OPEN ACCESS Freely available online
http://www.banglajol.info/index.php/BJID/index
Original Article
Bangladesh Journal of Infectious Diseases
December 2015, Volume 2, Number 2
ISSN (online) 2411-670X
ISSN (Print) 2411-4820

Diagnostic Microbiology: Present Status and Future Prospect

Shahanara Begum[1], Md. Abdullah Yusuf[2], Bhuiyan Mohammad Mahtab Uddin[3]

[1]Associate Professor, Department of Microbiology, Green Life Medical College, Dhaka, Bangladesh; [2]Assistant Professor, Department of Microbiology, National Institute of Neurosciences & Hospital, Dhaka, Bangladesh; [3]Assistant Professor, Department of Microbiology, Enam Medical College, Dhaka, Bangladesh

[Received: **1 January 2015**; Accepted: **15 July2015**; Published: **1 December 2015]**

Abstract

Diagnostic Microbiology is the tool that makes it possible to identify the exact pathogens of infectious diseases and the most optimal therapy at the level of individual patients. Conventional methods require time to grow the microbes in vitro under specific conditions and not all microbes can easily be cultured. This is followed by biochemical methods for identification which further makes the process lengthy. Transport of the specimens under less than ideal conditions, prior use of antibiotics and small number of organisms are among the factors that render culture-based methods less reliable. Newer methods depend on amplification of nucleic acids followed by use of probes for identification. This mitigates the need for higher microbial load, presence of metabolically active viable organisms and shortens the time. These methods can be used to detect antibiotic resistance genes directly from the specimen and help direct targeted therapy with efficacy. Since these methods will not fulfill all the diagnostic needs, a second approach is being used to shorten the time to identification after the organism has already grown. Mass spectrometry and bioinformatics are the tools making this possible. This review gives a historical perspective on diagnostic microbiology, discusses the pitfalls of current methodology and provides an overview of newer and future methods. [Bangladesh Journal of Infectious Disease 2015;2(2):42-47]

Keywords: Diagnosis; laboratory; molecular; rapid

[**How to Cite this article:** Begum S, Yusuf MA, Uddin BMM. Diagnostic Microbiology: Present Status and Future Prospect. Bangladesh J Infect Dis 2015;2(2):42-47]
Corresponding author: Dr. Shahanara Begum, Associate Professor, Department of Microbiology, Green Life Medical College, Green Road, Dhaka, Bangladesh**; Email:** shahanara210@gmail.com; **Cell no.:** +8801713228347
Conflict of Interest: Authors have declared no conflict of interest.
Contributions to authors: SB has contributed from literatures searches up to manuscript writing. MAY & BMMU have revised the manuscript.

Introduction

As the germ theory of disease became established and accepted, ways were sought to turn theory into practice by studying the microbes outside the body and in turn use that information for diagnosis and treatment of human and animal disease. The industrial and scientific revolution in the late 1800's led to wide use of microscopes to visualize microbes directly, to their classification into broad categories and to their cultivation in vitro. Once the methodologies for in vitro cultivation were standardized, it became necessary to connect the bench to the bedside. The pioneering work of Robert Koch resulted in Koch's postulates 1884. These became the cornerstone of clinical/diagnostic microbiology. In vitro cultivation of bacteria allowed for the testing of antimicrobial agents to treat infectious disease which for the first time in the history of medicine involved the concept of targeted therapy. Penicillin was noted as the most significant medical discovery of the millennium (1900-2000).

As the spectrum of microbes found to cause disease increased, it became clear that many of them have very specific growth requirements and some of the well-known pathogen have still not been cultivated in vitro, and therefore, do not fulfill the first two Koch's postulates. The prime examples include Treponema pallidum and Mycobacterium leprae. It is important to remember that Armauer Hansen discovered Mycobacterium leprae in 1873 before Koch's discovery of M. tuberculosis, but the inability to cultivate the leprosy bacteria in vitro allowed the credit of the germ theory to go to Koch[1].

