



Advances in The Diagnosis of Infection- What The Clinician Must Know

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140

A B S T R A C T

Comprehensive rapid and accurate tests in microbiology are extremely essential in infectious disease. Blood cultures are the gold standard in the diagnosis of infection and isolation of an organism from a sterile body site also allows for a susceptibility testing to be performed. Proper collection of these samples is vital to optimise results. Continuous monitoring systems of inoculated blood cultures improve on time to detection and isolation of organisms. In diseases like tuberculosis also, automated methods like BACTEC TB - 460 and Mycobacterial Growth Indicator Tube (MGIT) are replacing conventional traditional methods with improved isolation and better Turn Around Time. Antigen detection of certain pathogens in various body fluids as urine (*Streptococcus pneumoniae*, *Legionella pneumophila*), CSF (*S. pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*), stool (*Helicobacter pylori*, *Cryptosporidium parvum*, *Clostridium difficile* toxin A & B) etc. are also gaining importance. These are useful when prior antibiotic therapy has been initiated and culture results are negative after 24 hours of incubation. The detection time is faster in these assays upto a maximum of 2 hours, so therapeutic decisions can be made. Molecular approaches to infectious disease diagnosis are used for organisms that are slow growing, difficult to cultivate, or uncultivable microorganisms. Molecular tests such as PCR are a useful adjunct to confirm the presence or absence of an organism but their interpretation has to be done carefully. Validation data must be available to detail both the analytical and clinical specificity and sensitivity for every “home brewed” and commercial assay. Viral loads for hepatitis B, hepatitis C and HIV likewise are useful in monitoring therapy. Emerging technologies as DNA microarrays have the potential to assess and visualise thousands of nucleic acids simultaneously.

INTRODUCTION

Accurate, comprehensive, rapid and up-to-date microbiology diagnostics in India is the need of the hour. Significant developments have occurred in the last two decades in diagnostic testing that enhance the microbiologic workup for many infectious agents. Today, diseases like typhoid, dengue, malaria and tuberculosis and diarrheal disease continue to strike and together with HIV and other causes of immune suppression, our burden of infectious diseases surpasses that seen in developed countries.

Molecular-based methods are replacing culture-based or antibody-based methods. Heralded as the diagnostic tool the new millennium, interpretative issues have arisen in molecular testing of infectious disease.

Diagnosis of infectious diseases is divided into

1. Microscopy
2. Culture
3. Detection of specific antigen or antibody
4. Molecular techniques.

MICROSCOPY

An example of an advance in microscopy includes better sensitivity for mycobacteria with auramine/rhodamine fluorochrome staining over the traditional Ziehl-Neelson stain. The waxy mycolic acids in the cell walls of mycobacteria have an affinity for the fluorochromes, auramines and rhodamine. These dyes nonspecifically bind to nearly all mycobacteria. The mycobacterial cells appear bright yellow or orange against a greenish background.¹ This method can be used to enhance detection of mycobacteria directly in patient's specimens. The advantage of this method is that lower magnification can be used; so wider area is covered enabling rapid screening. However the cost of a fluorescent microscope and reagents are high and a highly trained technician should screen the smears.

CULTURE

In general the “culture” of performing cultures is not often forthcoming and antibiotics are started empirically.

Blood cultures have undergone advancement mainly from a perspective of improved automation. From a diagnostic standpoint,

positive blood cultures can establish an infectious etiology for an illness and provide a microorganism for susceptibility testing. It is often argued that cultures are a waste of money and they are sent only if there is a lack of therapeutic response but by then the patient is already initiated on antimicrobial and the window of opportunity is lost. It is vital to remember that the specificity for blood cultures being 100% they unequivocally are the gold standard and may actually be cheaper in the long run. However certain key principles have to be borne in mind in obtaining blood cultures. Proper skin antisepsis is vital to avoid skin flora and in selection of the best available site arterial blood is not better than venous blood. Blood should not be collected through an intravascular device and if this is done then it should be paired with a second culture from the peripheral venepuncture. Volume is a key variable for successful detection of blood stream infection. At least 20-30ml of blood should be collected per venepuncture from adults. Each ml of blood collected enhances the isolation of organisms by 3%.

There is data to substantiate that two or three blood cultures are adequate for detecting common pathogens. The recovery in the first culture is 80%, 88% in two cultures and 99% in all three cultures.²

Timing: Optimally at the onset of a shaking chill but in less urgent situations spaced at intervals of ½ - 1 hr. Ratio of blood to broth ideally should be 1:5 to 1:10. Organisms as Brucella, Legionella, some fungi and mycobacteria should be incubated for longer than the traditional 5 days.

Blood culture systems: Automated continuous monitoring blood culture systems certainly enhance not only recovery but also time to detection.³ In addition, various media can be optimally inoculated depending on whether the patient has received antibiotics, suspected of having a fungal or mycobacterial infection or is a child. Continuous monitoring systems as BACTEC / BacT Alert are available in India

TB Culture techniques

Conventional tests

Agar-based methods - Culture of mycobacteria is a much more sensitive test than smear examination and allows for biochemical identification of the species considering enhancing the specificity. Unfortunately the slow doubling time of *M. tuberculosis* makes culture on egg/agar-based solid media slow and time consuming. Agar-based media allow detection of colonies in 10-12 days, whereas the most commonly used Lowenstein Jensen medium (LJ) usually takes 18-24 days.

