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 decade. Now advanced techniques are available in the armamentarium of the laboratory scientist, which save time and give sensitive and specific results. Most of these advanced techniques area 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 are 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 sera or by tissue culture of genetic methods like PCR. In the modern era, however, identification of microbes has undergone a paradigm shift, and automation is being increasingly used for their identification. Such new techniques will be enumerated below.
2. **Chromogenic culture media:-** These have been used for the detection of bacteria like MRSA,  group B streptococcus, Enterococcus and Candida species. These selective media have one or more proprietary colorless chromogenic substrates which are broken down by enzymes produced by the microorganism of interest. This chemical reaction results in a product that is coloured, which thereafter imparts a colour to the entire colony as it grows on the medium. ChromID (bioMerieux, Marcy l'Etoile, France) is a selective chromogenic medium meant for S aureus , which targets the alpha-glucosidase enzyme and incorporates cefoxitin (at concentration of 4 mg/L). The growth of MSSA (Methicillin susceptible *Staphylococcus aureus*) is inhibited by the cefoxitin, whereas MRSA (Methicillin resistant *Staphylococcus aureus*) grows as green-colored colonies. CHROMagar MRSA (CHROMagar Microbiology, Paris, France; BD Diagnostics, Erembodegem, Belgium) also contains cefoxitin but shows a different chromogenic reaction, which yields rose to mauve colonies of MRSA.
3. **API/API-20:**  API test strip (or analytical profile index) is a smaller and standardized battery of biochemical tests It is used with complete identification databases, and the best known is the API 20E (named after the 20 common biochemical characteristics of erstwhile family Enterobacteriaceae).
4. **VITEK and VITEK-2**:- VITEK 2MS is also there. It also rapidly identifies bacteria by using cards and performs their susceptibility also. Results are matched with computer-generated databases and identification is confirmed.
5. **RCUT or Rapid carbohydrate utilization test:-**

 It is a rapid test which detects fermentation of sugars in buffer solution with indicator, within 1 or 2 hour by *Neisseria* spp. It is done commonly on a ELISA plate with a control well.

1. **BACTEC and BAC-T Alert:-** Both these systems have been used 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 detection and good bacterial recovery rate was seen in the BacT/ALERT® VIRTUO system when compared to other similar methods(1). BACTEC was modified to be known qs MGIT-960 (Mycobacterial growth indicator tube) which uses liquid media with several supplements. The principle is consumption of Oxygen and production of signal.
2. **MALDI TOF MS**:- The full name of MALDI-TOF MS is Matrix Assisted Laser Desorption Ionization-Time of flight Mass spectrometry. It is a very useful and rapid technique for identifying bacteria and fungi from colonies. When run in batches, it saves cost also. The MALDI-TOF MS is indeed the most promising technology for the present and future in microbiology laboratories. This is due to its prowess to analyse whole bacterial cells with practically no need of sample preparation or batching. The identification time of a positive culture can be improved ( about 10 – 20 seconds for acquisition of the protein spectra and 15 – 30 seconds for the comparison in the databank), starting from one colony(2). Microbial biomass can be utilized for MALDI-TOF MS as soon as the first faint or haze-like growth becomes prominent on solid media. It relies on protein analysis. Within a short span of time, MALDI-TOF MS system has almost completely substituted conventional biochemical identification schemes of pathogens in many laboratories. MALDI-TOF MS is well on the way to being used for antibiotic susceptibility testing also. First proposed in 1970s, the renowned German biophysicists Franz Hillenkamp and Michael Karas later developed the technique of MALDI-TOF MS, and rendered it suitable for routine usage(3). MALDI-TOF MS breaks down microbial, proteins, mainly ribosomal ones, and the data thence acquired is utilized to identify bacterial and fungi. The main plus-points of the technique are its extreme rapidity , literally in a matter of minutes, and the cost efficiency of individual assays, combined with high specificity. However, one 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 pathogens from whole blood also. In MALDI-TOF, the analyte is embedded in an acidic matrix material on a metal plate. Then nitrogen laser excitation is used to catalyze the charge transfer from the matrix to the analyte for desorption. Ions are separated based on their m/z ratio, and thereafter a mass analyzer is used for detecting and creating a spectral profile. Future directions for MALDI-TOF-MS are: antimicrobial susceptibility testing, microbial virulence assay, and glycans(4).

Before direct pathogen identification by means of MALDI-TOF MS from positive blood culture bottles , however, sample preparation has to be done using procedures like lysis–centrifugation , and then ethanol/formic acid extraction. However, this process is not universally accepted due to costs , not very high isolation rate and the complexity of the procedure.

1. **WGS (whole genome sequencing**) :- It is also a useful and rapid method to study and identify bacteria definitively. 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 next-generation sequencing(NGS) instrumentations have reduced the costs. Whole genome sequencing is now accessible as well as affordable. NGS is now useful in public health surveillance, and also utilised to track the source and spread of healthcare-associated infections caused by Staphylococcus aureus , Pseudomonas aeruginosa , Acinetobacter baumannii and Enterococcus faecium in order to guide infection prevention and control in hospitals(5). NGS offers rapid bacterial identification and can also differentiate between clones.
2. **PCR**:- PCR or Polymerase Chain reaction was discovered by Kary Mullis. It is used for definite identification of bacteria from samples as well as colonies. It depends on target gene amplification. Traditional PCR, which in the past often took days to carry out, has often been replaced by faster and more user-friendly methods like thermal cycler, rapid cycle or real-time PCR. These assays are carried out in a closed system, in which both amplification and detection pathways take place within the same reaction vessel(6).

