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Application of Molecular Biology in Clinical Microbiology

Ashish Bahal and Ashwin Dalal

Diagnostics Division, Centre for DNA Fingerprinting and Diagnostics, Hyderabad

Email: ashishmallika@gmail.com

Introduction

During the past 10 to 15 years, there has been extensive growth in the use of molecular biology techniques in the clinical laboratory and their various applications. As a result of this development, many laboratories are able to offer increased sensitivity of testing, faster turnaround times, and ultimately improved patient care.[1] The applications of molecular technology in clinical microbiology are enormous; some of these applications include:

1. Early detection and identification of pathogens from clinical specimens and cultures
2. Classification of micro organisms based on their genetic relatedness (genotyping)
3. Detection of antibiotic resistance and toxin production
4. Detection of fastidious, slow growing or small numbers of pathogens in clinical specimens
5. Finding the host and agent factors conferring susceptibility, protection or virulence.

Conventional methods of pathogen detection

They include microscopy, culture and immunological reactions. Microscopy with Gram staining from the specimen gives the first indication of the suspected organism. Some other techniques are utilized in other cases like hanging drop preparation to look for highly motile bacteria in cases of suspected cholera, Ziehl - Nielsen staining for acid fast bacilli in cases of pulmonary tuberculosis or split skin smears for cases of Hansen's disease. Microscopy is also utilized for rapid detection of parasitic infections like malaria, filaria and intestinal parasites. Culture and biochemical reactions are done subsequently for identification and speciation in cases of cultivable organisms. The time for culture varies from days to weeks; there has been advent of new automated culture and identification systems which have reduced this time to some extent.

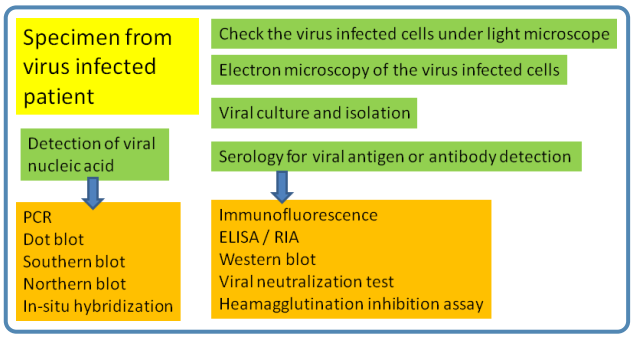

Figure 1 Processing of specimen for virological diagnosis (ELISA - Enzyme linked immunosorbent assay; RIA - Radioimmunoassay).

Microscopy has very limited usage in case of viral infections where serology and molecular techniques are the mainstay of diagnosis. After getting the specimen for a suspected case of viral infection it is further processed according to the schema shown in Figure 1, depending on the type of virus. For viruses such as Rubella and Hepatitis A, the onset of clinical symptoms coincides with the development of antibodies; here the detection of IgM antibody or rising titres of IgG antibody in the serum of the patient would indicate active disease. Some viruses produce clinical disease months or years after seroconversion e.g. human immunodeficiency

virus (HIV) and rabies virus. In the case of these viruses, the mere presence of antibody is sufficient to make a definitive diagnosis.

Viral cultures are done only at specialized centers. Viral culture techniques are less sensitive in some cases due to the low viral burden especially in encephalitis and also the presence of host neutralizing antibodies.

Types of specimen collected for microbiological studies
1. Respiratory tract infections: nasal and bronchial washings, throat and nasal swabs, sputum
2. Eye infections: throat and conjunctival swab/scraping
3. Gastrointestinal tract infections: stool and rectal swabs
4. Vesicular rash: vesicle fluid, skin scrapings
5. Maculopapular rash: throat, stool, and rectal swabs
6. CNS infection: stool, tissue, saliva, brain biopsy, cerebrospinal fluid
7. Genital infections: vesicle fluid or swab
8. Urinary tract infections: urine
9. Blood-borne infections: blood

Table 1 Types of specimen collected for microbiological studies.

