *Coliform bacilli*.

Material from ear is taken by swab sticks. Do a Gram stain and culture to a nutrient broth, blood agar and MacConkeys agar.

Eye Swab Cultures: Organisms isolated from the infections of the eyes are *Staphylococcus aureus, Neisseria gonorrhoeae, Pneumococci,* α and β hemolytic *Streptococci* and *Hemophilus*. Purulent material may be obtained from the conjunctiva with the help of a cotton swab stick. This should be inoculated to blood agar and chocolate agar and to thioglycollate media. Chocolate agar should be incubated in a 10% CO_2 jar.

GENERAL INSTRUCTIONS FOR MICROBIOLOGY

Storage of Organisms: It is unwise to maintain bacteria and fungi for long periods, in case they become contaminated. Therefore, organisms should be subcultured and checked for purity every 3 months or so to yield good results.

Precautions Before and during Testing: Before beginning practical work, hands should be washed with soap and warm water and so also after completing all testing procedures. Hand to mouth operations such, as chewing, sucking, or mouth pipetting should be avoided. Disposable plastic gloves should be worn during handling of infectious material.

Specimen Collection and Preparation: Collect specimen prior to use of antimicrobial agent. Wherever possible, indicate clearly that the patient is on antitubercular drugs. CSF: Collect as much as possible in a syringe, clean skin with alcohol before aspirating specimen. Body fluids: Disinfect the site and collect specimen with aseptic precautions. Sputum: Collect 5 to 10 ml in a sterile container from an early morning specimen of deep productive cough. For induced specimen, use sterile saline. Have patients rinse mouth with water to minimize specimen contamination with food particles, mouth wash or oral drugs. Urine: As organisms accumulate in the bladder overnight, first morning void provides best yield. Collect midstream clean catch urine, first morning catheterization/suprapubic taps in sterile containers.

Inoculation of Samples: The work surface should be swabbed with a suitable disinfectant before commencing the testing procedure as well as on completion of the testing procedure. Sterilize inoculating loops by flaming in a Bunsen burner flame to avoid contamination. Improper decontamination procedure may lead to erroneous results. Treat the unused specimen and contaminated containers by immersing in 2% activated Glutaraldehyde for at least two hours before incineration and disposal.

ALL CULTURE MEDIA ARE THESE DAYS AVAILABLE IN READY TO USE FORMATS.

Bacterial identification can be accomplished by any / all of the under mentioned methods.

Non Cultural Methods: Non cultural techniques these days employ – NAT PCR technologies and sero-immunological methods or direct observation by special stains or fluorescence based methods. Simple methods like colony characteristics, Grams stain, motility etc can sometimes suffice.

Serology: Bacteriologic diagnosis can also be confirmed by estimating antibodies to specific antigens of the bacteria. Examples: VDRL and Kahn tests for syphilis. ASO for beta-hemolytic streptococci and Widal for typhoid.

Cultural Characteristics: Bacteria grown artificially (*in-vitro*) on agar plates are described as colonies. These colonies vary in size, shape, pigment production, and hemolysis on blood agar depending on the type of media.

Colonies are described as: Shape: Circular, regular, radiating or rhizard. Surface: Smooth, rough, fine, granular shiny, dull, etc. Size: Usually colonies are 2-3 mm in diameter, smaller ones may be less than 1 mm. Contiguity: Colonies may be discrete or swarming. Consistency: May be mucoid, tenacious dry or adherent to the medium. Pigmentation: Some organisms produce pigmented colonies (Staphylococci, Pseudomonas).

Opacity: On nutrient agar they may be transparent, translucent or opaque. Elevation: Colonies may be raised, low convex, umblicated or dome shaped. Media Changes: Colonial growth may bring about color changes in the media themselves, e.g. hemolysis on blood agar by hemolytic *Streptococci*. With *Pseudomonas* the green pigment produced may diffuse into the medium.

BIOCHEMICAL REACTIONS

Organisms that are alike in microscopic and cultural characteristic are often differentiated by their reactions in various biochemical tests.

Sugar Fermentation: Specific carbohydrate fermentation is a property of some organisms when grown in sugar media. Sugars most frequently employed are glucose, sucrose, lactose, mannite, maltose and dulcite. Usually these are incorporated into peptone water, but for the more delicate organisms, Hiss's serum water must be used. Meningococci and gonococci will only react in solid serum-sugar media. Each sugar medium has a colored stopper and a set 'color scheme' may be established for the following sugars. Glucose (green), Lactose (red), Sucrose (blue), Mannite (mauve), Maltose (blue and white), Dulcite (pink). The organism ferments sugar and produces acid and, in certain groups, gas. Acid production is indicated by a color change of the medium, due to inclusion of a pH indicator. Gas production is shown by placing a small Durham's tube upside down in the medium during its production. Before inoculating the medium the tube should be completely filled with the medium. If gas is produced, small bubbles of gas will be seen in the inverted tube.

Other Biochemical Tests: Organisms may further be identified biochemically by their production of indole, change in pH (as shown by the methyl red test), by their utilization of citrate and by another test called the Voges-Proskauer reaction. These 4 tests are especially useful in the differentiation of intestinal pathogens.

CULTURES


A culture of *Bacillus anthracis*

A **microbiological culture,** or **microbial culture**, is a method of multiplying microbial organisms by letting them reproduce in predetermined culture media under controlled laboratory conditions. Microbial cultures are used to determine the type of organism, its abundance in the sample being tested, or both. It is one of the primary diagnostic methods of microbiology and used as a tool to determine the cause of infectious disease by letting the agent multiply in a predetermined media. For example, a throat culture is taken by scraping the lining of tissue in the back of the throat and blotting the sample into a media to be able to screen for harmful microorganisms, such as *Streptococcus pyogenes*. Furthermore, the term culture is more generally used informally to refer to "selectively growing" a specific kind of microorganism in the lab. Microbial cultures are foundational and basic diagnostic methods used extensively as a research tool in molecular biology. It is often essential to isolate a pure culture of microorganisms. A pure (or axenic) culture is a population of cells or multicellular organisms growing in the absence of other species or types. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another. For the purpose of gelling the microbial culture, the medium of agarose gel (Agar) is used. Agar is a gelatinous substance that is derived from seaweed. A cheap substitute for agar is Guar gum, which can be used for the isolation and maintenance of thermophiles.