Even with cultivable bacteria cultures fail to reveal an organism in 80% of patients with signs and symptoms consistent with an infectious process. There are multiple reasons for this under-detection including recent or current antimicrobial therapy, bacteria within biofilm especially in chronic tissue infections and device-related infections and slow growing or fastidious organisms for which necessary nutrients or cofactors for growth are not known. Even when the organism grows, it's simply not practical to fulfill Koch's postulates to distinguish 'false positive' from 'true positive' cultures. Both of these serious drawbacks have led to extensive empiric antibiotic use resulting in appearance of antibiotic response worldwide. This has been a significant collateral damage associated with rapid evolution of diagnostic microbiology and antimicrobial therapy in the past five decades. As with all good things, the pipeline of newer antimicrobial agents to treat resistant organisms created by the earlier therapies has become stagnant. Since antimicrobial agents in general are relatively safe drugs, this overuse has continued with worsening of resistance patterns and its global spread. It has become clear that even if more antimicrobial agents became available, bacteria will find ways to become resistant.

Conventional Microbiology

The evolution of the study of microbial genetics has led to new frontiers to recognize pathogenic microbes' in-situ within the host and in vitro without cultivation of the organism. These technologies can be applied to the recognition of the organism, its DNA/RNA, its antigenic components, specific virulence genes and antibiotic resistance genes. This has led to the proposal for molecular Koch's postulates. The development of nucleic acid-based amplification technologies (NAATs) including polymerase chain reaction (PCR) has enabled detection of microbial and host genetic sequences with high sensitivity and specificity[3]. *Tropheryma whipplei* and Hepatitis C virus are examples of uncultivable organisms first detected through molecular methods. In addition to detection and quantification of many bacterial, viral and fungal pathogens they have revolutionized the development of drugs for treatment of HIV, HBV and HCV infections. The highly significant collateral advantages are their utilization in guiding long term patient management and in public health surveillance.

NAATs involve extraction of the nucleic acid from the pathogen, amplification of the genetic target followed by detection of amplified sequences. The earlier labour intensive nucleic acid isolation and purification methods have now been replaced by automated platforms or are incorporated into single step cartridge-based devices[4]. Newer methods include loop-mediated amplification (LAMP), transcription mediated amplification, nucleic acid sequence-based amplification (NASBA) and strand displacement amplification (SDA). The LAMP, NASBA and SDA are isothermal and do not require expensive thermo cyclers. Turbidity, Chemiluminescence and solid or liquid bead-based microassays are used to detect amplified targets. Nucleic acids can also be detected directly using signal amplification methods such as branched DNA and probe amplification methods. Real-time nucleic acid detection relies on the use of fluorometric probes; for example, molecular beacons,

fluorescent energy transfer, TaqMan, scorpios and locked nucleic acids[5].

