Front. Med. 2012, 6(2): 173–186
DOI 10.1007/s11684-012-0195-5

REVIEW

Nucleic acid-based diagnostics for infectious diseases in public health affairs

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Abstract **Infectious diseases, mostly caused by bacteria and viruses but also a result of fungal and parasitic infection, have been one of the most important public health concerns throughout human history. The first step in combating these pathogens is to get a timely and accurate diagnosis at an affordable cost. Many kinds of diagnostics have been developed, such as pathogen culture, biochemical tests and serological tests, to help detect and fight against the causative agents of diseases. However, these diagnostic tests are generally unsatisfactory because they are not particularly sensitive and specific and are unable to deliver speedy results. Nucleic acid-based diagnostics, detecting pathogens through the identification of their genomic sequences, have shown promise to overcome the above limitations and become more widely adopted in clinical tests. Here we review some of the most popular nucleic acid-based diagnostics and focus on their adaptability and applicability to routine clinical usage. We also compare and contrast the characteristics of different types of nucleic acid-based diagnostics.**

Keywords nucleic acid-based diagnostics; infectious disease; PCR; NASBA; LAMP; microarray; LOAC; public health affairs

Introduction

According to the World Health Organization (WHO), infectious diseases account for more than 13 million deaths every year, one in two of which happen in developing countries with nearly two-thirds of all deaths among children under the age of 5. Poverty causes limited access to basic healthcare and has allowed deadly infectious diseases to gain ground. The poorest countries therefore pay the heaviest price. However, the substantial threats from infectious diseases do not recognize international boundaries. With globalization creating a new paradigm of population movement, living and non-living agents and materials capable of carrying infectious agents are inadvertently transported across vast distances. Modern transportation systems allow the spread of epidemics in a matter of hours. Huge increases in population mobility pose new challenges to traditional concepts of infection transmission and the relationship between populations and the global spread of disease [1].

This threat has grown and become more complex in the last decade, with many examples of changes in infectious disease patterns. New diseases have emerged: a typical example was provided in 2003 with the global alert on the Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) outbreak originating in Guangdong Province, China. It quickly spread from a localized area to become to a pandemic threat and, overall, 30 countries have reported a total of 8 098 probable cases of SARS. On the other hand, pathogens once viewed as declining in significance have resurged in importance; for example, the worldwide resurgence of dengue fever and the global spread of multidrug-resistant tuberculosis. The story is made even more complicated by the increasing awareness of the role of infectious agents in some cancers and chronic diseases once believed to be noninfectious. For example, *Helicobacter pylori* is now widely accepted as the causative agent of peptic ulcers, and perhaps of gastric malignancy, and human papillomavirus is likely to be the most important cause of invasive cervical cancer [2].

Changing epidemiology and understanding of infectious diseases calls for updated diagnostics. However, "correct diagnosis" is not enough, "timely" and "accurate" diagnosis

Received December 28, 2011; accepted March 3, 2012

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is critical to the global effort to fight infectious diseases. The loss of life and decrease in the quality of living caused by pandemics is still very large, mainly because of the lack of tools for fast and efficient diagnosis. The similarity of symptoms between pandemic diseases and the common cold can have severe social and economic impacts, increasing stress levels and hospital work-loads. The WHO has provided the "ASSURED" definition for an ideal diagnostic test (Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free and Delivered to those who need it). To maximize efficiency and minimize cost, an ideal diagnostic technology should also be multiplexed, able to test many disease targets and markers simultaneously. A multiplexed ASSURED diagnostic technology will provide a tool to control and contain infectious diseases at a sufficiently early stage.

Pathogen isolation and serological tests are the conventional diagnostic methods that, though not very sensitive, have been widely used to identify the cause of a disease. In the early 1990s, with the development of specific monoclonal antibody technology, molecular diagnostics became a powerful player in healthcare, representing a highly sensitive approach for clinical diagnosis. Although molecular tests have played an important role in the surveillance and control of infectious diseases, more accurate and more sensitive diagnostic tools are still required. Technological advances and personalized medicine are driving the rapid development of optimal patient diagnosis and treatment in healthcare institutions.