Bacterial culture: Microbiological cultures utilize petri dishes of differing sizes that have a thin layer of agar based growth medium in them. Once the growth medium in the petri dish is inoculated with the desired bacteria, the plates are incubated in an oven usually set at 37 degrees Celsius. Another method of bacterial culture is liquid culture, in which case desired bacteria are suspended in liquid broth, a nutrient medium. These are ideal for preparation of an antimicrobial assay. The experimenter would inoculate liquid broth with bacteria and let it grow overnight in a shaker for uniform growth, then take aliquots of the sample to test for the antimicrobial activity of a specific drug or protein (antimicrobial peptides).

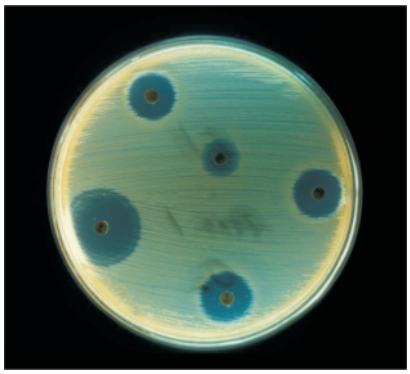
Virus and phage culture: Virus or phage cultures require host cells for the virus or phage to multiply in. For bacteriophages, cultures are grown by infecting bacterial cells. The phage can then be isolated from the resulting plaques in a lawn of bacteria on a plate. Virus cultures are obtained from their appropriate eukaryotic host cells.

Eukaryotic cell culture: Isolation of pure cultures: For single-celled eukaryotes, such as yeast, the isolation of pure cultures uses the same techniques as for bacterial cultures. Pure cultures of multicellular organisms are often more easily isolated by simply picking out a single individual to initiate a culture. This is a useful technique for pure culture of fungi, multicellular algae, and small metazoa, for example. Developing pure culture techniques is crucial to the observation of the specimen in question. The most common method to isolate individual cells and produce a pure culture, is to prepare a streak plate. The streak plate method is a way to physically separate the microbial population, and is done by spreading the inoculate back and forth with an inoculating loop over the solid agar plate. Upon incubation, colonies will arise and, hopefully, single cells will have been isolated from the biomass.

HAVING GROWN THE BACTERIA, THEY ARE TESTED AGAINST A PANEL OF ANTIBIOTICS TO CONCLUSIVELY EVALUATE AS TO WHICH ANTIBIOTIC WILL COMPLETELY ELIMINATE OR KILL THE PATHOGENIC BACTERIA. THIS IS KNOWN AS AN ANTIBIOGRAM.

An **antibiogram** is the result of a laboratory testing for the sensitivity of an isolated bacterial strain to different antibiotics. It is by definition an *in-vitro* sensitivity. In clinical practice, antibiotics are most frequently prescribed on the basis of general guidelines and knowledge about sensitivity: e.g. uncomplicated urinary tract infections can be treated with a first generation quinolone, etc. This is because *Escherichia coli* is the most likely causative pathogen, and it is known to be sensitive to quinolone treatment. Infections that are not acquired in the hospital, are called "community acquired" infections. However, many bacteria are known to be resistant to several classes of antibiotics, and treatment is not so straight-forward. This is especially the case in vulnerable patients, such as patients in the intensive care unit. When these patients develop a "hospital-acquired" (or "nosocomial") pneumonia, more hardy bacteria like *Pseudomonas aeruginosa* are potentially involved. Treatment is then generally started on the basis of surveillance data about the local pathogens probably involved. This first treatment, based on statistical information about former patients, and aimed at a large group of potentially involved microbes, is called "empirical treatment". Before starting this treatment, the physician will collect a sample from a suspected contaminated compartment: a blood sample when bacteria possibly have invaded the bloodstream, a sputum sample in the case of a ventilator associated pneumonia, and a urine sample in the case of a urinary tract infection. These samples are transferred to the microbiology lab, which looks at the sample under the microscope, and tries to culture the bacteria. This can help in the diagnosis. Once a culture is established, there are two possible ways to get an antibiogram: a semi-quantitative way based on diffusion (Kirby-Bauer method); small discs containing different antibiotics, or impregnated paper discs, are dropped in different zones of the culture on an agar plate, which is a nutrient-rich environment in which bacteria can grow. The antibiotic will diffuse in the area

surrounding each tablet, and a disc of bacterial lysis will become visible. Since the concentration of the antibiotic was the highest at the centre, and the lowest at the edge of this zone, the diameter is suggestive for the Minimum Inhibitory Concentration, or MIC, (conversion of the diameter in millimeter to the MIC, in µg/ml, is based on known linear regression curves). A quantitative way based on **dilution**: a dilution series of antibiotics is established (this is a series of reaction vials with progressively lower concentrations of antibiotic substance). The last vial in which no bacteria grow contains the antibiotic at the Minimal Inhibiting Concentration.



Kirby-Bauer's Diffusion method of performing antibiotic sensitivity testing

Once the MIC is calculated, it can be compared to known values for a given bacterium and antibiotic: e.g. a MIC > 0,06 µg/ml may be interpreted as a penicillin-resistant *Streptococcus pneumoniae*. Such information may be useful to the clinician, who can change the empirical treatment, to a more custom-tailored treatment that is directed only at the causative bacterium.

