

Molecular tools in infectious diseases

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Abstract: Molecular tools have been found to be extremely useful in the diagnosis of infectious diseases. Molecular diagnosis is very important and practical in the case of those infectious agents that are difficult to detect, identify or test for susceptibility with conventional methods. Since the target nucleic acids may be present in a very small amount in the clinical samples, many types of nucleic acid testing techniques for the early detection of infectious agents have been developed, e.g. nucleic acid probes, signal amplification and target/ nucleic acid amplification techniques. Availability of the Food and Drug Administration (FDA) approved commercial test kits have improved the testing conditions. These tests have also been successfully used to detect specific antimicrobial drug resistance genes in many organisms.

Introduction

Worldwide infectious diseases (ID) are a major threat to humankind. The diagnosis of ID is still based on the old diagnostic techniques, i.e. detection of aetiological microorganisms depending upon their phenotypic characteristics, such as morphology and biochemical characterization, etc.¹ These techniques are expensive and time consuming, and may even lose their relevance by the time definite tests are completed. For the monitoring and treatment of ID, especially those caused by fastidious microorganisms such as *Mycobacterium tuberculosis*, *Chlamydia*, *Listeria*, *Mycoplasma*, etc. and hard to detect viruses such as human immunodeficiency virus (HIV) and arboviruses, more rapid, accurate and advanced diagnostic tests are required. Automation in diagnostic microbiology has improved the methods of microbial diagnosis but it still has certain limitations, especially in cases of small sample size, low-degree bacteraemia, past infection, drug-resistant strains, patients already on treatment, etc. Immunological assays have played an important role in the diagnosis of ID. However, in case of a window period, these may escape detection before seroconversion, making these assays unreliable substitutes for culture methods and biochemical identification.^{2,3} Therefore with the advent of better diagnostic technology, it was thought that the development of diagnostic techniques based on the genetic make up of aetiological agents would overcome the above lacunae in the field of microbial diagnosis.

Types of molecular tools

The new molecular techniques are primarily based on the detection of nucleic acids (DNA/RNA) of the target organism. Nucleic acid amplification (NAA) has introduced new avenues for the detection, identification and characterization of pathogenic organisms in the field of clinical microbiology. The

three main types of nucleic acid testing techniques are: (i) nucleic acid probes, (ii) signal amplification and (iii) NAA.

NUCLEIC ACID PROBES

This is the simplest of three techniques. It can distinguish between two or more species, determine a particular strain within a given species, or identify differences between genes.² It is helpful in the early diagnosis and management of tuberculosis. DNA probes may have an advantage, especially for the detection of certain viruses, e.g. human papillomavirus (HPV), hepatitis B virus (HBV), Epstein-Barr virus (EBV), etc. Hybridization techniques employing nucleic acid probes help in quick diagnosis and early management of infections, especially those caused by organisms with fastidious growth requirements or difficult to culture, e.g. *Mycobacteria*, *Legionella*, *Mycoplasma*, *Borrelia burgdorferi*, HIV and other sexually transmitted diseases. Nucleic acid probes have also been tested successfully in malaria. A specific probe can detect as little as 10 pg of purified DNA of *Plasmodium falciparum* or 1 ng of DNA of *P. falciparum* in blood.⁴ Detection of DNA probes for fungal infections, e.g. *Histoplasma capsulatum*, *Blastomyces dermatidis*, *Cryptococcus neoformans* along with culture confirmation has many advantages over conventional means of identification.⁵ Almost all known organisms can be detected using this technique.

SIGNAL AMPLIFICATION TECHNIQUES

There are mainly two types of such kits available commercially.