Sequencing

Molecular finger printing technologies such as pulsed-field gel electrophoresis (PFGE) and multiple locus sequence typing (MLST) were used to study relatedness in outbreak isolates. However, whole-genome sequencing (WGS) has shown that multiple strains with the same PFGE and MLST patterns have significantly different genetic contents and these methods lack the resolution to provide strain specific diagnostics at this point[6]. Ultrasequencing or pyro-sequencing has demonstrated multiple strains of HIV-1 within a single individual and identification of minority mutants in the quasi-species. Whole genome sequencing as a diagnostic tool is based on the fact that the vast majority of bacterial pathogens possess a supra-genome that is much larger than the genome of individual strains[7]. This species supra-genome contains three different sets of genes; (i) the core genes that are found in every strain of the species, (ii) the distributed genes found in a subset of strains in the species and (iii) the unique genes found in only a single strain of the species. The significant ramifications of this genomic plasticity on diagnostics include; (a) association of different genes and gene combinations with different disease phenotypes, (b) microbial populations in most chronic infections are polyclonal and/or polymicrobial and grow in a biofilm mode. Biofilm formation provides a metabolic and physiologic metamorphosis and serves as a population level virulence factor. Large numbers of recombinants created in the process have increased virulence and potential antibiotic resistant genes. WGS gives the microbial profile as it stands in situ and the computational systems that perform the analysis of WGS data allow for rapid turnaround time. WGS has been used for identification from cultured isolates or directly from clinical specimens e.g. fresh-frozen or paraffin embedded tissue. Sequencing can detect pathogens in culture negative species due to prior antibiotic therapy or other causes. Generally, genus identification is provided for >90% and species identification for 65-85% of the isolates tested. Sequencing is also used to demonstrate antibiotic resistance and identify known or new mutations. At this point the approach is restricted to large university and reference laboratories because of the complexity and cost. The next generation sequencing' (NGS) provides increased accuracy and is less labour-intensive like XPRIZE. However, NGS requires extensive bioinformatics for data interpretation. For diagnostic testing, compact NGS systems with a small footprint and fast turnaround time have been developed. Once time and cost prohibitive, bacterial sequencing can now be performed for less than 100 USD in less than one day. These features make NGS highly competitive with any currently available technology including conventional methods of cultivating bacteria in vitro. NGS could potentially replace complex multifaceted conventional microbiological identification procedures for phenotype identification in addition to detecting genetic markers of virulence and antibiotic resistance[8]. The potential of missing new and uncharacterized genetic elements responsible for phenotype resistance will need to be minimalized by performing extensive phenotypic/ genotypic comparisons and gene expression analysis for detecting complex phenotypes. Additionally, for diagnostic utility, NGS system need easy, concise, low cost data management and interpretative software and access to commercially available vetted databases. Sequencing will facilitate tracking nosocomial infections and the spread of successful clones and tracking of epidemics, zoonotic events, bioterrorism attacks and food borne epidemics. The direction of norovirus transmission in linked patients in a children's bone marrow transplantation unit was recently identified using NGS[9]. The ability to identify single base changes or single gene acquisitions associated with novel clinical syndromes is of particular significance in immunocompromized patients. The recent sequence based discovery of Bradyrhizobium enterica, a novel bacteria led to finding the aetiology of 'cord colitis syndrome' seen as a complication of haemopoetic stem cell transplantation[10]. The rapid growth of metagenomic sequencing largely attributed to the decreased cost of DNA sequencing on high throughput next generation sequencing platforms holds great promise for studies of bacterial ecosystems[11]. Single genome sequencing is the latest advance in microbial genome and metagenome sequencing. The term meta-genomics was coined in 1998 and this new field explored the scale of genetic variability within natural microbial populations and associated phage. Meta-genomics required the cloning of environmental DNA into vectors followed by expensive Sanger sequencing and fragment assembly. The pairing of emergent pyro-sequencing technology with meta-genomics marked the beginning of the meta-genomics revolution in microbial ecology. Obviously the power of observational science lies not just in data collection

but in the analysis and experimentation. The data analysis provided by bioinformatics is the key to the anticipated success of microbial genomics as a science and as a diagnostic modality. Computational subtraction of human host sequences to identify microbial sequences was first implemented as Amazon EC2 (Elastic Compute Cloud) environment in the form of Pathseq software[12]. Rapid identification of non-human sequences (RINS) is an alternative open source workflow available on desktop computers. A platform called CAPSID to store and visualizes the identified non-human sequences were described recently. READSCAN is a fast and highly scalable and efficient tool to analyze ultra-high volume of data produced by the latest sequences like Illumina Hiseq that can produce 3 to 6 billion short reads in a single run.

