FUTURISTIC TRENDS IN DIAGNOSTIC AND MOLECULAR MICROBIOLOGY

Abstract

Diagnostic microbiology has evolved very fast over the last few decades. Now many advanced techniques are available in the armamentarium of the laboratory scientist, which save time and provide sensitive and specific results. Most of these advanced techniques are available in medical bacteriology and mycology, but medical virology is also witnessing rapid advancements. The authors will elaborate these in this chapter. However the interested reader is also referred to the references listed at the end of this chapter for developing a more holistic notion of this subject.

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Author

Dr. Sayan Bhattacharyya
Associate Professor
Department of Microbiology
All India Institute of Hygiene and Public Health (AIIH&PH)
Kolkata, India.

I. INTRODUCTION

Traditionally bacteria and fungi can be identified by overnight culture and observation of colonies, the staining and putting up of biochemicals, taking 2 or 3 days in the process(1). Viruses are detected by detecting antigen and antibody in patients' sera or by tissue culture , or gene detection like PCR. In the modern era, however, identification of microbes has undergone a total paradigm shift, and automation is being increasingly put in vogue for their identification. Some of these new techniques will be enumerated below.

1. **Chromogenic Culture Media:** These media have been employed for detecting bacteria like MRSA (Methicillin resistant *Staphylococcus aureus*), *Streptococcus agalactiae*, *Enterococcus* and yeasts like *Candida* spp. These selective media contain one or more undisclosed colorless chromogenic substrates which are broken down by the enzymes that are produced by the microbe of interest. This chemical reaction produces a product that is coloured. It thus therefore imparts a colour to the whole colony as it forms colony on this medium. Chrom ID (produced by bioMerieux in Marcy l'Etoile in France) is a selective and chromogenic medium , designed to culture*Staphylococcus aureus*. It detects the alpha-glucosidase enzyme present in the bacterium and also has cefoxitin (at a concentration of 4 mg per Litre) to test Methicillin resistance. Colonies of MSSA (Methicillin susceptible *Staphylococcus aureus*) are inhibited by cefoxitin, while MRSA (Methicillin resistant *Staphylococcus aureus*) grows in this medium in the form of green colonies. CHRO Magar MRSA (produced by CHROMagar Microbiology, Paris, in France along with BD Diagnostics, Erembodegem, in Belgium) also contains cefoxitin. However, it demonstrates a different colour-forming reaction, which produces colonies of MRSA that are rose to mauve in colour.

2. **API/API-20:** API test strip (full name being analytical profile index) is a smaller and standardized battery of biochemical tests. It is used along with complete identification databases, and the best such known database is the API 20E (named after the 20commonly seen biochemical traits of members of erstwhile family Enterobacteriaceae).

3. **VITEK and VITEK-2:** VITEK 2MS is also used commonly for bacterial identification. It also rapidly identifies bacteria by using cards and performs their susceptibility as well. Results are matched with computer-generated databases and identification is thereby confirmed.

4. **RCUT or Rapid carbohydrate utilization test:** It is a rapid test which detects fermentation of different sugars in buffer solution with indicator, within 2 or 3 hours by *Neisseria* spp. It is done commonly on a ELISA plate or microtitre plate with a control well. *Escherichiacoli* is employed commonly as positive control since it breaks down most sugars.

5. **BACTEC and BAC-T Alert:** Both these systems have been in use for many years now, and rely on Oxygen consumption or Carbon dioxide production or other metabolic indications. It has been found that a shorter time to detect the grown bacteria with reasonably good rate of bacterial recovery is seen in the BacT/ALERT® VIRTUO

apparatusin comparison with other similar methods(1). BACTEC was modified later as MGIT-960 (Mycobacterial growth indicator tube) which uses liquid medium called Middlebrook's 7H9 with several supplements like SIRE and antibiotics. The principle is consumption of Oxygen and production of signal by dissociation of Oxygen from Ruthenium.