Nucleic acid-based diagnostics provide us not only fast, accurate and sensitive detection and diagnosis of pathogens that cause infectious disease, but also knowledge of the epidemiology of the disease. These diagnostic methods usually involve two critical steps: sample pre-treatment and genetic analysis. The sample pre-treatment, such as extraction and purification of the target nucleic acids, is a time consuming manual process. This step is essential, and the methodology used should be standardized to minimize discrepancies between tests and between testing centers. Potential contamination should also be kept to a minimum to increase the accuracy of the tests. A full discussion of these factors is outside the scope of this review (see Refs. [3] and [4]). For the genetic analysis, there is often a nucleic acid amplification step. While reverse-transcriptase polymerase chain reaction (PCR) is most commonly used, a number of other amplification methods, including nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and displacement amplification have shown their importance in clinical applications. Recently, multiplex PCR assays have been introduced to detect as many as 20 different pathogens simultaneously and several commercial multiplex assays are now available [5]. These highly sensitive nucleic acid-based diagnostics are gradually being adopted for routine clinical tests.

New tests and diagnostic systems must demonstrate clinical value and validity, and be easy to use, in order to attract widespread adoption. Nucleic acid-based amplification technologies could provide promising tools in the quest for an ideal diagnostic. Here we review the main nucleic acid-based diagnostic tools, focusing on their pros and cons for routine clinical usage. The review is written for medical health professionals and will not explore in-depth technical issues of nucleic acid-based diagnostics.

Polymerase chain reaction-based amplification

PCR is an *in vitro* enzymatic reaction that allows the production of large quantities of a specific fragment of DNA through repeated cycles of replication driven by DNA polymerase. It has become a very popular and basic practical technique used in laboratories for DNA cloning and other molecular procedures like Southern blotting, DNA sequencing, and recombinant DNA technology. This easy-to-carry-out but vital tool has revolutionized molecular biology and clinical diagnostics. Kary Mullis was awarded the Nobel Prize in Chemistry in 1993 for this discovery [6].

The portion of DNA to be amplified is known as the "template." "Primers" are short pieces of artificially synthesized DNA sequences that bind to the template to provide sites for initiation of DNA replication. There are three major steps involved: (1) Denaturation: The double-stranded DNA is subjected to high temperature to "unzip" it into single strands, and the heating also stops all enzymatic activities; (2) Annealing: When the temperature is reduced, the primers identify and bind to the segment of the targeted region to be amplified; (3) Extension: At an appropriate temperature, DNA polymerase catalyzes the formation of a DNA molecule complementary to the template sequence. These three steps are usually repeated for 30 to 40 cycles. The specificity of PCR is provided by the sequences of the primers which are complementary to only one region of the template DNA. Only the sequence between the two primer-bound sites is amplified. Primers bound at positions with no exact match will not hydrogen-bond properly and will not result in extension of the fragment.

The DNA polymerase used in the original protocol was not heat-resistant and therefore had to be replenished after the denaturation step of each cycle. This problem was solved with the discovery of *Taq* polymerase [7], a DNA polymerase purified from *Thermus aquaticus*, a thermophilic bacterium living naturally in hot springs [8]. *Taq* polymerase, being stable and heat-resistant, allowed an automatic thermocycler-based process for DNA amplification to be implemented. The repeated PCR cycles can now be performed on an automated cycler which rapidly heats and cools the reaction mixtures.