SENSITIVITY

Introduction: Resistance to antimicrobial agents (AMR) has resulted in morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that emergent antibiotic resistance is a serious global problem. Appropriate antimicrobial drug use has unquestionable benefit, but physicians and the public frequently use these agents inappropriately. Inappropriate use results from physicians providing antimicrobial drugs to treat viral infections, using inadequate criteria for diagnosis of infections that potentially have a bacterial aetiology, unnecessarily prescribing expensive, broad-spectrum agents, and not following established recommendations for using chemo prophylaxis. The availability of antibiotics over the counter, despite regulations to the contrary, also fuel inappropriate usage of antimicrobial drugs in India. The easy availability of antimicrobial drugs leads to their incorporation into herbal or "folk" remedies, which also increases inappropriate use of these agents. Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance. The association between increased rates of antimicrobial use and resistance has been documented for nosocomial infections as well as for resistant community acquired infections. As resistance develops to "first-line" antibiotics, therapy with new, broader spectrum, more expensive antibiotics increases, but is followed by development of resistance to the new class of drugs. Resistance factors, particularly those carried on mobile elements, can spread rapidly within human and animal populations. Multidrug-resistant pathogens travel not only locally but also globally, with newly introduced pathogens spreading rapidly in susceptible hosts. Antibiotic resistance patterns may vary locally and regionally, so surveillance data needs to be collected from selected sentinel sources. Patterns can change rapidly and they need to be monitored closely because of their implications for public health and as an indicator of appropriate or inappropriate antibiotic usage by physicians in that area. 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, and is revised periodically. The principles of determining the effectivity of a noxious agent to a bacterium were well enumerated by Rideal, Walker and others at the turn of the century, the discovery of antibiotics made these tests (or their modification) too cumbersome for the large numbers of tests necessary to be put up as a routine. The ditch plate method of agar diffusion used by Alexander Fleming was the forerunner of a variety of agar diffusion methods devised by workers in this field. The Oxford group used these methods initially to assay the antibiotic contained in blood by allowing the antibiotics to diffuse out of reservoirs in the medium in containers placed on the surface. With the introduction of a variety of antimicrobials 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 type used. The disc diffusion method of AST is the most practical method and is still the method of choice for the average laboratory. Automation may force the method out of the diagnostic laboratory but in this country as well as in the smaller laboratories of even advanced countries, it will certainly be the most commonly carried out microbiological test for many years to come. It is, therefore, imperative that microbiologists understand the principles of the test well and keep updating the information as and when necessary. All techniques involve either diffusion of antimicrobial agent in agar or dilution of antibiotic in agar or broth. Even automated techniques are variations of the above methods.

METHODS OF ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include:

Diffusion	Dilution	Diffusion&Dilution
Stokes method	Minimum Inhibitory Concentration	E-Test method
Kirby-Bauer method	i) Broth dilution ii) Agar dilution	

Disk Diffusion

Reagents for the Disk Diffusion Test

Müeller-Hinton Agar Medium: Of the many media available, Müeller-Hinton agar is considered to be the best for routine susceptibility testing of nonfastidious bacteria for the following reasons: It shows acceptable batch-to-batch reproducibility for susceptibility testing. It is low in sulphonamide, trimethoprim, and tetracycline inhibitors. It gives satisfactory growth of most nonfastidious pathogens. A large body of data and experience has been collected concerning susceptibility tests performed with this medium. Although Müeller-Hinton agar is reliable generally for susceptibility testing, results obtained with some batches may, on occasion, vary significantly. If a batch of medium does not support adequate growth of a test organism, zones obtained in a disk diffusion test will usually be larger than expected and may exceed the acceptable quality control limits. Only Müeller-Hinton medium formulations that have been tested according to, and that meet the acceptance limits described in, NCCLS document M62-A7- Protocols for Evaluating Dehydrated Müeller-Hinton Agar should be used.

Preparation of Müeller-Hinton Agar: Müeller-Hinton agar preparation includes the following steps. Müeller-Hinton agar should be prepared from a commercially available dehydrated base according to the manufacturer's instructions. Immediately after autoclaving, allow it to cool in a 45 to 50°C water bath. Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm. The agar medium should be allowed to cool to room temperature and, unless the plate is used the same day, stored in a refrigerator (2 to 8°C). Plates should be used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize drying of the agar. A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35°C for 24 hours or longer.

Disc diffusion methods: The Kirby-Bauer and Stokes' methods are usually used for antimicrobial susceptibility testing, with the Kirby-Bauer method being recommended by the NCCLS. The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedures as described here. NCCLS is an international, interdisciplinary, non-profit, non-governmental organization composed of medical professionals, government, industry, healthcare providers, educators etc. It promotes accurate antimicrobial susceptibility testing (AST) and appropriate reporting by developing standard reference methods, interpretative criteria for the results of standard AST methods, establishing quality control parameters for standard test methods, provides testing and reporting strategies that are clinically relevant and cost-effective. Interpretative criteria of NCCLS are developed based on international collaborative studies and well correlated with MIC's and the results have corroborated with clinical data. Based on study results NCCLS interpretative criteria are revised frequently. NCCLS is approved by FDA-USA and recommended by WHO.

Procedure for Performing the Disc Diffusion Test

Inoculum Preparation

Growth Method: The growth method is performed as follows: At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a suitable broth medium, such as tryptic soy broth. The broth culture is incubated at 35°C until it achieves or exceeds the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours). The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain a turbidity optically comparable to that of the 0.5 McFarland standard. This results in a suspension containing approximately 1 to 2 x 10^8 CFU/ml for *E.coli* ATCC 25922. To perform this step properly, either a photometric device can be used or, if done visually, adequate light is needed to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.

Direct Colony Suspension Method: As a convenient alternative to the growth method, the inoculum can be prepared by making a direct broth or saline suspension of isolated colonies selected from a 18- to 24-hour agar plate (a nonselective medium, such as blood agar, should be used). The suspension is adjusted to match the 0.5 McFarland turbidity standard, using saline and a vortex mixer. This approach is the recommended method for testing the fastidious organisms, *Haemophilus spp., N. gonorrhoeae*, and *Streptococci*, and for testing staphylococci for potential methicillin or oxacillin resistance.