6. **MALDI TOF MS:** MALDI-TOF MS is the short form of Matrix Assisted Laser Desorption Ionization-Time of flight Mass spectrometry. Regarded as a very useful and rapid modern technique, it can help identify bacteria as well as fungi from pure colonies. When run in batches, it saves cost also.The MALDI-TOF MS is now regarded as very promising and upcoming in diagnostic medical microbiology laboratories. This can be attributed due to its unique prowess to analyse whole cells of bacteria. There is practicallyno need of sample preparation or batching here. The time to identify a positive culture can be improved (which is about 10 – 20 seconds for acquiring the protein spectra and then 15 to 30 seconds for comparing data with that in the databank), when beginning with a single colony(2). Biomass of the microbes may be utilized for MALDI-TOF MS study just as the initial faint or hazycolonies become prominent on solid media.This assay hence relies on protein analysis. Within no time, MALDI-TOF MS system has nearly fully substituted extant conventional biochemical identification systems of pathogenic bacteria in many advanced Government as well as private laboratories. MALDI-TOF MS can also be used for antibiotic susceptibility testing. First proposed in 1970s, the renowned biophysicists of German origin, Franz Hillenkamp and Michael Karas later devised the full-fledged technique of MALDI-TOF MS. They later rendered it suitable for routine usage(3). MALDI-TOF MS disintegrates the microbial proteins, mainly the ribosomal ones. Data there by generated is utilized for identifying the bacterial or fungal pathogens. The main plus-points of this technique are its fastness , literally in a few minutes, and also the cost effectiveness of single assays, along with high specificity. However, one major disadvantage is the high cost of the device and also the need of expert trained staff. MALDI-TOF can now be used for direct DNA-Based diagnosis of the pathogens from whole blood specimens also. In MALDI-TOF, the analyte or microbe to be tested, is first fixed in an acidic matrix material resting on a metallic plate. Then Nitrogen laser excitation is employed for catalyzing the transfer of charge from the matrix on to the analyte of interest, for the purpose of desorption. The ions are then segregated on the basis of their m/z (mass divided by charge number) ratio. After that,a mass analyzer is put in useto detect and create a spectral profile. Newer applications for MALDI-TOF-MS are:-testing of antimicrobial susceptibility, study of virulence factors of microbes, and assay of glycans(4).

   Before the direct identification of pathogens with the help of MALDI-TOF MS from blood culture bottles which are positive, however, prior sample preparation is to be carried out using procedures like lysis–centrifugation , and then ethanol/formic acid extraction. However, this method is still not universally accepted due to hefty costs , not very good isolation rate and the overall complex nature of the procedure.

7. **WGS (whole genome sequencing)**: It is also a useful and rapid method to study and identify bacteria with certainty. It is widely used now, not only in diagnostics but also to study the molecular epidemiology, virulence traits and antibiotic resistance of bacteria.

Earlier sequencing was costly but now the advent ofnext-generation sequencing(NGS) instrumentations have reduced the costs. Whole genome sequencing assays are now widely accessible as well as affordable. NGS is now also useful in public health surveillance. It may also be utilised to find out the source and mode of spread of nosocomial pathogens like *Staphylococcus aureus*, *Acinetobacter baumannii* , *Pseudomonasaeruginosa*and *Enterococcus faecium*. It hence aids in infection prevention and control in hospitals(5). NGS ensures rapid bacterial identification and can also help differentiate between clones.

8. **PCR:** PCR or Polymerase Chain reaction was discovered some decades ago by Kary Mullis. It is used for definitive identification of bacteria present inclinical specimens, as well as from colonies. It depends on the amplification of the target gene. Traditional or conventional PCR assay, which previously often consumed days to be accomplished, has now been replaced by quicker and easier methods like thermal cycler, rapid-cycle and Real-time PCR. These tests can generally be carried out in a closed system. In real time PCR, the amplification and also the detection pathways can quicklybe carried outin the same reaction vessel(6).