Traditionally, PCR is performed in a tube and the amplified DNA can only be visualized after the reaction is completed by subsequent analysis with gel electrophoresis. Although it has become the most important technique for DNA study, PCR

cannot be used for quantitative analysis. Although computer software may be utilized to quantify the intensity of the band to determine the relative amount of PCR product, the sensitivity and accuracy of the process require further evaluation. A great advancement of the PCR technique was the invention of quantitative real-time PCR (RT-PCR or qPCR). RT-PCR detects the initial amount of the template rather than the final product [9]. By monitoring the amount of fluorescence emitted during the PCR reaction as an indicator of the amount of DNA amplification during each cycle, the progress of the reaction in "real time" can be visualized using an RT-PCR machine. Thus, RT-PCR is commonly used to determine not only the presence, but also the original copy number of a specific DNA target.

Extensive application of PCR in clinical laboratory tests requires a high level of sensitivity and accuracy of detection. RT-PCR has a very high degree of precision compared to standard PCR. Unless a standardized molecular test with high sensitivity and specificity becomes available, the risk of false-negative test results (where the test is negative while the virus is present) exists, leading to escape of infected patients/carriers from isolation and control measures. When SARS broke out in 2003, scientists had to devise a more sensitive detection method because it was difficult to identify the virus at an early stage of infection [10]. When the regular primer sets failed, a group of Chinese scientists designed a nested set of primers and successfully amplified shorter genomic fragments for sequencing [11]. This nested PCR procedure, combined with RT-PCR, evolved into an enhanced real-time PCR (ERT-PCR) technique (Fig. 1), which is more sensitive, reliable and accurate in the detection of SARS-CoV [12,13]. Statistical results from 120 clinical samples showed that the limit of detection (LOD) of ERT-PCR was 10^3-fold higher than the standard RT-PCR and 10^7-fold higher than traditional PCR assays [12]. The increased sensitivity of the assay may help to control the spread of infection during future outbreaks of SARS and other infectious diseases [10,14].

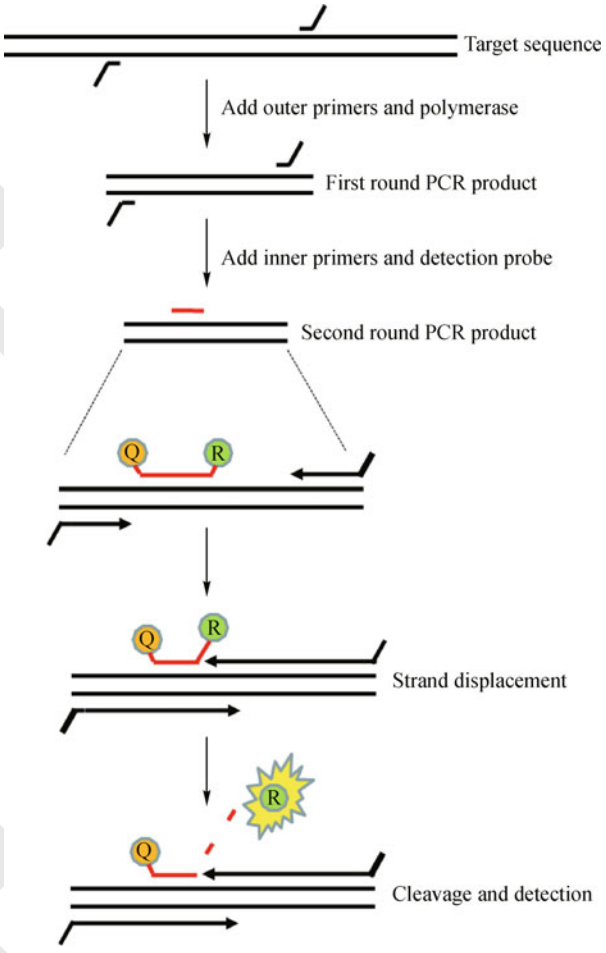

Fig. 1 Enhanced real-time PCR (ERT-PCR). The template sequence (RNA or DNA) is amplified with a set of specific primers. The PCR product is then used as a template for a second round of amplification using a second set of nested primers. During this second round of PCR a detection probe is included to quantitate the reaction in real-time. The probe contains a fluorescent reporter (R) and quencher (Q). When the reporter and quencher are close together there is no fluorescent signal. As the PCR continues the probe is displaced and cleaved, releasing the reporter which emits a detectable fluorescent signal.