Proteomics

It is unlikely that the conventional diagnostic methodology particularly in vitro cultivation will be given up completely in preference to advancing genome capabilities. Much progress has been made in the past two decades to modify/enhance culture media that would improve the yield and shorten the time to growth. The most significant is the improved culture methods for slow growing organisms especially Mycobacterium tuberculosis. The slow turnaround time for cultures is impacted by the time needed for identification of the growth in addition to time to growth. Mass spectrometry and bioinformatics are revolutionizing the identification of microbial growths with much reduced time requirements[13-14]. Matrix-assisted laser desorption/ionization time-of-flight Mass spectrometry (MALDI-TOF-MS) has application in biochemistry, polymer chemistry and proteomics. The newer and easy to use MLADI-TOF-MS platforms can be used routinely in diagnostic microbiology laboratories for rapid identification of bacteria, fungi, mycobacteria and parasites[15-16]. For most bacteria, testing by MALDI-TOF-MS is relatively simple and only requires transferring a portion of an isolated colony into a well in a designated disposable or reusable target slide. The target slide is loaded into the instrument after the inoculated well is covered with a chemical matrix, generally α-cyano-4 hydroxycinnamic acid and dried. Vacuum is applied to the chamber and laser is used to pulse the target slide well converting the sample into an ionic gas composed of small molecules, small proteins and peptides[17]. The positively charged molecules in the ionization chamber are accelerated through an electric field before they enter the time-of-flight mass analyzer. Smaller particles reach the particle detector faster. The flight times measured by the particle detector are converted into mass/velocity values and plotted on a mass spectrogram. Validation is done by comparison to a library of spectrograms using proprietary algorithms specific to each manufacturer. The generated mass spectrum provides a profile ('fingerprint') of the unknown organism[18]. Use of calibrations and controls ensures appropriate performance of the equipment and the process. It takes about 10 to 20 minutes to get the first result and 1 minute to get each new subsequent result. The colonies growing in culture are inexpensively and accurately identified in minutes without the need for prior knowledge of the organism type. Organisms like mycobacteria and fungi require an additional pretreatment step to ensure non-viability before manipulation and extraction of proteins[19]. The MALDI-TOF-MS systems fulfill a number of essential characteristics for an ideal test. They are also amenable to integration into automated platforms that can inoculated the MALDI-TOF-MS slide and collate the identification with antimicrobial susceptibility results. The cost effectiveness of the system lies in low reagent cost, short technical time and the clinical benefit of providing identification in about 20 units resulting in appropriate antibiotic selection. These offset the initial and the yearly maintenance costs for the equipment. Antimicrobial resistance detection applications of MALDI-TOF-MS are in early stages of development.

So far, preliminary studies show that MALDI-TOF-MS can detect some types of antibiotic resistance mechanism that result in structural modifications of the antibiotics[20]. Upon hydrolysis, beta lactamases cause a mass shift in the size of the original beta lactam which occurs within hours of exposure of the drug to the organism being tested. The ultimate goal is to couple MALDI-TOF-MS rapid identification with rapid susceptibility testing in the diagnostic laboratory. A combination of PCR and T2 magnetic resonance has recently been reported to achieve nanoparticle-mediated rapid detection of candida in whole blood[20]. Using this technology, candida species can be detected directly from as little as 1 ml of whole blood which is the same as the sensitivity range of 1 to 3 colony-forming units (CFU) per ml of blood. This technology circumvents the need for waiting for the blood culture bottle to be flagged as positive resulting in a significant reduction in detection time.

Significance

Diagnostic microbiology is in the process of a much needed shift from conventional methods first used in 1850 to grow bacteria on culture media produced from seaweed (agar) to the molecular methods for rapid pathogen detection and identification. The molecular methods involve both genomics and proteomics in concert with bioinformatics. The FDA has now approved NAATs for multiple viruses and bacteria[21]. The presence of these organisms usually indicates diseases because they are not normally found on human body surfaces or tissues. Many bacteria encountered commonly in clinical practice such as *Staphylococci* and *Escherichia coli* are frequently a part of the commensal flora. It is far more challenging to interpret results showing such bacteria. Either quantitation as in the case of urinary tract infections or recovery from a normally sterile site such as blood is needed to make the detection meaningful. The technology is now being used with blood culture and for blood directly for multiple organism groups. Multiplex screenings for blood-borne viral, bacterial and protozoan parasites using an open array platform have been currently described [22]. The open array platform customized with real-time PCR assays demonstrates a high level of multiplicity with sensitivity and specificity for detection of four viral, two bacterial and three protozoan blood-borne pathogens. Similar multiple real-time PCR assays are being used for detection of bacterial toxins[23]. A number of FDA-approved molecular diagnostic are available for rapid detection of nosocomial pathogens like MRSA, URE and *Clostridium difficile*[24]. Rapid detection of multiple antibiotic resistant genes is another recent addition to the clinical and infection transmission prevention aspects of microbiologic detection.