Inoculation of Test Plates: Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab. The dried surface of a Müeller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed. The lid may be left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks. NOTE: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

Application of Discs to Inoculated Agar Plates: The predetermined battery of antimicrobial discs is dispensed onto the surface of the inoculated agar plate. Each disc must be pressed down to ensure complete contact with the agar surface. Whether the discs are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center. Ordinarily, no more than 12 discs should be placed on one 150 mm plate or more than 5 discs on a 100 mm plate. Because some of the drug diffuses almost instantaneously, a disc should not be relocated once it has come into contact with the agar surface. Instead, place a new disc in another location on the agar. The plates are inverted and placed in an incubator set to 35°C within 15 minutes after the discs are applied. With the exception of *Haemophilus spp.*, *Streptococci* and *N. gonorrhoeae*, the plates should not be incubated in an increased CO_2 atmosphere, because the interpretive standards were developed by using ambient air incubation, and CO_2 will significantly alter the size of the inhibitory zones of some agents.

Reading Plates and Interpreting Results: After 16 to 18 hours of incubation, each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc. Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate. The petri plate is held a few inches above a black, nonreflecting background and illuminated with reflected light. If blood was added to the agar base (as with *Streptococci*), the zones are measured from the upper surface of the agar illuminated with reflected light, with the cover removed. If the test organism is a *Staphylococcus* or *Enterococcus* spp., 24 hours of incubation are required for vancomycin and oxacillin, but other agents can be read at 16 to 18 hours. Transmitted light (plate held up to light) is used to examine the oxacillin and vancomycin zones for light growth of methicillin-or-vancomycin-resistant colonies, respectively, within apparent zones of inhibition. Any discernable growth within zone of inhibition is indicative of methicillin or vancomycin resistance. The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. However, discrete colonies growing within a clear zone of inhibition should be subcultured, re-identified, and retested. Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus* spp., the thin veil of swarming growth in an otherwise obvious zone of inhibition should be ignored. When using blood-supplemented medium for testing streptococci, the zone of growth inhibition should be measured, not the zone of inhibition of hemolysis. With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore,

disregard slight growth (20% or less of the lawn of growth), and measure the more obvious margin to determine the zone diameter. The sizes of the zones of inhibition are interpreted by referring to the flyer given along with this issue. Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement, and the organisms are reported as either susceptible, intermediate, or resistant to the agents that have been tested. Some agents may only be reported as susceptible, since only susceptible breakpoints are given.

DISC DIFFUSION FOR FASTIDIOUS ORGANISMS

Determination of MIC for Fastidious organisms

The Agar dilution method

Standardization of inoculum: The inoculum should be an actively growing culture diluted in saline to 10^4 to 10^5 microorganism per ml. For *S. pneumoniae* – Direct colony suspension from a 12-15 hour culture from TSBA medium is to be used. The colonies are suspended in 0.5ml of normal saline and the opacity adjusted to McFarland 0.5. A 1/10 dilution of this suspension is made and within 15 minutes of making the diluted suspension the test plates should be inoculated with either a platinum loop calibrated to deliver 0.001ml or multipoint inoculator. For *N. gonorrhoeae* and *H. influenzae*- Similar to the procedure described above for *S. pneumoniae*.

Inoculation of test plate: In general the inoculum should be applied as a spot that covers a circle about 5-8mm in diameter. A platinum loop calibrated to deliver 0.001ml of the inoculum is used to spot inoculate the cultures. Appropriate ATCC quality control organism(s) should be included along with each test. Inoculated plates are left undisturbed until the spots of inoculum have dried.

Incubation: After the spots of inoculum have dried, the plates are incubated at 35°C for 16 to 18 h in an inverted position in a 5% CO_2 incubator. A candle extinction jar may be used if a CO_2 incubator is not available.

Reading: The control plate should show the growth of the QC test organism. The MIC of the quality control strain should be in the expected quality control range. The end point is the lowest concentration of antibiotic that completely inhibits growth. A barely visible haziness or single colony should be disregarded. Results are reported as the MIC in micrograms or units/ml. Interpretation is made in accordance to the guidelines laid down in the NCCLS M100-S12: Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement (MIC Interpretative Standards) as susceptible, intermediate and resistant.

ANTIBIOTICS USAGE IN ANTIBIOGRAMS

Selectivity: Clinically effective antimicrobial agents all exhibit selective toxicity toward the bacterium rather than the host. It is this characteristic that distinguishes antibiotics from disinfectants. The basis for selectivity will vary depending on the particular antibiotic. When selectivity is high the antibiotics are normally not toxic. However, even highly selective antibiotics can have side effects.

Therapeutic Index: The therapeutic index is defined as the ratio of the dose toxic to the host to the effective therapeutic dose. The higher the therapeutic index the better the antibiotic.

Categories of Antibiotics: Antibiotics are categorized as bactericidal if they kill the susceptible bacteria or bacteriostatic if they reversibly inhibit the growth of bacteria. In general the use of bactericidal antibiotics is preferred but many factors may dictate the use of a bacteriostatic antibiotic. When a bacteriostatic antibiotic is used the duration of therapy must be sufficient to allow cellular and humoral defense mechanisms to eradicate the bacteria. If possible, bactericidal antibiotics should be used to treat infections of the endocardium or the meninges. Host defenses are relatively ineffective at these sites and the dangers imposed by such infections require prompt eradication of the organisms.

Antibiotic Susceptibility Testing: The basic quantitative measures of the *in-vitro* activity of antibiotics are the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The MIC is the lowest concentration of the antibiotic that results in inhibition of visible growth (i.e. colonies on a plate or turbidity in broth culture) under standard conditions. The MBC is the lowest concentration of the antibiotic that kills 99.9% of the original inoculum in a given time. Figure 1 illustrates how to determine the MIC of an antibiotic. For an antibiotic to be effective the MIC or MBC must be able to be achieved at the site of the infection. The pharmacological absorption and distribution of the antibiotic will influence the dose, route and frequency of administration of the antibiotic in order to achieve an effective dose at the site of infection. In clinical laboratories, a more common test for antibiotic susceptibility is a disk diffusion test (figure 1). In this test the bacterial isolate is inoculated uniformly onto the surface of an agar plate. A filter disk impregnated with a standard amount of an antibiotic is applied to the surface of the plate and the antibiotic is allowed to diffuse into the adjacent medium. The result is a gradient of antibiotic surrounding the disk. Following incubation, a bacterial lawn appears on the plate. Zones of inhibition of bacterial growth may be present around the antibiotic disk. The size of the zone of inhibition is dependent on the diffusion rate of the antibiotic, the degree of sensitivity of the microorganism, and the growth rate of the bacterium. The zone of inhibition in the disk diffusion test is inversely related to the MIC. The test is performed under standardized conditions and standard zones of inhibition have been established for each antibiotic. If the zone of inhibition is equal to or greater than the standard, the organism is considered to be sensitive to the antibiotic. If the zone of inhibition is less than the standard, the organism is considered to be resistant. Figure 1 also illustrates how the disk diffusion test is done.