For diarrhoea, PCR can also be used for diagnosing the pathogen responsible. Molecular techniques have been introduced for routinely diagnosing diarrhoea in a number of microbiology laboratories. Generally and broadly, these techniques are classified into two groups: (a) those that use PCR to find out one or several genes belonging to any one microbe (like detecting *Clostridium difficile* or viruses like norovirus), and (b) assayswhich use multiplex PCR to find out the bacteria, viruses and parasites causing gastroenteritis which can be found together(7). Recently, real-time PCR assay meant for diagnosing CDAD (*Clostridioidesdifficile*-associated diarrhoea) has been okayed by the FDA, and this is coined as the BD GeneOhm Cdiff Assay (marketed by BD GeneOhm, based in San Diego, California, USA). It can amplify a highly conserved region belonging to the *tcdB* gene present in the bacterium.

PCR has also been used successfully over the years for diagnosis of sexually transmitted infections (STI) also. NAATs, especially the real-time polymerase chain reaction (or RT-PCR) methods, and the multiplex PCR techniques allow several microorganisms involved in a particular infection to be detected. They thus fulfil the needs for establishing rapid microbial diagnosis for several STIs to be met.

These applications are

- Detection of *C. trachomatis*, *Mycoplasma genitalium* , *Neisseria gonorrhoeae*and *Ureaplasma* spp. in patients having proctitis, cervicitis, urethritis and pelvic inflammatory disease or PID;
- Detection of *T. vaginalis* and *Candida* spp. in patients with vaginitis or diagnosing bacterial vaginitis by help of techniques which can measure the presence or absence of *Lactobacillus* spp.,*Mycoplasma hominis*, *Atopobium vaginae* , *G. vaginalis* , and *Mobiluncus* spp.
- Finding *Treponema pallidum*,*Haemophilus ducreyi*, and the L1,L2, and L3 strain types of *C. trachomatis* which cause sexually transmitted lymphogranuloma, and the

Herpes simplex virus (HSV) types 1 and 2 , which are responsible forrectal, genital and pharyngeal ulcers(7)

9. **CBNAAT/TrueNAT:** These are also nucleic acid amplification methods and take a few hours. They are used mostly in diagnosis and analyzing multi-drug resistance in pulmonary Tuberculosis. The full form of CBNAAT is Cartridge-based Nucleic acid amplification, and it takes about 3 hours for final results. True NAT has been developed in India, and operated with the help of batteries. The Truenat™ (marketed by Molbio Diagnostics, based in Goa, India) testing system employs systems that are portable and operated by battery to rapidly identify bacteria in the *Mycobacterium tuberculosis* complex (or MTBC) . It can also detect resistance against Rifampicin. This assay uses two major devices: (a)the Trueprep® AUTO v2 Universal Cartridge-based Sample Prep Device for automated extraction as well as purification of the DNA, and (b)the True lab® Real Time micro PCR Analyzer to carry out the real-time polymerase chain reaction (or RT-PCR) assay *per se*. By this one can semi-quantitatively detect the MTBC. The system uses reagents which are stable at room temperature(called Trueprep™ AUTO Sample Pre-treatment and Prep kits) and also chips for Truenat™ micro PCR. Hence the need of electricity and air-conditioning is abrogated, and it is a portable device also.

Figure 1: CBNAAT assembly (image credit: Dr A. Sarfraz, AIIMS Patna)

10. **Biofire Film Array:** With its own integrated sample preparation, amplification, detection, and analysis steps, the BIOFIRE System employs basically multiplex PCR technique to simultaneously look for a comprehensive set of targets in about an hour (8).The new BioFire Film Array Meningitis/Encephalitis panel (marketed by bioMerieux), for example, has been cleared by FDA, and it is a multiplex PCR assay. It can detect 14 different pathogens from any CSF (Cerebrospinal fluid)specimen within 1 hour.

11. **Species-specific detection by DNA Microarray:** Microarray is a very common modern technique useful in medical Microbiology. The principle behind microarrays is that complementary sequences will bind to each other.