Inference

On the proteomics side, MALDI-TOF-MS has been used for a few years in European clinical microbiology laboratories. It is being adopted world-wide and is likely to antiquate most biochemical microbial identification in the near future. The current biochemical test panels performed manually or on automated instruments identify more organisms and are less cumbersome than the previously used in-house biochemical tests. However, they have longer turnaround times, use costly consumables and require knowledge of organism type being tested. Broad range PCR followed by sequencing accurately identify bacteria and fungi, but has a long turnaround time in addition to its expense and limited availability. MALDI-TOF-MS is a product of advances in mass spectrometry and bioinformatics. The colonies growing in culture are identified inexpensively, accurately and within minutes[25].

In general the molecular approaches for diagnosis of invasive fungal infections are still in infancy. The need for these tests for detection of fungi and identification of antifungal drug resistance is immense mostly due to an enlarging immunocompromised patient population and their role in infection-associated mortality[26]. A current study compared a standard diagnostic strategy of culture and histology with a ranked biomarker based strategy (aspergillus galactomannan and PCR) for directing use of antifungal therapy of invasive aspergillosis in high risk hematology patients[27]. The study showed that the use of galactomannan and PCR reduced the empiric use of antifungal agents.

Another strategy involving algorithms use of a biomarker glutamate dehydrogenase (GDH); toxin detection by immunoassay; and bacterial gene detection by PCR has been adopted for diagnosis of *Clostridium difficile* infection. Recently, a novel subtyping assay was described for detection of *Clostridium difficile* virulence genes[28].

Conclusion

It is evident from the current body of literature and availability of molecular testing for infectious diseases that a combination of genomics and proteomics is the future of diagnostic microbiology. What remains to be seen is how long it will take to evolve tests that fulfill the last three characteristics that will allow their use in resource-constrained settings that include the developing world but also the under-served inner city and rural areas within developed countries. For use in settings with significant resource limitations, useful diagnostic assays should require little or no electricity, minimal technical skills, high portability and minimal cold chain storage requirement, stability to temperature extremes and power surges and point of care applicability.