Combination Therapy: Combination therapy with two or more antibiotics is used in special cases: To prevent the emergence of resistant strains. To treat emergency cases during the period when an etiological diagnosis is still in progress. To take advantage of antibiotic synergism. Antibiotic synergism occurs when the effects of a combination of antibiotics is greater than the sum of the effects of the individual antibiotics. Antibiotic antagonism occurs when one antibiotic, usually the one with the least effect, interferes with the effects of another antibiotic.

Antibiotics and Chemotherapeutic agents: The term antibiotic strictly refers to substances that are of biological origin whereas the term chemotherapeutic agent refers to a synthetic chemical. The distinction between these terms has been blurred because many of our newer "antibiotics" are actually chemically modified biological products or even chemically synthesized biological products. The generic terms to refer to either antibiotics or chemotherapeutic agents are antimicrobic or antimicrobial agent. However, the term antibiotic is often used to refer to all types of antimicrobial agents.

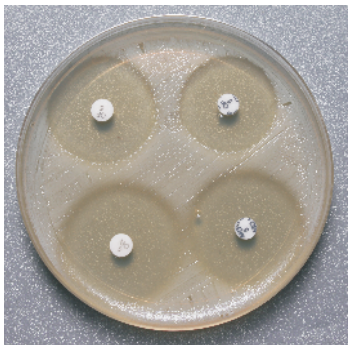


Fig 1. Antibiotic susceptibility testing

TROUBLESHOOTING

QUALITY ASSURANCE IN ANTIBIOTIC SUSCEPTIBILITY TESTING

ANTIBIOTIC SUSCEPTIBILITY testing has become a very essential step for properly treating infectious diseases and monitoring antimicrobial resistance in various pathogens. The choice of antibiotic needs to be made taking into consideration the susceptibility profile of the pathogen, pharmacology of the antibiotic, the need for antibiotic therapy, and its cost effectiveness.

Indications for routine susceptibility testing: A susceptibility test may be performed in the clinical laboratory for two main purposes: To guide the clinician in selecting the best antimicrobial agent for an individual patient. To accumulate epidemiological information on the resistance of microorganisms of public health importance within the community.

Susceptibility test as a guide for treatment: Susceptibility tests should never be performed on contaminants or commensals belonging to the normal flora, or on other organisms that have no causal relationship to the infectious process. These should be carried out only on pure cultures of organisms considered to be causing the infectious process. The organisms should also be identified since not every microorganism isolated from a patient with an infection requires an antibiogram. Routine susceptibility tests are not indicated when the causative organism belongs to a species with predictable susceptibility to specific drugs. This is the case for *Streptococcus pyogenes* and *Neisseria meningitidis*, which are still generally susceptible to penicillin. If resistance of these microorganisms is suspected on clinical grounds, repres-entative strains should be submitted to a competent reference laboratory.

Susceptibility test as an epidemiological tool: Routine susceptibility tests on major pathogens (e.g. *S.typhi*, shigellae) are useful as part of a comprehensive programme of surveillance of enteric infections. These are essential for informing the physician of the emergence of resistant strains (chloramphenicol resistant *S.typhi,* co-trimoxazole resistant and ampicillin resistant shigellae) and indicate a need to modify standard treatment schemes. Continued surveillance of the results of routine suscepti-bility tests is an excellent source of information on the preva-lence of resistant staphylococci and Gram-negative bacilli that may be responsible for cross-infections in the hospital. Periodic reporting of the susceptibility pattern of the prevalent strains is an invaluable aid to forming a sound policy on antibiotic usage in the hospital by restriction and/or rotation of life-saving drugs, such as the aminoglycosides and cephalosporins.

Choice of drugs: The choice of drugs used in a routine antibiogram is governed by considerations of the antibacterial spectrum of the drugs, their pharmacokinetic properties, toxicity, efficacy, and availability, as well as their cost to both the patient and the community. Among the many antibacterial agents that could be used to treat a patient infected with a given organism, only a limited number of carefully selected drugs should be included in the susceptibility test. Table 1 indicates the drugs to be tested in various situations. These agents are divided into two sets. Set 1 includes the 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.

Direct versus indirect susceptibility tests: In the standardized method, the inoculum is prepared from colonies on a primary culture plate or from a pure culture. This is called an "indirect sensitivity test". In certain cases, where a rapid answer is important, the standardized inoculum may be replaced by the pathological specimen itself, e.g. urine, a positive blood culture, or a swab of pus. For urine specimens, a microscopic examination of the sediment should first be made in order to see if there is evidence of infection, i.e. the presence of pus cells and/or organisms. The urine may then be used as the inoculum in the standard test. Likewise, susceptibility tests may be performed on incubated blood cultures showing evidence of bacterial growth, or a swab of pus may be used as a direct inoculum, when a Gram stained smear shows the presence of large numbers of a single type of organism. This is called a "direct susceptibility test"; its advantage over the indirect test is that a result is obtained 24 hours earlier. The disadvantage is that the inoculum cannot be properly controlled. When the susceptibility plate shows too light or too heavy growth, or a mixed culture, the results should be interpreted with caution or the test repeated on pure cultures.