Clinical applications of PCR

Watson and Crick's model of the DNA double helix structure [15] has opened the path for DNA technology which has revolutionized both science and medicine. It is now easy to amplify DNA by PCR and conduct functional analysis of the genes [16]. Medical research and clinical medicine benefit from PCR technology via the diagnosis of hereditary diseases, the identification of genetic fingerprints (used in forensic sciences) and paternity testing [17].

The PCR technique is faster and more accurate than antibody-based detection or bacterial incubation. The antibody-based test used for virus detection is based on the fact that an infected individual produces specific antibodies against viral antigens which can be detected in the laboratory. Unfortunately, it takes a period of time, the window period, for the body to produce sufficient antibodies to yield a positive result. Therefore, during this window period, carriers can be misdiagnosed (a false-negative result) and the disease can be transmitted to other people. PCR can be used to detect the presence of virus directly in the early infectious cycle. Bacterial/viral incubation used in diagnosis also has a window period, which may last for several days to weeks. For example, tubercle bacillus, the bacterium causing tuberculosis, multiplies slowly, with a reproduction cycle every 24 h. Doctors can only order treatment based on the limited information of the disease symptoms while waiting for a definitive result. In this situation, the PCR technique can be used to amplify genes very rapidly, and so greatly increase the sensitivity of detection and greatly reduce the overall

detection time. Some diseases, like Lyme disease which is caused by a tick-borne spirochete, are not easily diagnosed. In this case, the spirochetes are too small to be seen under the light microscope and nearly impossible to culture in the laboratory. Traditionally, diagnosis is made on the basis of symptoms, epidemiological considerations, and serological tests. None of these methods is completely reliable, and without accurate diagnosis treatments have to be delayed. Recently, the Federal Rocky Mountain Laboratory successfully used PCR to amplify the spirochetal DNA, and used DNA probes to detect the pathogen in patients' blood [18]. Influenza is a very common infectious disease that has been widely ignored. Since the epidemics of SARS [12,13], H1N1 [19] and H5N1 [20], fast nucleic acid tests (PCR or PCR-related) for diagnosis of influenza have been reported. RT-PCR has also been used to screen a congenital virus infection in new-borns [21].

PCR has not been limited to detection of infectious organisms, but is also useful in blood screening. The WHO recommends all donated blood used for transfusion should be screened for human immunodeficiency virus (HIV), hepatitis B, hepatitis C and syphilis, at a minimum. The traditional screening methods, immunoassays which detect antibodies to viruses or viral antigens, have the same risk of missing the infection during the "window period." PCR detects the presence of viral infection by directly probing for viral nucleic acids, and can be used to screen whole blood and plasma samples. The benefits of using PCR technology to monitor the blood supply include a decreased window period during which the infectious agent is undetectable by traditional screening methods and the ability to perform comprehensive and combined blood screening for several pathogens. Combination or multiplex PCR-based tests in blood donor screening programs are expected to decrease the turnaround time and reduce the risk of virus transmission.

PCR has also been used to gain insights into the molecular basis of cancer and its genetic factors [22]. However, since clinical biomarkers and gene detections of predictive value are not yet accurate and reliable for cancers, PCR is not currently of much diagnostic value in this area [23]. Nevertheless, as more biomarkers become available PCR will become an increasingly valuable tool in the fight against cancer.