References

1. Renault CA, Ernst JD. Mycobacterium leprae. In: Mandell, Douglas and Bennett's Principles and Practice of Infectious

Diseases. 7th ed. Philadelphia: Churchill Livington Elsevier; 2010. p. 3165-76
2. Caliendo MA, Gilbert DN, Ginnochio CC, Hanson KE, May L, Quinn TC, et al.; Infectious Diseases Society of America (IDSA). Better tests, better care: Improved diagnostics for infectious diseases. Clin Infect Dis 2013;57:S139-70
3. Hiller NL, Ahmed A, Powell E, Martin DP, Eutsey R, Earl J, et al. Generation of genetic diversity among Streptococcus pneumoniae strains via horizontal gene transfer during chronic polyclonal pediatric infections. PloS Pathog 2010;6:e1001108
4. Ehrlich GD, Post C. The time is now for gene and genome-based bacterial diagnostics: "You say you want a revolution". JAMA Intern Med 2013;173:1405-6
5. Reuter S, Ellington MJ, Cartwright EJ, Köser CU, Török ME, Gouliouris T, et al. Rapid bacterial whole-genome sequencing to enhance diagnostic and public health microbiology. JAMA Intern Med 2013;173:1397-404
6. Ehrlich GD, Ahmed A, Earl J, Hiller NL, Costerton JW, Stoodley P, et al. The distributed genome hypothesis as a rubric? For understanding evolution in situ during chronic infectious processes. FEMS Immunol Microbiol 2010;59:269-79
7. Ahmed A, Earl J, Retchles A, Hillier SL, Rabe LK, Cherpes TL, et al. Comparative genomic analysis of 17 clinical solates of Gardnerella vaginalis provide evidence of multiple genetically isolates consistent with subspeciation into genovars. J Bacteriol 2013;194:3922-37
8. Hogg JS, Hu F, Janto B, Boissy R, Hayes J, Keefe R, et al. Characterization and modeling of the Haemophilus Influenzae core and supra-genosmics based on the complete genomic sequences of Rd and 12 clinical nontypeable strains. Genome Biol 2007;8:R103
9. Kundu S, Lockwood J, Depledge DP, Chaudhry Y, Aston A, Rao K, et al. Next-generation whole genome sequencing identifies the direction of norovirus transmission in linked patients. Clin Infect Dis 2013;57:407-14
10. Bhatt AS, Freeman SS, Herrera AF, Pedamallu CS, Gevers D, Duke F, et al. Sequence-based discovery of Bradyrhizobium enterica in cord colitis syndrome. N Engl J Med 2013;369:517-28
11. Anderson SG, Goodman AL. Bacterial genomes: Next generation sequencing technologies for studies of bacterial ecosystems. Curr Opin Microbiol 2012;15:603-4
12. Naeem R, Rashid M, Pain A. READSCAN: A fast and scalable pathogen discovery program with accurate genome relative abundance estimation. Bioinformatics 2013;29:391-2
13. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, et al. Ongoing revolution on bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 2009;49:543-51
14. Patel R. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry in clinical microbiology. Clin Infect Dis 2013;57:564-72
15. Muang AM, Newton D, Kunapuli A, Gandhi TN, Washer LL, Isip J, et al. Impact of rapid organism identification via matrix-assisted laser desorption/ionization time-of-flight combined with antimicrobial stewardship team intervention in adult patients with bacteremia and candidemia. Clin Infect Dis 2013;57:1237-45
16. Chen JH, Yam WC, Ngan AH, Fung AM, Woo WL, Yan MK, et al. Advantages of using matrix-assisted laser desorption ionization-time of flight mass spectrometry as a rapid diagnostic tool for identification of yeasts and mycobacteria in the clinical microbiological laboratory. J Clin Microbiol 2013;51:3981-7
17. Bizzini A, Greub G. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clin Microbiol Infect 2010;16:1641-9
18. Welker M. Proteomics for routine identification of microorganisms. Proteomics 2011;11:3143-53
19. Clerc O, Prod'hom G, Vogne C, Bizzini A, Calandra T, Greub G. Impact of matrix-assisted laser desorption ionization time-of-flight mass spectrometry on the clinical management of patients with gram-negative bacteremia: A prospective observational study. Clin Infect Dis 2013;56:1101-7
20. Sparbier K, Schubert S, Weller U, Boogen C, Kostrzewa M. Matrix-assisted laster desorption ionization time-of-flight mass spectrometry-based functional assay for rapid detection of resistance against beta-lactam antibiotics. J Clin Microbiol 2012;50:927-37
21. Neely LA, Audeh M, Phung NA, Min M, Suchocki A, Plourde D, et al. T2 magnetic resonance enables nanoparticle- mediated rapid detection of candidemia in whole blood. Sci Transl Med 2013;5:182ra54
22. Krishna NK, Cunnion KM. Role of molecular diagnostics in the management of infectious disease emergencies. Med Clin North Am 2012;96:1067-78
23. Grigorenko E, Fisher C, Patel S, Chancey C, Rios M, Nakhasi HL, et al. Multiiplex screening for blood-borne viral, bacterial, and protozoan parasites using an OpenArray platform. J Mol Diagn 2014;16:136-44
24. Lefterova MI, Slater KA, Budvytiene I, Dadone PA, Banael N. A sensitive multiplex, real-time PCR assay for prospective detection of shiga toxin-producing escherichia coli from stool samples reveals similar incidences but variable severities of non-O157 and O157 infections in Northern California. J Clin Microbiol 2013;51:3000-5
25. Currie B. Impact of molecular diagnostics on infection control. Infect Dis 2011:11-5
26. Zhang SX. Enhancing molecular approaches for diagnosis of fungal infections. Future Microbiol 2013;8:1599-611
27. Morrissey CO, Chen S, Sorrell TC, Milliken S, Bardy PG, Bradstock KF, et al. Galactomannan and PCR versus culture and histology for directing use of antifungal treatment for invasive aspergillosis in high-risk haematology patients: A randomized controlled trial. Lancet 2013;13:519-27
28. Angione SL, Sarma AA, Novikov A, Seward L, Fieber JH, Mermel LA, et al. A novel subtyping assay for detection of clostridium difficile virulence genes. J Mol Diagn 2014;16:244-52