Table 1: Basic sets of drugs for routine susceptibility tests

	Set 1	Set 2
Staphylococcus	Benzylpenicillin 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 Co-trimoxazole Tetracycline Cefalotin Gentamicin	Cefuroxime Ceftriaxone Ciprofloxacin Piperacillin Amikacin
Pseudomonas aeruginosa	Piperacillin Gentamicin Tobramycin	Amikacin

GENERAL PRINCIPLES OF ANTIMICROBIAL SUSCEPTIBILITY TESTING: 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 dilution method:** For quantitative estimates of antibiotic activity, dilutions of the antibiotic may be incorporated into broth or agar medium, which is then inoculated with the test organism. The lowest concentration that prevents growth after overnight incubation is known as the minimum inhibitory concentration (MIC) of the agent. The MIC value is then compared with known concentrations of the drug obtainable in the serum and in other body fluids to assess the likely clinical response. **The diffusion method:** Paper discs impregnated with a defined quantity of antimicrobial agent are placed on agar medium uniformly seeded with the test organism. A concentration gradient of the antibiotic forms by diffusion from the disc and the growth of the test organism is inhibited at a distance from the disc that is related among other factors to the susceptibility of the organism. The recommended method for intermediate and peripheral laboratories is the modified Kirby-Bauer method, the methodology of which is given below: This method has been recommended by National Committee on Clinical Laboratory Services (NCCLS-USA) Subcommittee on Antimicrobial Susceptibility Testing. This is the most thoroughly described disc diffusion method for which interpretive standards have been developed and which is supported by laboratory and clinical data.

The Modified Kirby-Bauer Method: Reagents: **Mueller-Hinton agar:** 1. Mueller-Hinton agar should be prepared from a dehydrated base according to the manufacturer's recommendations. The medium should be such that control zone sizes within the standard limits are produced. It is important not to overheat the medium. 2. Cool the medium to 45-50°C and pour into the 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. 3. When the agar has solidified, dry the plates for immediate use for l0-30 minutes at 36°C by placing them in an upright position in the incubator with the lids tilted. 4. Any unused plates may be stored in a plastic bag, which should be sealed and placed in the refrigerator. Plates stored in this way can be kept for 2 weeks.To ensure that the zone diameters are sufficiently reliable for testing susceptibility to sulfonamides and co-trimoxazole, 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

33l86) 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. **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°C; the freezer compartment of a home refrigerator is convenient. A small working supply of discs can be kept in the refrigerator for upto 1 month. On removal from the refrigerator, the containers should be left at room temperature for about I 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 g/L) solution of barium chloride dihydrate into a l00-mL graduated cylinder, and filling to l00 mL with 1% (10 mL/L) sulfuric 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 6 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 a 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 the confluent growth is similarly suspended in saline. 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° 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 1 disc placed in the centre of the plate. Each disc should be gently pressed down 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 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 them with standard tables. The measurements can be made with a ruler on the under surface of the plate without opening the lid. The endpoint 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* species 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.

Clinical definitions of terms resistant and susceptible: the three-category system: 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 recognizes three categories of susceptibility and it is important that both the clinician and the laboratory worker 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: This term 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 in 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 this 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 of the location of the infection. For testing the response of staphylococci to benzylpenicillin, only the categories 'susceptible' and 'resistant' (corresponding to the production of b-lactamase) are recognized. Factors influencing zone size and common problems encountered in performing susceptibility test are shown in Tables 2 and 3.

Table 2: Factors influencing zone size in antibiotic susceptibility testing

Factor	Influence
Inoculum density	Larger zones with light inoculum and vice versa
Timing of disc application	If after application of disc, the plate is kept for longer time at room temperature, small zones may form
Temperature of incubation	Larger zones are seen with temperatures <35°C
Incubation time	Ideal 16-18 hours; less time does not give reliable results
Size of the plate	Smaller plates accommodate less number of discs
Depth of the agar medium	Thin media yield excessively large inhibition zones and vice versa
Proper spacing of the discs	Avoids overlapping of zones
Potency of antibiotic discs	Deterioration in contents leads to reduced size
Composition of medium	Affects rate of growth, diffusion of antibiotics and activity of antibiotics
Acidic pH of medium	Tetracycline, novobiocin, methicillin zones are larger
Alkaline pH of medium	Aminoglycosides, erythromycin zones are larger
Incubation in the presence of CO_2	Increases zone size of tetracycline and methicillin
Addition of thymidine to medium	Decreases activity of trimethoprim
Addition of defibrinated blood	Decreases activity of sulfonamides
On chocolate agar, decreased activity of	Sulfonamides, trimethoprim, aminoglycosides
Reading of zones	Subjective errors in determining the clear edge
Chelating agents such as calcium, magnesium and iron	Decreases diffusion of tetracycline and gentamicin

Table 3: Troubleshooting guide for disc diffusion test in antibiotic susceptibility testing

Aberrant results	Probable cause
Tetracycline zone too small	pH of medium too low
Aminoglycoside zone too small	pH of medium too high
Aminoglycoside zone too large	Ca^{2+} and/or Mg^{2+} level too high in medium Ca^{2+} and/or Mg^{2+} level too low in medium
Too large zone on control plates	Inoculum too light Nutritionally poor medium Slow growing organisms (not seen with controls) Improper medium depth (too thin)
Zone universally too small on control plates	Inoculum too heavy
Methicillin zone indeterminant in disc test	Methicillin degraded by strong β-lactamase producing staphylococci

Carbenicillin zone disappears with *Pseudomonas* control	Resistant mutant has been selected for testing
Single disc result above or below control limits	Error in reading, fuzzy zone edge, transcription error, bad disc Disc may not be pressed firmly onto agar surface
Colonies within zone of inhibition	Mixed culture Resistant mutants within zone
Zones overlap	Discs too close together
Zones indistinct	Poorly streaked plates
Zone within zone phenomenon	Swarming *Proteus* species Feather edge of zones around penicillin or ampicillin discs usually with b lactamase negative strains of *Staph. aureus*
Enterococcus appears sensitive to aminoglycoside discs	Assessment of aminoglycosides inaccurate in disc test