Disadvantages of conventional methods

The gold standard in bacteriology largely remains culture, primarily due to cost factors and the complex nature of infections but there could be shortcomings like:

1. Minute quantities of pathogen may not be detected
2. The use of antibiotics before specimen collection reduces chances of pathogen detection
3. Some pathogens are difficult to culture, identify, or less amenable to susceptibility testing with conventional methods.
4. Need for speed – sometimes there is a need for timely intervention but the laboratory results of etiologic diagnosis take time

Similarly there could be limitations in cases of serological tests like:

1. Many viruses often produce clinical disease before the appearance of antibodies such as viruses causing respiratory and diarrhoeal diseases; hence, serology is not useful in these cases
2. Long length of time required for diagnosis for paired acute and convalescent sera
3. Mild local infections such as genital herpes may not produce a detectable antibody response
4. Antigenic cross-reactivity between related pathogens e.g. Herpes simplex virus (HSV) and Varicella zoster virus, Japanese B encephalitis and dengue, may lead to false positive results
5. Immuno-compromised patients often give a reduced or absent antibody response
6. Patients with infectious mononucleosis and those with connective tissue diseases such as SLE may react non-specifically giving a false positive result
7. Patients given blood or blood products may give a false positive result due to the transfer of antibody

Molecular methods for pathogen detection and quantification

There are a large number of molecular techniques which can be used for detection and quantitation of pathogens.[2] Some of these techniques include:

- Nucleic acid amplification: Nucleic acid amplification includes not only polymerase chain reaction (PCR) and its variants, but also alternate technologies, such as strand displacement amplification and transcription-mediated amplification. The technique involves selecting a specific genomic target in the pathogen of interest and designing specific primers for amplification of this target using Taq polymerase. Presence of a band of amplification of a specific size would indicate presence of the organism (Figure 2).

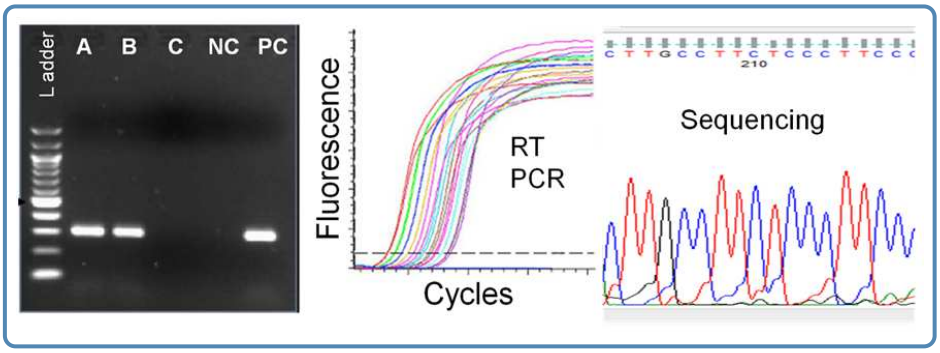

Figure 2 Examples of various molecular techniques used in clinical microbiology (Lanes A and B show presence of target nucleic acid, PC- positive control, NC- negative control).

- Real Time PCR: Real time PCR is mainly used as a method for quantification of viruses. Here the amount of amplification is checked at every cycle of PCR by incorporation of fluorescent dyes in the PCR mix or by designing fluorescent probes. The amount of fluorescence produced at every cycle is directly proportional to the initial quantity of the target nucleic acid (i.e. number of viral particles) (Figure 2).

- Sequencing: Molecular techniques come in handy when traditional phenotypic methods of microbial identification and typing are insufficient or time-consuming. Specific examples include the 16S rRNA sequencing for identification and subtyping of bacteria. 16S rRNA is a component of the 30S small subunit of prokaryotic ribosomes. The gene coding for it is referred to as 16S rDNA and is approximately 1500 nucleotides long. The 16S rDNA gene is highly conserved between different species of bacteria and archaea.[3] Viral genomes are relatively small, so they were among the first organisms to be fully sequenced. The complete DNA sequence of the Epstein-Barr virus was determined as early as 1984.