(1) Branched DNA (bDNA): The bDNA probe technique designed by Chiron Corporation is the commonly used signal amplification method.⁶ Several studies have shown its sensitivity to be in the range of 10^3 to 10^5 DNA molecules. bDNA assays have been developed for HIV-1, hepatitis C virus (HCV) and cytomegalovirus (CMV) for which specific

Table 1. Various amplification techniques

Amplification Method	Nucleic acid target	Detection Method	Commercial Source	Different kits available for
Polymerase chain reaction (PCR)	DNA and RNA	Enzyme immunoassay (EIA)	Roche Diagnostics	HIV-1 (monitor*), HIV-1 (amplicor) HCV*, <i>M. tuberculosis</i> , <i>C. trachomatis</i> *, <i>N. gonorrhoeae</i> , CMV, HTLV-1/2, etc.
Nucleic acid sequence based amplification (NASBA) assay	RNA	Electrochemiluminescence (ECL)	BioMerieux, formerly Organon Teknika	HIV-1, HIV-1QT, CMV pp67, basic kits for: <i>M. tuberculosis</i> , <i>Toxoplasma</i> , rubella, dengue fever, malaria, etc. (interactive website)
Transcription-mediated amplification (TMA)	RNA and DNA	Hybridization protection assay (HPA-ECL)	Gene-Probe Incorporate	<i>M. tuberculosis</i> *, <i>C. trachomatis</i> *
Ligase chain reaction (LCR)	DNA	EIA	Abbot Laboratories	<i>C. trachomatis</i> *, <i>N. gonorrhoeae</i> *
Strand displacement amplification (SDA)	DNA		Chiron Corporation	<i>C. trachomatis</i> , <i>N. gonorrhoeae</i>
Branched DNA (bDNA)	DNA and RNA	EIA	Becton Dickinson and Company	HIV-1, HCV, hepatitis B

PCR: Polymerase chain reaction; HIV-1: Human immunodeficiency virus type 1; HCV: Hepatitis C Virus; CMV: cytomegalovirus; HTLV-I/II: Human T-lymphocyte virus I or II. *FDA approved

antiviral therapies are available.² Thus, bDNA provides quantitative detection over a range of several orders of magnitude. It is also useful for monitoring therapeutic response to α -interferon (hepatitis B and C), azidothymidine (HIV) and ganciclovir (CMV).

(b) *Digene-hybrid capture system*: This is another assay based on signal amplification, which uses a solution hybridization antibody capture assay and chemiluminescent detection.⁶ Here the target DNA hybridizes with an RNA probe. This system is useful for the detection of HPV infection in samples such as cervical biopsy and cervical swabs. The disadvantage of this method is that it is comparatively less sensitive than amplification methods and has a detection limit of 5×10^2 to 2×10^5 nucleic acid target molecules.

NUCLEIC ACID AMPLIFICATION METHODS

These are *in vitro* methods of enzymatic amplification of a target molecule (DNA) to levels at which it can be readily detected. These systems are capable of simultaneously identifying a pathogen and providing a replica of the target sequence which can be further characterized. There are many types of NAA techniques, e.g. polymerase chain reaction (PCR), transcription, based amplification, ligase chain reaction, strand displacement amplification, etc.

Polymerase chain reaction :

PCR is an *in vitro* method for replicating a target DNA sequence, so that its amount is increased exponentially. PCR can amplify single DNA copy to a million copies within a few hours. It can be used to selectively target sequences that are present in low abundance in a background of genomic DNA. This feature makes it potentially useful for the diagnosis of pathogens present in small numbers. PCR is a widely used technique because of its simplicity and flexibility. One of the best examples is the detection of proviral sequences of HIV type 1 (HIV-1) with a low prevalence in human mononuclear cells for virus specific sequence.

Several comparative studies of the PCR and conventional culture detection of HIV-1 in the peripheral blood of infected individuals have confirmed the sensitivity of nucleic acid-based techniques. At the same time, PCR is faster, less expensive and potentially less hazardous than culture. In the past few years, the availability of PCR reagents in kit form has enabled the synthesis of oligonucleotides at a low cost and the tremendous increase in availability of nucleotide sequence data have increased the application of PCR technology in the diagnosis of ID.