Advantages and disadvantages of PCR

PCR enables small amounts of genetic material to be detected and, with species-specific primers, allows unique identification of virus/bacteria or biomarkers. It is fast, accurate and specific, requires little sample and is easy to perform. Therefore, PCR is well suited for clinical use to provide early diagnosis and in-time treatment for patients. The major problem with PCR is the possibility of false-negative/positive results. The presence of inhibitors which prevent DNA amplification will cause a false-negative test result. Other aspects leading to false-positive or false-negative results include contamination, mismatched target gene and primer sequences, variable experimental conditions, DNA extraction procedures and PCR product detection methods [24]. Thus, the set up of the experiment, primer design and interpretation of PCR results requires a well-trained professional.

Isothermal amplification

Isothermal nucleic acid amplification technologies utilize a single reaction temperature for the amplification phase, which allows less complex and less expensive instrumentation to be used, compared to the specially designed thermal cyclers required for PCR amplification. Isothermal amplification can be performed in water baths, using simple resistive heaters, or via exothermic chemical heating [25].

Current isothermal amplification methods can be grouped based on reaction mechanisms. Nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification are based on RNA transcription [26,27]; both are well-established and implemented in commercial laboratories. Helicase-dependent amplification is an example of DNA replication with enzymatic duplex melting and primer annealing [28]. Methods based on DNA-polymerase-mediated strand displacement from linear or circular targets include loop-mediated isothermal amplification (LAMP) and rolling circle amplification [29]. Many isothermal amplification methods, such as strand-displacement amplification, use polymerase extension in conjunction with a single-strand cutting event [30,31]. In this section, we focus on the most widely used isothermal technologies, NASBA and LAMP, and compare their clinical applications.

Nucleic acid sequence-based amplification (NASBA)

NASBA is a continuous, isothermal, enzymatic RNA-based nucleic acid amplification technique developed in 1991 [26,27]. Because the reaction is isothermal it can be carried out in a simple water bath and does not require a thermocycler (Fig.2). It employs a mixture of reverse transcriptase, ribonuclease-H, RNA polymerase and two specially designed DNA oligonucleotide primers. The forward primer has a 5′ extension containing the promoter sequence for bacteriophage T7 DNA-dependent RNA polymerase. The reverse primer has a 5′ extension containing a complementary binding sequence for a DNA oligonucleotide detection probe labeled with a ruthenium-based electrochemiluminescent (ECL) tag. During the amplification process, the 5′ primer extensions are incorporated fully into the amplified sequence, allowing both highly efficient production of complementary RNA template (directed by the RNA polymerase) and specific detection by the ECL-tagged probe [32]. This method of detection requires the use of a chemiluminescence detector. Under optimum conditions, it is