Need for quality control in susceptibility test: The final result of a disc diffusion test is influenced by a large number of variables. Some of the factors, such as the inoculum density and the incubation temperature, are easy to control, but a laboratory rarely knows the exact composition of a commercial medium or the batch-to-batch variations in its quality, and it cannot take for granted the antimicrobial content of the discs. The results of the test must, therefore, be monitored constantly by a quality control programme which should be considered part of the procedure itself. The precision and accuracy of the test are controlled by the parallel use of a set of control strains, with known susceptibility to the 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. When 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. **Standard procedure for quality control:** The quality control 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. **Standard Strains:** These 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 the refrigerator. These should be subcultured onto fresh slants every 2 weeks. **Frequency of quality control testing:** Salient features of quality control in antibiotic susceptibility testing are: Use antibiotic discs of 6 mm diameter, Use correct content of antimicrobial agent per disc, Store supply of antimicrobial discs at -20°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°C, Space the antibiotic discs properly to avoid overlapping of inhibition zone, Use inoculum size that produces 'near confluent' growth, Ensure even contact of the antibiotic disc with the inoculated medium, Measure zone sizes precisely, Interpret zone sizes by referring to standard charts. The zone size produced by an antimicrobial agent indicates its activity against the organism. However, zone sizes of two agents to which the organism is sensitive are not comparable and should not give an erroneous impression that the test organism is more sensitive to the drug which has yielded a bigger zone size.

BOUQUET

In Lighter Vein

-Life Insurance : A contract that keeps you poor all your life so that you can die Rich.

-CAR SICKNESS: The feeling you get when the car payment is due.

-Alimony - the cost of falling in love.

-Capitalism - the survival of the fattest.

-In the corridor of a govt office was a signboard that read,"Do not make a noise"Someone added the following"Otherwise we may wake up"...

-"I told the doctor I broke my leg in two places. He told me to quit going to those places.

-My friend has a fine watch dog. At any suspicious noise he wakes the dog and the dog begins to bark.

-I removed L from LOVER…….n now its all OVER !!!

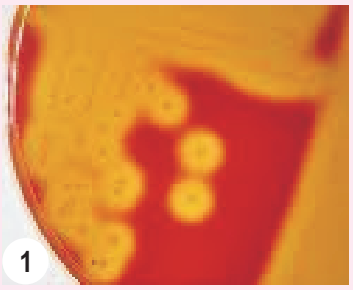
-Intel inside…….fool is out side.

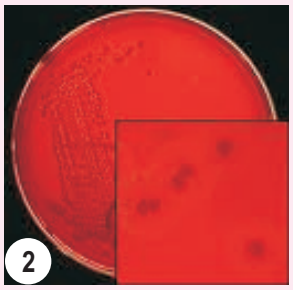
Wisdom Whispers

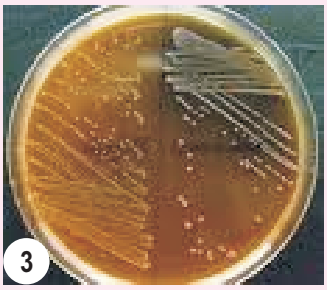
- Excuses are the easiest things to manufacture, and the hardest things to sell.
- Love sought is good, but given unsought, is better.
- Some see a hopeless end, while others see an endless hope"
- Procrastination has its good side. You always have something to do tomorrow
- Flattery - an insult in gift wrapping
- People with narrow minds usually have broad tongues.
- If at first you don't succeed, you'll get a lot of free advice from folks who didn't succeed either.
- Freedom is not worth having if it does not include the freedom to make mistakes.
- Many people lose their tempers merely from seeing you keep yours.
- Buy land. They've stopped making it.
- Immorality: The morality of those who are having a better time.

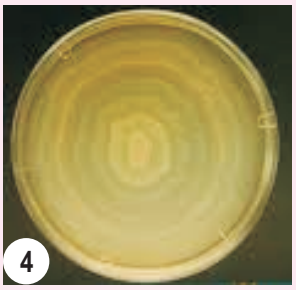
Brain Teasers

Identify the following bacteria from the growth pattern images









Answers: 1. Beta hemolytic Streptococci, 2. Alpha hemolytic Streptococci, 3. *Pseudomoas aeruginosa*, 4. *Proteus vulgaris*.

INTERPRETATION

CD4 COUNTS, HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) AND HIV MANAGEMENT

(Continued from previous issue)