One of the most interesting applications of PCR has been the analysis of archived paraffin embedded tissue specimens⁷ and mucous specimens. Roche Diagnostics System Incorporated (USA) has developed PCR-based kits for the diagnosis of various infectious diseases (Table 1). In addition to this, many other DNA amplification-based techniques have been developed and applied for the detection of microbial infection, identification of clinical isolates and strain subtyping.¹ These tests are valuable for identifying cultured and non-cultivable organisms. PCR is also designed for rapid identification and determination of species/sub-species of *M. tuberculosis*.

In addition to the amplification of a target DNA sequence by a target PCR procedure, several specialized types of PCR have been developed for specific applications. Some of them are relevant to diagnostics (Table 2). Multiplex PCR has been shown to be a valuable method for the identification of various viruses such as HIV-1 and -2, human T lymphotropic virus-1 and -2 (HTLV), Herpes simplex virus (HSV)-1 and -2, CMV, EBV, etc. and many bacteria, e.g. *Neisseria gonorrhoeae*, *C. trachomatis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Mycoplasma* spp., *Campylobacter coli*, *Ureaplasma*, etc. and parasites such as *Giardia lamblia*, *Cryptosporidium parvum*, *Leishmania* spp. etc.⁸

Real-time PCR :

It is capable of screening genetic activity within hours, using a minimal amount of sample material and can detect a single molecule of DNA or RNA.⁹ Another advantage of real-time

Table 2 . Types of polymerase chain reaction (PCR) methods available and their applications

Amplification method	No. Primers	Target	Applications
PCR	Two	DNA	HIV-1, HCV, CMV, <i>C.trachomatis</i> , <i>Neisseria gonorrhoeae</i> , HTLV-I/II, enterovirus, <i>Mycobacterium tuberculosis</i>
Nested PCR	One	DNA	Designed mainly to increase the sensitivity of PCR tests
Reverse transcriptase (RT) PCR	Two or more (multiple)	RNA	RNA viruses, detection of viable <i>Mycobacterium</i> , monitoring antimicrobial therapy
Multiplex PCR	One	DNA/RNA	Detects more than one organism in a single specimen. More helpful in human & cancer genetics
Arbitrarily primed PCR	One	DNA	To differentiate strains of various species, and subtypes in a serotype, mainly for epidemiological work
Broad-range PCR	One	DNA/RNA	Rapid bacterial identification
Expression cassette PCR	One	DNA	Used for the generation of proteins with N and/or C terminal
Quantitative PCR	One	DNA/RNA	Used in HCV, HIV-1, CMV, etc. monitor kits for viral quantification
Membrane-bound PCR	One	DNA	Useful in the cases where there is a small amount of DNA
PCR		DNA/RNA	Improves end point analysis in PCR assays and also prevents contamination due to closed tube detection

HIV-1: Human immunodeficiency virus type 1; HCV: hepatitis C virus; CMV: cytomegalovirus; HTLV-I/II: human T-lymphotropic virus I or II.

PCR is that the PCR tubes do not need to be opened after the amplification reaction is complete. This prevents contamination by PCR products and reduces false-positive results.

Transcription-based amplification

It is a non-PCR target amplification system based on amplification by *in vitro* transcription. There are mainly two types of transcription-based amplification tests; NASBA (nucleic acid sequence-based amplification) assay and TMA (transcription-mediated amplification).

(i) Nucleic acid sequence-based amplification assay

Nucleic acid sequence-based amplification (NASBA) assay amplifies RNA in a DNA background.¹⁰ The NASBA (bioMerieux) system called NucliSens is used for measuring the load of CMV and HIV besides approximately 72 other applications, both viral and bacterial. In our laboratory we use NASBA for the detection of CMV (pp67, mRNA) and have found it very useful for the diagnosis of active CMV disease, especially in renal transplant cases¹¹. This assay detects messenger RNAs coding for matrix tegument protein pp67 of CMV that is only expressed during viral replication. The detection of CMV pp67 mRNA indicates whether the patient is suffering from an active infection or not. In tuberculosis, the problem of detecting non-viable organisms by PCR can be overcome by detecting mRNA instead of DNA. NASBA is also helpful in assessing bacterial viability by detecting RNA. It is very helpful in differentiating between active and past infections. Basic kits of NASBA are also available for tuberculosis, toxoplasmosis, malaria, dengue, enterovirus, etc. (Table 1). We use it for tuberculosis, HCV, malaria and dengue. The NASBA kit developed for quantitative testing of HIV-1 has shown promising results. A completely automated system called NucliSens is available as a work station for handling bulk samples. It does all the steps of NASBA in a single tube with its robotic action and completes the assay in 3 hours.