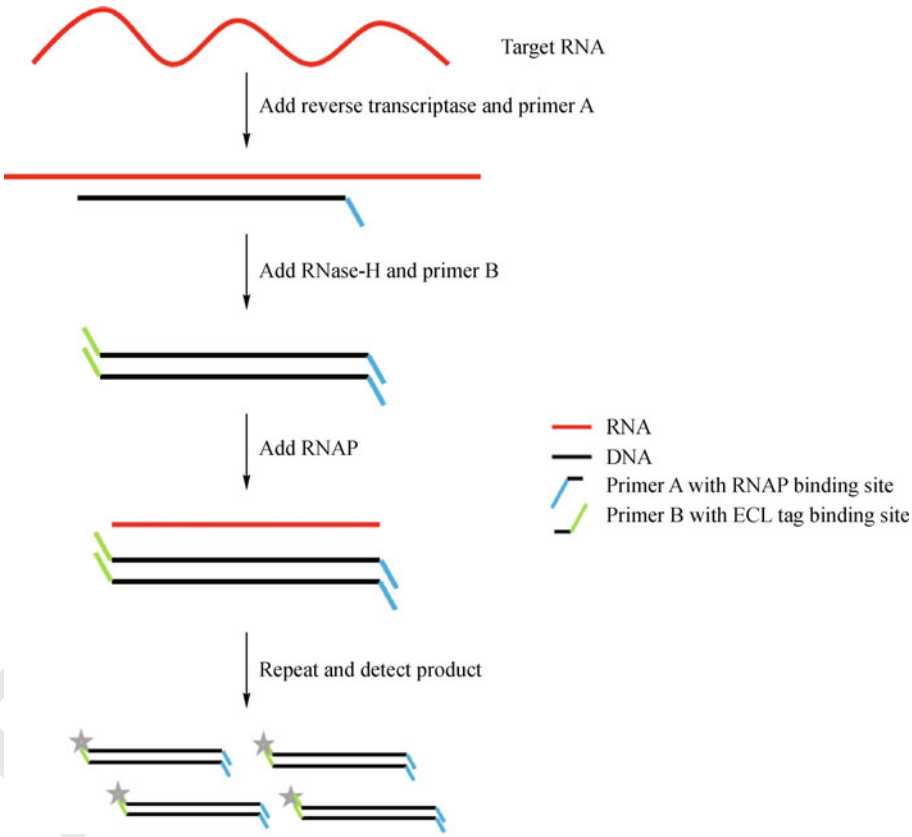

Fig. 2 Nucleic acid sequence-based amplification (NASBA). An RNA sequence of interest is amplified with reverse transcriptase and a sequence-specific primer. The DNA/RNA hybrid is then treated with RNase-H to hydrolyze the RNA. Reverse transcriptase and a second primer are then used to generate a double stranded DNA with incorporated RNA polymerase (RNAP) and ECL (or EOC) binding sites (see text for details). RNAP produces an RNA template of the target sequence which is used to repeat the whole cycle, allowing large amounts of the target sequence to be generated and detected.

possible to achieve 10^{12}-fold amplification, versus the 10^{9}-fold amplification accomplished by PCR [33].

Being highly sensitive, specific, accurate and rapid for nucleic acid amplification, the applicability of ECL-NASBA is only limited by the high cost of the equipment required for the ECL detection. An alternative, more applicable procedure, the enzyme-linked oligonucleotide capture (EOC) detection, was then developed [34]. In the EOC-NASBA detection method, the amplicons are immobilized by hybridization onto a biotinylated oligonucleotide capture probe bound to a streptavidin-coated surface. Detection is then achieved by a digoxigenin (DIG)-labeled detection probe and an anti-DIG antibody-alkaline phosphatase conjugate which produces a colorimetric end product that can be read by standard 96-well microtiter plate spectrophotometers. EOC-NASBA allows relatively inexpensive and high throughput analyses of amplicons, providing an affordable option for highly sensitive and specific molecular diagnostics in many laboratories. Since only simple equipment, such as a water bath and ELISA plate reader, are required, EOC-NASBA becomes a more versatile application compared to ECL-NASBA and RT-PCR, especially in locations where field-sample testing is crucial, such as monitoring potential contagious diseases crossing country borders [35]. NASBA detection methods have also been adapted to real-time format [36]. Real-time NASBA uses DNA hybridization probes that fluoresce upon hybridization with their amplicon targets. The probes have a stem-loop structure and contain a fluorophore and a quencher group. In its normal state, the stem keeps the fluorophore and the quencher together, preventing emission of fluorescence. Upon hybridization of the loop sequence, the probe unfolds, the quencher no longer absorbs photons emitted by the fluorophore, and the probe starts to fluoresce. The whole process of amplification and detection runs in a fluorescent reader. Real-time NASBA assays are suitable for high-throughput applications, reducing the assay time and limiting potential contamination between samples [37].

Loop-mediated isothermal amplification (LAMP)

LAMP is another isothermal DNA amplification method first reported in 2000. It employs two sets of primers and a DNA polymerase with strand displacement activity to amplify target DNA [38]. The four different primers identify six

distinct regions on the target gene. A pair of outer primers displaces the amplified strand with the help of the DNA polymerase to release a single-stranded DNA which then forms a hairpin to initiate the starting loop for a cyclic amplification. The amplification proceeds in cyclical fashion, with each strand being displaced during elongation with the addition of new loops in each cycle. The final products are stem-loop DNAs with several inverted repeats of the target sequence. The addition of a primer set that anneals to the LAMP amplicon loop structure increases the efficiency and specificity of the reaction [39].