Applications of molecular techniques in clinical microbiology

The applications of molecular techniques in clinical microbiology depend on the organism being studied. The various applications are detailed below:

- For bacterial diseases: In case of fastidious bacteria like *Mycobacterium tuberculosis, Chlamydia trachomatis, Neisseria gonorrheae* and *Bordetella pertussis*, molecular testing has reduced the time taken by conventional culture to allow early detection and treatment. Although molecular methods have helped mycobacteriology, it is important to note that conventional culture still remains more sensitive. Despite this limitation, molecular detection of *M. tuberculosis* allows confirmation of acid-fast bacilli (AFB) seen on microscopy with up to 98% sensitivity within a day compared to approximately four weeks using phenotypic methods.

In the management of sexually transmitted diseases (STDs), traditional screening methods require invasive methods which are less acceptable as they cause embarrassment and discomfort, thus reducing compliance. Molecular methods offer more convenience and acceptance, enhancing the compliance. This has indirectly led to increase in laboratory confirmed cases of STDs. Molecular methods have the advantage of being performed on dry swabs with little degradation of the DNA (DNA is stable) during transit compared to the difficulties in maintaining viability during transport, which make them very useful for samples collected from remote and rural areas. In addition, molecular methods can detect multiple pathogens such as *C. trachomatis, N. gonorrhoeae, Haemophilus ducreyi* and the genital mycoplasma from the same swab.

Some bacteria can only be detected by molecular means as culture is either difficult or represents a significant occupational risk to the laboratory personnel. Examples include Whipple's disease due to *Tropheryma whipplei*, cat scratch disease due to *Bartonella henselae*, Q fever due to *Coxiella burnetii*, and male urethritis due to *Mycoplasma genitalium*. Molecular methods have the advantage here. In case of meningococcal disease, detection

can be done on the same day from specimens arising from sterile sites. Similarly, multiplex PCR methods have been developed for detection of other common bacterial causes of meningitis like *Streptococcus pneumoniae* and *Haemophilus influenza* type B.[4]

• For mycology and parasitology: Molecular testing can be helpful in certain circumstances. The diagnosis of *Pneumocystis jiroveci* pneumonia in immunosuppressed patients is limited to microscopy of respiratory tract specimens. Immunofluorescence is more sensitive than microscopy but is more expensive and needs specialized facilities. PCR can be useful but the specificity of PCR is limited because this organism is a ubiquitous commensal and can be detected in the absence of pneumonia. Another example is the use of 18S rRNA gene PCR to detect *Aspergillus* species infection in neutropenic patients.

In parasitological diagnosis, *Toxoplasma gondii* can be detected by PCR from amniocentesis fluid to confirm fetal infection and from cerebrospinal fluid (CSF) to diagnose toxoplasma encephalitis. Microscopy remains the mainstay of malaria diagnosis but *Plasmodium* species PCR, because of its superior sensitivity compared to microscopy, can diagnose malaria in patients whose thick and thin blood films test negative due to administration of chemoprophylaxis or partial immunity. *Plasmodium* species PCR can also detect mixed infections that can be difficult to detect microscopically.

• For viral diseases: a. Meningitis and encephalitis – The diagnosis of HSV encephalitis previously required brain biopsy in certain cases due to the low sensitivity of cerebrospinal fluid (CSF) culture and serology. PCR now allows the detection of HSV DNA from CSF with 95% sensitivity.[5] HSV PCR can be multiplexed with other pathogens responsible for meningitis.

b. Blood borne virus infection – Active hepatitis C viral (HCV) infections are diagnosed by the presence of HCV RNA, since the detection of antibody to HCV cannot distinguish between past and present infection. Early HIV infection and vertical transmission of HIV infection can be detected by the presence of HIV pro-viral DNA. Some blood banking services screen pooled samples from all donations for HIV and HCV using transcription mediated amplification assays, reducing the window period from 22 and 66 days to 9 and 7 days respectively.[6]

c. Genital /intrauterine infections - Cytomegalovirus (CMV), rubella, varicella zoster virus, and genital ulceration due to HSV type 2 infection are now routinely being detected by PCR.