Automated liquid culture methods

BACTEC TB – 460

It is a sensitive, specific and rapid culture method for smear positive respiratory as well as non-respiratory specimens. Time for detection of *M. tuberculosis* complex from smear-negative clinical specimens is 13-15 days. In specimens, which are difficult to obtain such as tissue biopsies and body fluids, the use of LJ media and BACTEC TB 460 may be justified to maximize isolation of mycobacteria. Different studies of the sensitivity of TB-BACTEC in monitoring mycobacterial growth showed that an inoculum of 200 viable *M. tuberculosis* bacilli could be

detected in 12-13 days while as few as 20 viable bacilli could be detected if one waited for 14-17 days.^{4,5}

Mycobacterial Growth Indicator Tube (MGIT) 960

The test employs a new state of the art fluorescent technology that enables result towards positivity as rapidly as 7-10 days. It is based on oxygen quenching of mycobacteria with a fluorescent dye.⁶

TB- Phage assay

The ability of mycobacteriophages to lyse and destroy mycobacteria has been explored for rapid diagnosis of TB. The assay uses specific mycobacteriophages (DNA viruses specific for *M. tuberculosis* complex) as a reporter to reflect the presence of viable TB bacilli in the clinical specimens within 48 hours.⁷

DETECTION OF ANTIGEN

- NEWER METHODS

Today assays are available for rapid detection of bacterial antigens in various body fluids. These are useful when prior antibiotic therapy has been initiated and culture results are negative after 24 hours of incubation. The turn around time is faster in these assays to a maximum of upto 2 hours. The following fluids / samples can be assayed.

CSF

Latex agglutination for *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *E. coli* and *Streptococcus agalactiae* (Group B).

Cryptococcus neoformans latex agglutination test is useful in patients suspected of having cryptococcosis and can also be performed in the serum of HIV patients for diagnosis in case for some reason a lumbar puncture is contraindicated .

Serum

Plasmodium falciparum and *P. vivax* are detected at levels of 100-200 parasites /µl. The histidine-rich protein (hrp 2) has a sensitivity of 92 % and a specificity of 95% for *P. falciparum*. Similarly the p LDH (parasite lactate dehydrogenase) has a sensitivity of 76% and a specificity of 97%.

Urine

In patients with community-acquired pneumonia, antigen testing is useful in patients on treatment to establish the etiological agent. Urinary antigens of *S. pneumoniae* (pneumococci) are detected and their sensitivity which surpasses that of Gram stain ranges from 75 –95 %. Their clinical usefulness depends on the geographic prevalence of the specific serogroups detected.⁸

Legionella pneumophila group 1 is also excreted in the urine and the sensitivities of the antigen detection by Enzyme Immuno Assay ranges from 70 % to 90%.⁹

In cases of histoplasmosis with disease limited to the respiratory tract or disseminated beyond the primary respiratory tract, a positive antigen test is available in 30% and 90% respectively. Unfortunately the antigen may cross react with *B. dermatidis* and *C.immitis*, limiting the specificity of the test in areas of endemicity.

Stool

Antigen assays are available for detection of *Helicobacter pylori* which has a sensitivity of 96% and a specificity of 95%.¹⁰

Clostridium difficile associated diarrhoea is a cause of infectious diarrhoea in hospitals. With enzyme immuno assays (EIA) Toxin A and B can be detected with a specificity approaching 100%

Cryptosporidium parvum and *Giardia lamblia* are also detected by an EIA test in stool.

DETECTION OF ANTIBODIES

A plethora of antibody tests are available. Most antibody assays employ EIA-based technologies. A rising titer is difficult to extrapolate when readings are taken as Optical Densities. IgG avidity testing is a method to distinguish past infection from recent infection and is based on the premise that newer antibodies do not bind avidly enough. A high avidity denotes a past infection, and a low avidity, a recent one. This is useful in infections in pregnancy.

MOLECULAR METHODS

Advances in molecular medicine have provided the research and diagnostic laboratory with tools that are revolutionising its function. Molecular methods of diagnosis are exciting new developments and seem to be the answer for organisms that growth characteristics that are slow (mycobacteria), difficult (viruses, Chlamydia) or fastidious (Mycoplasma). They also are highly sensitive methods that can detect low pathogen numbers as in meningitis and are attaining importance in monitoring response to treatment (viral load assays for hepatitis B, hepatitis C and HIV). As these tests move from the bench to the bedside it is vital that clinicians have a working knowledge of the principles and more importantly develop a framework for their limitations.