CD4 count monitoring in resource-limited settings: One of the many challenges in deciding when to start and how to monitor patients on HAART in resource-limited settings (RLS) is the inaccessibility and expense of laboratory tests, including CD4 counts and viral load, which are standard of care in the developed world. Since viral-load testing is not widely available, the WHO has developed clinical and immunologic criteria that can be used to define treatment failure in RLS. Clinical failure is defined as the development of a new or recurrent WHO stage 4 condition. Immunologic failure is defined as a persistent CD4 count of under 100 cells/mm^3, a fall in CD4 count of more than 50% from the on-treatment peak value or a fall in CD4 count to below the pretreatment value. Since in some locations the availability of CD4 testing is limited, numerous researchers have looked at whether the total lymphocyte count (TLC) can be used as a surrogate marker for CD4 counts with variable results. The correlation between TLC and CD4 appears to be poor in children. The correlation in adults may be slightly better; one study of HIV-infected patients in Nairobi, Kenya found that a TLC cut-off of 1900 cells/mm^3 was 81% sensitive and 90% specific in detecting patients with a CD4 count below 200 cells/mm^3. Another study in Ethiopia found that a TLC cutoff of 1780 cells/mm^3 was 61% sensitive and 62% specific in identifying adults with a CD4 count of less than 200 cells/mm^3. The TLC generally increases while on HAART but is not a reliable test to monitor the efficacy of such treatment. Recently, there has been a great deal of effort towards finding simpler and more cost-effective means of measuring CD4 counts. Novel techniques have been devised, utilizing more affordable flow cytometry methods as well as technologies other than flow cytometry, which requires significant operator expertise in addition to the expense. One promising technology is the modified Dynabeads® method for measuring CD4 counts; the cost for this test is less than US$3/sample, compared with $17/sample for the older Capcellia technology. Owing to these developments, CD4 counts are increasingly being measured in many RLS at baseline and for monitoring purposes. However, viral loads remain largely inaccessible and/or cost prohibitive – this can lead to the late identification of virologic failure and development of resistance. This is particularly problematic in RLS, where there are often limited treatment options; therefore, the prevention of resistance remains of the utmost importance. Unfortunately, several studies in RLS have shown that changes in CD4 count cannot be used alone to predictably identify patients with virologic failure. A prospective study in South Africa investigated the relationship between CD4 count and virologic failure and found that a negative CD4 count slope was only 53% sensitive and 64% specific for identifying patients with virologic failure. A study in Botswana found that CD4 count increases have a low predictive value for identifying patients with suppressed viral loads; an increase in the absolute CD4 count of more than 50 cells/mm^3 after starting HAART was 93% sensitive in identifying patients who had achieved virologic suppression but only 62% specific. Another study by Keiser et al., analyzing outcomes from 10 antiretroviral treatment programs in Africa and South America, demonstrated that the sensitivity of the WHO criteria for immunologic failure in detecting virologic failure among patients on HAART was only 12.6–17.1% in these settings, although it was approximately 97% specific. This raises concerns as presumably the use of immunologic monitoring without concurrent viral-load monitoring would lead to a later detection of treatment failure and delayed switching to a second-line regimen, thus facilitating the development of viral resistance. The converse hazard – premature switching to a second-line regimen based on immunologic criteria in individuals who actually have viral suppression – exists as well. A study of a Namibian population on first-line ART, who met immunologic or clinical criteria for treatment failure, demonstrated that 79% of patients actually had a suppressed viral load when viral-load testing was performed. If the decision to switch to second-line therapy was based on clinical and immunologic criteria alone, many patients would be switched to more expensive and possibly less well-tolerated second-line regimens unnecessarily. As a result, there has been a considerable interest as to whether medication adherence can be used in addition to the CD4 count for patient monitoring. Assessment of medication adherence may add value to the CD4 count in terms of detecting virologic failure or may even be a better predictor of virologic failure than the CD4 count alone. A study by Bisson et al. found that medication adherence, as measured by pharmacy refills, was independently associated with virologic failure, although the median level of adherence among patients with virologic failure was greater than 90%. Although identifying virologic failure early is important in preventing resistance, it may not have as much of an effect on clinical outcomes in the developing world as might be expected. Resources expended on viral-load monitoring may be better spent on providing more patients with HAART. The recently released results from the Development of Antiretroviral Therapy in Africa (DART) study in Uganda and Zimbabwe demonstrated minimal differences in outcomes between a group of patients on HAART who were clinically monitored versus those receiving both laboratory and clinical monitoring (including CD4 count, complete blood count and chemistries every 12 weeks, but excluding viral loads). Among the clinical monitoring group, the 5-year survival rate was 87%, compared with 90% in the laboratory and clinical monitoring. This high 5-year overall survival is somewhat encouraging, albeit surprising, as the study group had advanced disease at the time of initiation on HAART. The difference in outcomes only became apparent after the second year of treatment in the 5 year study, indicating that clinical monitoring alone may be feasible during the first 2 years of treatment and that CD4 counts could be reserved for monitoring treatment beyond this point. Similarly, a computer simulation model developed by Phillips et al. showed minimal differences in outcomes between patients monitored by clinical status alone and those monitored with viral load and CD4 count or CD4 count alone.

Future perspective: In the next 5–10 years absolute CD4 counts and CD4 percentages will most likely remain the cornerstone for initiating and monitoring patients on therapy. Other markers, such as T-cell subsets as well as activation, inflammatory and/or coagulation biomarkers, may become increasingly important for evaluating disease progression, although their anticipated cost for RLS will pose a formidable challenge. Serious non-AIDS-related diseases, such as liver, cardiovascular, renal and non-AIDS malignancies will contribute to the majority of morbidity and mortality among HIV-infected patients who are stable on HAART. Higher CD4 counts have already been shown to reduce these rates. As antiretroviral treatment continues to improve with fewer side effects and less frequent dosing, the CD4 count threshold for starting therapy is likely to increase. With recent data supporting the cost–effectiveness of starting therapy earlier in South Africa, future guidelines on starting antiretroviral therapy at higher CD4 counts should include RLS. **CD4 count as a predictor in patient outcome:** The baseline CD4 count is a significant predictor for HIV disease progression, survival and treatment outcome. Individuals with higher baseline CD4 counts at HAART initiation have the best chance for full immune reconstitution. Patients with lower absolute CD4 counts and percentages at baseline have a higher risk of developing immune reconstitution inflammatory syndrome. Patients with lower CD4 counts are at risk for both AIDS-and non-AIDS-related events. **Absolute CD4 count versus CD4 percentage:** Absolute CD4 counts may fluctuate among individuals or be influenced by factors including illness and/or medications. CD4 percentages can provide additional information regarding prognosis in individuals with CD4 counts above 200 cells/mm^3. **Discordant CD4 & viral-load response to HAART:** Individuals with discordant responses (immunological-only or virological-only responders) do worse than individuals with complete response, but better than individuals with no response. **CD4 counts in resource-limited settings:** CD4 counts are becoming cheaper to obtain and more readily available. In resource-limited settings clinical monitoring alone may be an option for the first 2 years of treatment.

TULIP NEWS

Microxpress™ Biogram™

Antimicrobial Susceptibility Discs

Product Range:

- All 79 antimicrobials as per C.L.S.I.
- 123 antimicrobials with customized concentration levels
- Individual Disc dispenser

Pack Size : 5,10 Cartridges

Contents:

- Each Pouch contains single cartridge with dessicant.
- Each cartridge contains 50 susceptibility discs.
- Single disc dispensing device.
- Package insert.

Engineered to Perform as per International Recommendations!

Printed and published by D.G. Tripathi, Edited by Dr. R.J. Sood M.D. (path.) for and on behalf of Tulip Diagnostics Private Ltd, Gitanjali, Tulip Block, Dr. Antonio Do Rego Bagh Alto Santacruz, Bambolim Complex Post Office, Goa - 403202, INDIA.
Fax: (0832) 2458544, E-mail: sales@tulipgroup.com. Website: www.tulipgroup.com