d. Respiratory viral pathogens - Molecular detection of respiratory viral pathogens is cost-effective due to avoidance of unnecessary testing and hospitalization. It also helps reduce unnecessary antibiotic use. New assays of multiplex PCR are now available for testing all the common respiratory viruses along with fastidious bacterial causes of pneumonia. Uncommon viruses such as the severe acute respiratory syndrome- associated coronavirus (SARS-CoV) and influenza A/H5N1 (avian influenza) virus can also be incorporated into these tests.

e. Viral diarrhea - Viruses cause more infectious diarrhoea worldwide than bacteria and other pathogens. The method of choice for microbiological diagnosis of rotavirus from stool samples is PCR. Norovirus, responsible for large outbreaks both in the community and health care facilities is amenable for diagnosis by PCR, which is the most sensitive and rapid method. PCR is also the most sensitive method for the diagnosis of astroviruses and enteric adenoviruses.

• For treatment monitoring: Monitoring viral DNA or RNA load has become the standard of care for several chronic viral infections and is an integral component of the management of HIV, HCV and hepatitis B virus (HBV) infections. Measurement of the viral load is performed by real-time PCR. Patients who remain negative for HCV RNA 6 months after completing combination therapy for HCV infection almost always remain free of the virus.

• For sensitivity testing:

Bacterial pathogens: Detection of antibiotic resistance by molecular methods has become routine in many laboratories. This is done by detection of mutations in the organisms which confer resistance. Examples of genes studied include:

1. *mecA* gene in methicillin resistant *Staphylococcus aureus* (MRSA)
2. *vanA / vanB* genes in vancomycin resistant enterococci (VRE)
3. Extended spectrum β-lactamases (ESBL) in *Escherichia coli* and *Klebsiella pneumoniae*

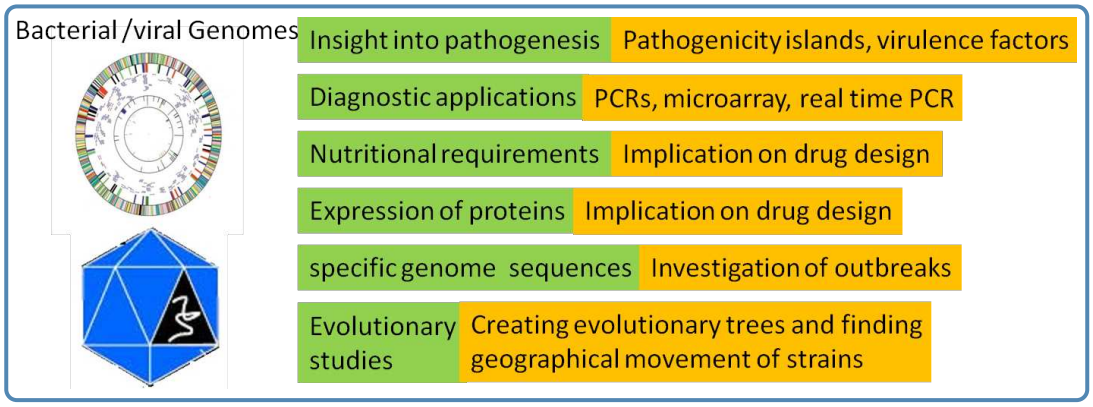

Figure 3 Applications of genome sequencing in bacteria and viruses.

