Futuristic Trends in Medical Sciences

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**Futuristic trends in Diagnostic and Molecular Microbiology**

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**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. These will be elaborated in the 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.

**Keywords**:- PCR, Microarray, MALDI-TOF, CBNAAT.

1. **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.
2. **Chromogenic culture media:-** These media have been employed for detecting bacteria like MRSA (Methicillin resistant *Staphylococcus aureus*),  group B streptococcus, Enterococcus and 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 microorganism of concern. This chemical reaction forms a product that is coloured. It thus therefore leaves a colour for the entire colony as it grows on this medium. ChromID (produced by bioMerieux, Marcy l'Etoile, France) is a selective medium which is chromogenic and designed to culture Staphylococcus aureus. It targets the alpha-glucosidase enzyme of 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. CHROMagar MRSA (produced by CHROMagar Microbiology, Paris, France and BD Diagnostics, Erembodegem, Belgium) also contains cefoxitin. However, it demonstrates a different chromogenic reaction, that produces colonies of MRSA which are rose to mauve in colour.
3. **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 20 commonly seen biochemical traits of members of erstwhile family Enterobacteriaceae).
4. **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.
5. **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. *Escherichia* *coli* is employed commonly as positive control since it breaks down most sugars.

1. **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 system as compared to 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.
2. **MALDI TOF MS**:- MALDI-TOF MS stands for Matrix Assisted Laser Desorption Ionization-Time of flight Mass spectrometry. It is a very useful and rapid modern technique for identifying bacteria as well as fungi from pure colonies. When run in batches, it saves cost also. The MALDI-TOF MS is now the most promising and upcoming technology in diagnostic medical microbiology laboratories. This can be attributed due to its unique prowess to analyse whole cells of bacteria. There is practically no need of sample preparation or batching here. The time to identify a positive culture can be improved ( which is about 10 – 20 seconds for acquisition of the protein spectra and then 15 – 30 seconds for the comparison in the databank), when beginning from a single colony(2). Biomass of the microbes can be utilized for MALDI-TOF MS study just as the initial faint or hazy growth becomes prominently visible on solid media. This assay hence relies on protein analysis. Within a short period of time, MALDI-TOF MS system has almost fully replaced extant conventional biochemical identification systems of pathogenic bacteria in many advanced Government and 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 breaks down the microbial proteins, mainly the ribosomal ones. The data thereby acquired is utilized for identifying the bacterial or fungal pathogens. The main plus-points of this technique are its rapidity , literally in a matter of minutes, and also the cost effectiveness of single assays, together 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 embedded in an acidic matrix material on a metallic plate. Then Nitrogen laser excitation is used for catalyzing the charge transfer from the matrix on to the analyte for desorption. The ions are then separated based on their m/z (mass divided by charge number) ratio. After that,a mass analyzer is employed for detecting and creating a spectral profile. Newer directions for MALDI-TOF-MS are:- antimicrobial susceptibility testing, study of microbial virulence factors, and assay of glycans(4).

Before the direct identification of pathogens with the help of MALDI-TOF MS from positive blood culture bottles , 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.

1. **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 of next-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 , *Pseudomonas* *aeruginosa* 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.
2. **PCR**:- PCR or Polymerase Chain reaction was discovered some decades ago by Kary Mullis. It is used for definitive identification of bacteria from clinical specimens as well as from colonies. It depends on the amplification of the target gene. Traditional or conventional PCR assay, which in the past often consumed days to be accomplished, has often been replaced by quicker and more user-friendly methods like thermal cycler, rapid cycle or real-time PCR. These assays can generally be carried out in a closed system. In real time PCR, both amplification and detection pathways can quickly be carried out in 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 detect one or several genes belonging to the same microbe (like detecting *Clostridium difficile* or norovirus), and (b) assays which 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 detecting CDAD (*Clostridioides* *difficile*-associated diarrhoea) has been okayed by the FDA, and this is coined as the BD GeneOhm Cdiff Assay ( marketed by BD GeneOhm, San Diego, CA, 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, in particular the real-time polymerase chain reaction (or RT-PCR) methods, like the multiplex PCR techniques permit several microbes involved in a given infection to be detected. They thus fulfil the needs for establishing rapid microbial diagnosis for several STIs to be met. These applications are (i) detection of *C. trachomatis*, *Mycoplasma genitalium* *, Neisseria gonorrhoeae* and *Ureaplasma* spp. in patients having proctitis, cervicitis, urethritis and pelvic inflammatory disease or PID; (ii) detecting *T. vaginalis* and *Candida* spp. in patients having vaginitis or diagnosing bacterial vaginitis by techniques which are able to measure the presence or absence of  *Lactobacillus* spp.and *G. vaginalis* , *Mycoplasma hominis*, *Atopobium vaginae* and *Mobiluncus* spp. (iii) detecting *Treponema pallidum*, the L1-L2-L3 strains of *C. trachomatis* which cause venereal lymphogranuloma, *Haemophilus ducreyi*, and the Herpes simplex 1 and 2 virus, which produce genital, rectal and pharyngeal ulcers(7).

1. **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. TrueNAT has been developed in India, and operated with the help of batteries. The Truenat™ (marketed by Molbio Diagnostics, Goa, India) testing system employs portable systems operated by battery to rapidly detect *Mycobacterium tuberculosis* complex bacteria (or MTBC) and also Rifampicin resistance. This system uses two major devices: (a)the Trueprep® AUTO v2 Universal Cartridge based Sample Prep Device for the automated extraction and purification of DNA, and (b)the Truelab® Real Time micro PCR Analyzer to perform the real-time polymerase chain reaction (PCR) assay *per se*. This results in the semi-quantitative detection of 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.



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

1. **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 BioFire FilmArray Meningitis/Encephalitis panel (which is marketed by bioMerieux), for example, is an FDA-cleared, multiplex PCR assay. It can detect 14 different pathogens from any CSF (Cerebrospinal fluid) specimen within 1 hour.

**k. 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 DNA molecules are cut into smaller fragments by the help of restriction endonuclease enzymes. Then, fluorescent markers are bound to these DNA fragments. They are thereafter allowed to react with probes of the DNA chip. Following this, the target DNA fragments together with the complementary sequences will bind to the DNA probes. The remaining DNA fragments will be washed away. The target pieces of DNA can thereafter be identified by their fluorescence emission pattern after passing a laser beam. A computer can be used to note the specific pattern of fluorescence emission and hence the identification of DNA (9).

**l. RAPID ELISA:-** It is useful for detecting *Clostridioides difficile*. This rapid enzyme immunoassay (EIA) test has been used more commonly in the laboratories, owing to its quicker turnaround time and also ease of operability. The EIA tests have variable sensitivity (ranging from 50%-99%) and specificity (70%-100%), which also depends on the study and the reference standard.

**m. 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 diagnosis many 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.

**n. Artificial intelligence (AI) in diagnostic Microbiology**:-

 AI can also be used in modern diagnostic medical Microbiology. AI assays like Machine learning algorithms, neural networks and deep learning techniques are 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.

**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 DNA fragments is produced on a gel. It is then compared to a database. The extent of that database can be variable, depending 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 is basic local alignment search tool. It can detect regions of similarity between various biological sequences. This assay compares different nucleotide or protein sequences and then finds out the statistical significance of the found matches. BLAST can 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).
4. **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.

1. **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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