    Here, the unknown molecules of DNA are split into smaller pieces with the help of restriction endonuclease enzymes. Then, fluorescence-emitting markers would be bound to these DNA fragments. They are there after permitted to react with the probes of the chip of DNA. Following this, the target fragments of DNA along with the complementary sequences will stick to the DNA probes. The leftover DNA fragments will be washed off. The pieces of DNA targeted, can thereafter be identified with the help of their pattern of fluorescence emission after passage of a laser beam. The specific pattern of fluorescence emission can be noted by a computer, and hence helps identify the DNA (9).

12. **Rapid Elisa:** It is useful for detecting *Clostridioides difficile*. This rapid enzyme immunoassay (EIA) test has been in vogue more often in the laboratories, owing to its faster turnaround time coupled withease of operability. The EIA assayshave variable sensitivity (ranging from 50%-99%) with a specificity of 70%-100%, which also depends on the procedure adopted and the standard reference used.

13. **ICT or Immunochromatographic tests:-** Theses new ICT or lateral flow assays are now widely used for rapid and accurate diagnosis of many infections, like Typhoid (Typhidot assay) and Malaria (many commercial kits). Using ICT for malaria, one can detect specific antigens of individual species of Malaria parasite like Lactate dehydrogenase and HPR-2 (Histidine rich protein 2). Thus one may avoid the need of microscopy for parasitological diagnosismany a times, though ICT for *P. falciparum* is often falsely and persistently positive. For malaria, currently, however, there is no ICT for *Plasmosidium knowlesi*, and results cross-match with *Plasmodium falciparum* or *P. malariae*. ICT is available to detect qualitatively the enzymes glutamate dehydrogenase (or GDH) and toxins A and B (QAB) of *Clostridioides difficile* in stool, which is called the CDIFF Quik Chek Complete assay. It has got a considerable negative predictive value but simultaneously low positive likelihood ratio (PLR).

    ICT is now also available for detecting COVID in nasopharyngeal swabs with reasonably good sensitivity and specificity, except possibly the newer SARS-CoV2 variants.

14. **Artificial intelligence (AI) in diagnostic Microbiology:** AI can also be used in modern diagnostic medical Microbiology. AI assays like Machine-based learning algorithms, neural networking and deep learning methodologies may be able to analyze large amounts

of data from many sources in order to identify specific patterns and thereafter detect the presence of a specific pathogen in a specimen.

II. MOLECULAR TYPING

It is often done to assess the relatedness between isolates.

A few such techniques have been listed below.

1. **PFGE:** PFGE or pulsed field gel electrophoresis is used to assess the similarity between different isolates of bacteria like *Staphylococcus aureus*. It is used to study the relationship between different strains of the same species of microorganisms. Large DNA fragments can be separated after digestion with unique restriction enzymes(10).In PFGE, a particular fingerprint, which is also called pulsotype of pieces of DNA is produced on a gel. Comparison is then performed with a database. Extent of that database can vary, which also depends on the species of bacteria, so as to identify the isolate of bacteria (11).

2. **MLST:** In MLST or Multi Locus sequence typing, a number of housekeeping genes are sequenced in part(12). MLST has been applied for some time for studying many different bacteria and eukaryotic microorganisms for epidemiological analysis and surveillance of a number of pathogenic microorganisms. It can also be used to investigate their population structure and evolution.

3. **BLAST:-**The full form of BLAST is basic local alignment search tool. It can detect regions of similar sequences between various biological sequences.This assay measures similarity and differences different nucleotide or protein sequences and then finds out the statistically significant matches. BLAST may also be utilized to deduce the functional as well as evolutionary relationship between different sequences. It can also help in identifying members of the gene families(13).

III. DISCUSSION

Many new methods and assays of are coming up in the field of diagnostic microbiology. Clinicians and laboratory scientists need to keep themselves updated continuously of these new assays. These new rapid techniques are quite sensitive and specific, can save time and effort but often need expertise. With time more new information is likely to emerge from these novel techniques.

IV. CONCLUDING REMARKS

New tests are the need of the hour in diagnostic microbiology. Laboratory scientists need to understand which test to apply for which pathogen, where and when.

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