4. Mutations in an 81-bp rifampin resistance-determining region (RRDR) in the *rpoB* gene in *M. tuberculosis*[7]

Summary of uses for molecular methods
1. Identification - nearly all possible pathogens
2. Viral load monitoring - Cytomegalovirus, Epstein-Barr virus, Hepatitis B, Hepatitis C, HIV
3. Viral genotyping - HIV, Hepatitis B, Hepatitis C, Human papillomavirus
4. Bacterial resistance detection - MRSA, VRE, ESBL producing E. coli, *K. pneumonia, M. tuberculosis*
5. Bacterial genotyping - *M. tuberculosis, N. meningitides*
6. Broad-range PCR - Infective endocarditis, bacterial meningitis

MRSA: Methicillin resistant staphylococcus aureus
VRE: Vancomycin resistant enterococcus
ESBL: Extended spectrum beta lactamase

Table 2 Summary of uses for molecular methods.

Viral pathogens: HIV genotyping for the detection of drug resistance is the standard of care to guide antiretroviral therapy and complements viral load assessment. Standard sequencing methodology and hybridization-based technology are the two principal methods used for HIV-1 genotyping. Genotyping is also critical to the management of chronic viral hepatitis. There are six HCV genotypes prevalent; of these, genotype 2 or 3 HCV infections have higher response to therapy compared to genotype 1 HCV infection.[8] Similarly, human papilloma virus (HPV) genotypes are also classified as either low or high-risk for the causation of cervical cancers.

Recent advances in pathogen discovery

• Microarrays in clinical microbiology: Microarrays using DNA chips have been extensively used for research, drug discovery and diagnostics. The most common use of DNA microarrays is for monitoring expression levels of transcripts from cells, viruses and bacteria in order to know which genes are being transcribed at a point of time. This method becomes useful to predict the function of uncharacterized genes or for analyzing the expression of virulence-associated genes (Figure 3). There could be extension of this application when response of a host to an invading pathogen is sought to throw light on the relation between host and pathogen as well as providing clues into the mechanisms of microbial pathogenicity.[9] Microarrays can be used to identify individuals who are more susceptible to infection and to determine prognostic markers for the outcome of the infection.

• Detection of new viral pathogens and vectors: This an upcoming application for which microarrays have potential. Here, an array containing the highly conserved sequences from fully sequenced reference viral genomes is designed. The aim is to detect a wide range of known viruses as well as novel members of existing viral families. The currently developed arrays include around 10,000

Limitations of Molecular Methods
1. Unlike bacterial culture, which can detect a large number of cultivable bacteria without initially knowing the specific organism, PCR can only detect the organism whose DNA is complementary to the primers used.
2. Differentiation between infection and disease, since the presence of nucleic acid does not necessarily mean the presence of viable microorganisms
3. Molecular tests are often subject to false positive results due to their high sensitivity
4. High level of staff training and skill is required for performing and interpreting these tests

Table 3 Limitations of Molecular Methods.

oligonucleotide probes capable of detecting almost 1,000 viruses in a single experiment. Another application for arrays could include the identification of distinct subspecies of vectors and reservoirs that harbour zoonotic pathogens (Figure 3).[10]

- Investigation of epidemic: Scientists in Taiwan identified and subtyped Influenza A virus as the H6N1 subtype, based on sequences of the genes encoding haemagglutinin and neuraminidase which were highly homologous to chicken H6N1 viruses. This virus had a G228S substitution in the haemagglutinin protein that might increase its affinity for the humans. The analysis was done in a few days as compared to long times needed for epidemic investigation by conventional methods.

- Revolution in bacteriology - identification of bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF): There has been recent interest in bacterial identification using this technique which has been found to be rapid, inexpensive, and accurate. In one study 95.4% of the bacterial isolates were correctly identified in less than 1 hour. Of these bacterial isolates 84.1% were identified at the species level, and 11.3% were identified at the genus level.[11]

Conclusion

Molecular technology has gone beyond the era of research and has now become an integral part of any microbiological laboratory. The introduction of molecular methods in clinical microbiology laboratories not only depends on the performance of the test for each individual microorganism, but also on the clinical relevance of the diagnosis, the disease load and whether the new methods are supplementary to the procedures in use or their replacements. Therefore, strategies have to be devised for each infectious agent or clinical syndrome based on the available phenotypic information.

Acknowledgements

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