Nucleic acid probes

Nucleic acid hybridization is a powerful and widely used technique which exploits the ability of complementary sequence in single-strand (ss) DNA or RNA to pair with each other to form a duplex. The ss nucleic acid probes used for hybridization should be complementary to the amplified sequence or the region of interest on the gene to be identified. In situ hybridization is used to detect and locate specific DNA or RNA sequence(s) in tissues or chromosomes by making use of radioactive or fluorescent labeled DNA/RNA probes complementary to the required sequence. However in paucibacillary states like tubercular meningitis and pleural effusion the number of bacilli is too low to be picked up by this technology. This technique is commercially available for the identification of *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. kansasii* and *M. goodii*. Nucleic acid probes form a useful adjunct to BACTEC cultures for confirmatory identification.

Nucleic acid amplification

These tests can be divided into three groups

1. Target amplification
2. Signal amplification

3. Probe amplification

Target Amplification

Polymerase chain reaction (PCR)

PCR is an in vitro method for amplifying specific DNA sequence. Starting with extremely minute amounts of a particular nucleic acid sequence from any source, PCR enzymatically generates millions or billions of exact copies thereby making genetic analysis of tiny samples a relatively simple process. The target DNA acts as a template and in the presence of nucleotides, primers and thermostable DNA polymerase and generates copies by alternate heating and cooling for denaturation, annealing and extension.

Real Time PCR

This technology offers rapid PCR with simultaneous amplicon detection. A fluorescent signal is used for real time monitoring of PCR amplicon production. Today, a dramatic reduction in the time required for the assay performance is a significant advance.¹¹

Transcription-mediated amplification (TMA)

TMA uses a species specific sequence of ribosomal RNA (rRNA) as the target for reverse transcriptase.¹² The advantage of this technology being that the dead cells have no transcription machinery hence only viable cells are picked up and amplified.

Nucleic Acid Sequence Based Amplification (NASBA)

It is also like TMA isothermal amplification, wherein RNA is transcribed into cDNA and then RNA copies are synthesized using RNA polymerase.

Signal amplification

This is an alternative to enzymatic duplication of target nucleic acid to increase the signal generated from a hybridised probe molecule. Commonly used technologies include branched DNA (bDNA) and hybrid capture assays

Probe amplification

The end-product of the reaction is an amplified version of the original components used to detect the target and include the ligase chain reaction LCR, Q-beta replicase and cycling probe technology. In LCR, a phenotypic change in the organism such as virulence or drug resistance can be detected.

DNA amplification technology can amplify minute quantities of DNA to levels that can be readily seen following routine agarose gel electrophoresis. But amplification can amplify even minute quantities of contaminating DNA. False positive results are the major concern. Also, the mere presence of an organism in a clinical specimen does not necessarily indicate active disease. DNA-based assays do not differentiate dead from living organisms. False-negative results may originate from very low copy numbers of the microorganism at the site of infection and from inhibitors in the sample.

In tuberculosis, results of molecular tests demonstrate the arbitrariness of TB latent infection disease and may have a useful but limited place in evaluating persons for TB. A negative PCR never eliminates TB as a diagnostic possibility and a positive result does not confirm it. In theory, nucleic acid amplification (NAA) tests are capable of amplifying a single copy of the target

genomic sequence. Validation data should be available to detail both the analytical and clinical specificity and sensitivity for every “home-brewed” and commercial assay. In practice, however, these tests may have only modest sensitivity in specimens that have a low bacillary load. Data from several studies that have shown a significantly lower sensitivity in smear-negative sputum specimens, compared with smear-positive specimens, support this argument. Since specimens such as cerebrospinal fluid (CSF) and pleural fluid often have low bacillary load (i.e. they tend to smear-negative), it is possible that NAA tests can amplify the target DNA or RNA. In a separate meta-analysis assessing the accuracy of NAA tests for tuberculous pleuritis, the summary estimate of sensitivity was only 0.62 for pleural fluid specimens tested by commercial NAA tests. Similarly in another metaanalysis the diagnostic accuracy of NAA for TBM was a low sensitivity of 0.58 and high specificity of 0.98.¹³ However, a major concern with the evaluation of any test for tuberculous meningitis is the lack of a definitive reference standard. None of the available molecular tests can be considered “gold” standards.

In herpes simplex virus encephalitis however the usefulness of molecular testing is well recognised. and has a sensitivity of 95 % with a specificity of 99 to 100%.

Quantitative assays or viral loads for hepatitis B, hepatitis C and HIV likewise are useful in monitoring therapy. In an effort to standardise quantitative testing laboratory collaboration with the World Health Organisation have established International Standards with concentrations expressed as IU/ml which can be used to calibrate, validate and compare quantitative assays.

Emerging technologies : DNA microarray technology i.e. biochip, DNA chip, gene array etc., have the potential to assess and visualise thousands of nucleic acids simultaneously. The solid platform contains genetic material arrayed in a predetermined fashion. When the chip is probed with cDNA (from RNA of the clinical sample) hybridisation can be detected. The applications of this technology are unlimited, especially in detection of drug resistance.

Thus, significant developments have occurred in the past two decades in the diagnostic arena. The large scale global use of

antibiotics with consequent antimicrobial resistance in pathogens and opportunistic microorganisms stress the importance of rapid, accurate, comprehensive and up-to-date microbiology diagnostics. Physicians must continue to apply traditional microbiology with newer molecular testing to the understanding and application of clinical medicine.

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