 For diarrhoea PCR can also be used for diagnosis. Molecular techniques have been introduced into the routine diagnosis of diarrhoea in many microbiology laboratories across the world. Generally, these techniques can be classified into two groups: (a) using PCR to detect one or several genes from the same microbe (like detecting *Clostridium difficile* or norovirus), and (b) using multiplex PCR to find out gastroenteritis-causing bacteria, viruses and parasites which occur together(7). Recently, the FDA has approved a real-time PCR assay for detection of CDAD (Clostridioides difficile-associated diarrhoea),termed the the BD GeneOhm Cdiff Assay (BD GeneOhm, San Diego, CA, USA). It amplifies a highly conserved region of the tcdB gene of the bacterium.

 PCR has also been used successfully for diagnosis of Sexually transmitted infections (STI) also. NAATs, particularly the real-time polymerase chain reaction (rt-PCR) methods, like the multiplex PCR techniques which permit several microbes involved in a given infection to be detected, are fulfilling the needs for a rapid microbiological diagnosis for several STIs to be met. These applications are (i) detecting *Neisseria gonorrhoeae*, *C. trachomatis*, *Mycoplasma genitalium* and *Ureaplasma* spp. in patients with urethritis, proctitis, cervicitis and pelvic inflammatory disease[25](https://www.elsevier.es/en-revista-enfermedades-infecciosas-microbiologia-clinica-english-428-articulo-methods-rapid-diagnosis-in-clinical-S2529993X17300163#bib0355); (ii) detecting *T. vaginalis* and *Candida* spp. in patients with vaginitis or diagnosing bacterial vaginitis by techniques which can measure the presence or absence of *G. vaginalis* and *Lactobacillus* spp. ,*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. TrueNAT has been developed in India, and operated with the help of batteries. The Truenat™ (Molbio Diagnostics, Goa, India) testing system uses portable, battery-operated systems to quickly detect *Mycobacterium tuberculosis* complex bacteria (MTBC) and rifampicin resistance. The system uses two main devices: the Trueprep® AUTO v2 Universal Cartridge based Sample Prep Device for the automated extraction and purification of DNA, and the Truelab® Real Time micro PCR Analyzer to perform real-time polymerase chain reaction (PCR). This results in the semi-quantitative detection of MTBC. The system uses room temperature stable reagents ( called Trueprep™ AUTO Sample Pre-treatment and Prep kits) and Truenat™ micro PCR chips. 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 multiplex PCR technique to simultaneously test for a comprehensive group of targets in about an hour.8). The BioFire FilmArray Meningitis/Encephalitis panel (marketed by bioMerieux) is an FDA-cleared, multiplex PCR assay which can detect 14 different pathogens from CSF (Cerebrospinal fluid) specimen in 1 hour.

**k. Species-specific detection by DNA Microarray:-**

The principle behind microarrays is that complementary sequences will bind to each other.

Here, the unknown DNA molecules are cut into fragments by restriction endonucleases. Then, fluorescent markers are attached to these DNA fragments. These are then allowed to react with probes of the DNA chip. Then the target DNA fragments along with complementary sequences will bind to the DNA probes. The remaining DNA fragments get washed away. The target DNA pieces can thereafter be identified by their fluorescence emission by passing a laser beam. A computer is used to record the pattern of fluorescence emission and DNA identification(9).

**l.RAPID ELISA:-** It is useful for detecting *Clostridioides difficile*. This rapid enzyme immunoassay (EIA) testing has been employed more commonly in clinical laboratories, owing to its faster turnaround time and ease of operability. The EIA tests vary widely in sensitivity (50%-99%) and specificity (70%-100%), depending 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 kits). Using ICT for malaria, we can detect specific antigens of individual species of Malaria parasite, and thereby one may preclude the need of microscopy for 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 glutamate dehydrogenase (GDH) and toxins A and B (QAB) of Clostridium difficile in stool, called the CDIFF Quik Chek Complete assay. It has got a high negative predictive value but low positive likelihood ratio (PLR).

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

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

 AI assays like Machine learning algorithms, deep learning techniques and neural networks are able to analyze large amounts of data from many sources in order to identify specific patterns and thereafter detect the presence of a 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 similarity between different isolates of bacteria like *Staphylococcus aureus*. It is used to study the relationship between different strains of same species. Large DNA fragments are separated after digestion with unique restriction enzymes(10). In PFGE, a fingerprint or pulsotype of DNA fragments is produced on a gel and compared to a database. The extent of that database can vary more or less, depending on the bacterial species, so as to identify the bacterial isolate(11).
2. **MLST**:- Here in Multi Locus sequence typing, a number of housekeeping genes are sequenced in part(12). MLST has since been applied for a number of different bacteria and eukaryotic microorganisms as a tool for epidemiological analysis and surveillance of pathogens as well as to investigate their population structure and evolution.
3. **BLAST:-** It is basic local alignment search tool. It finds regions of similarity between biological sequences. The program compares the nucleotide or protein sequences and calculates the statistical significance of the matches. BLAST can be utilized to deduce the functional and evolutionary relationships between sequences , and also help identify members of the gene families(13).
4. **Discussion:-**

Many new methods and assays of are coming up in diagnostic microbiology, and clinicians and laboratory scientists need to keep themselves updated continuously of these new assays. These new rapid techniques are quite sensitive and specific, 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, where and when.

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