(ii) Transcription-mediated amplification

A transcription-mediated amplification (TMA)-based assay has been introduced in the market as Gene-Probe (BioMerieux) for the detection of *M. tuberculosis* in smear-positive as well as negative sputum specimens and also for *C. trachomatis* infection. A TMA assay for the quantification of HIV-1 RNA was recently shown to be more sensitive than RT-PCR or bDNA.¹² This is the only assay that is approved by the US Food and Drug Administration (FDA) for direct detection of *M. tuberculosis* from both smear-positive as well as negative sputum samples.

Ligase chain reaction

Ligase chain reaction (LCR) is a probe-based amplification system. Several studies of FDA-approved LCR diagnostic tests for *C. trachomatis* infection have shown comparable clinical sensitivity to PCR.¹³ A combination LCR kit for the detection of both *C. trachomatis* and *N. gonorrhoeae* has been introduced by Abbot Laboratories. Although convenient and readily automated, one potential drawback of LCR is the difficult inactivation of post-amplification products.

Strand displacement amplification

A semi-automated system (Probetech ET, Becton Dickinson) to carry out strand displacement amplification (SDA) with real-time fluorescence detection was introduced for the detection of *C. trachomatis* and *N. gonorrhoeae*. This system has been shown to be of equivalent sensitivity and specificity to LCR.¹⁴

APPLICATIONS OF MOLECULAR TOOLS

Molecular techniques have revolutionized the epidemiological investigations in ID. Besides this, the application of these techniques in the clinical microbiology laboratory are: - (i) prompt and accurate diagnosis of microorganisms, (ii) identification of unusual bacteria, (iii) disease monitoring through organism quantification, (iv) detection of antimicrobial drug resistance genes in many organisms e.g.

mec A resistance gene in methicillin-resistant staphylococci, (v) detection of epidemics, (vi) subtyping of microorganisms, etc. The most important application of 16S rRNA sequencing is the identification of previously unrecognized and new species and their molecular epidemiology.^{13,15}

Future prospects

Introduction of automation in these tests can overcome most of the problems associated with the first-generation assays. In particular, automating the sample preparation only can eliminate most of the contamination problems. Gene-Probe Incorporate has developed a fully automated instrument for the detection of NAA called Tigiris. These second-generation instruments are fully automated. They can process 500 tests in 8 hours and the VITROS system developed by Johnson and Johnson can detect an infectious agent in less than 2 hours. A triplex TMA assay for screening blood for simultaneous detection of HIV-1, HCV and HBV currently under trial in the USA, has been found useful in identifying pre-seroconversion infectious blood units. Despite their cost-effectiveness, these tests are expected to become the standard techniques in transfusion medicine. The introduction of microelectrochemical system-based microfluids (lab-on-a-chip) for DNA analysis is also very promising for the simultaneous identification and genotypic determination of drug-resistant microorganisms. Scientists are trying to make a universal drug resistance chip that will contain oligonucleotide arrays specific for all the known drug resistance genes on a single chip. DNA nanotechnology, molecular beacons, invasive cleavage assays, rolling circular amplification, peptide nucleic acid technology, data bank, online applications and ordering facilities such as Eurogenetics Clinical Investigation, Life Technologies, etc. are some of the upcoming technologies, which will prove to be some of the milestones in the

development of DNA diagnostics. What is required at the moment is the availability of universal standards & methods for quality control (QC) of NAA. Until QC for various NAA tests is in place it may not be easy to interpret or extrapolate the results of various laboratories conducting these assays.

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