Several detection methods can be used to identify positive LAMP reaction products. The byproducts of a LAMP reaction, pyrophosphate ions, bind to magnesium ions to form white magnesium pyrophosphate precipitates which can be easily visualized, especially for large reaction volumes [40], or via simple detection approaches capable of measuring real-time turbidity which can quantify 2×10^3 to 2×10^9 copies of initial template DNA [41]. Alternatively, LAMP products can be visualized by incorporation of SYBR Green I stain which has high binding affinity for DNA [42]. A sequence-specific visual detection method that utilizes the unique nature of low molecular weight polyethylenimine (PEI) has also been described [43]. PEI forms an insoluble complex with high molecular weight DNA, such as a LAMP product, but not with a single-stranded anionic polymer of low molecular weight, such as a DNA probe. Since the amount of LAMP amplicon is large, the LAMP-PEI precipitate is of a size that can be detected visually. For multiplex nucleic acid target detection, DNA probes can be labeled with different fluorescent dyes, and the precipitate would emit a different fluorescence for each distinct fluorescent DNA probe hybridized to the corresponding LAMP target. As a result of these characteristics, minute amounts of nucleic acid targets can be detected simply through visual observation of the color of the LAMP-PEI precipitate [43].

Clinical applications of isothermal amplification

NASBA is particularly well suited for the amplification of single-stranded RNA and has been successfully used in the detection of numerous different viruses such as HIV type 1 [27], simian immunodeficiency virus [44], avian influenza virus [34], foot-and-mouth disease virus [45], Newcastle disease virus, classical swine fever virus, porcine reproductive and respiratory syndrome virus [32]. NASBA protocols have also been described for bacteria, fungi, parasite and cytokine detection [46]. Examples include macrophage-derived chemokine mRNA [47] and *Salmonella enterica* [48]. LAMP has been applied to detect a variety of pathogens, including viruses and bacteria, and can also amplify RNA with the addition of reverse transcriptase [49]. Detection of food-borne disease bacteria such as *Salmonella* [50], *Escherichia coli* [51] and *Noroviruses* [52] employing LAMP technology has been reported. Clinical applications employing the LAMP protocol, including detection of SARS-CoV [53], H5N1 [54], *Mycobacterium tuberculosis* [55], avian influenza subtypes, influenza subtype 1, influenza subtype 3 and influenza B virus, have been reported [56].

Advantages and disadvantages of isothermal amplification

NASBA has several advantages over PCR-based diagnostics. Foremost, it requires only a water bath and so developing countries and budget-restricted laboratories can afford to perform NASBA-based molecular diagnostics. Besides, it provides accurate and consistent results with high sensitivity, even surpassing RT-PCR [14] and comparable to the "gold standard" virus culture approach which takes days to be completed [57]. Moreover, because of its isothermal nature, NASBA methods can be standardized easily. On the contrary, although RT-PCR is currently the commonly used molecular method for the detection of various viruses and other pathogens, variations in reaction temperatures, signal-generating chemistry and detection technologies, and the availability of many different reagents and cycling equipment make the standardization of PCR conditions almost unfeasible. The main disadvantage of NASBA is its limitation to the detection of RNA pathogens, since it is an RNA-based amplification procedure. Although it is possible to amplify DNA using different enzymes in the NASBA protocol, in practice this is not done.

The LAMP assay has high specificity because amplification only proceeds when all six regions within a target DNA are correctly recognized by the primers. However, the difficulty of designing workable primers discourages practical usage of LAMP [49]. Real-time turbidity detection by a cost-effective photometer with an incubation function has enabled the kinetic analysis of the LAMP reaction without the need for any detection reagents such as a fluorescence intercalator [40].

Unlike PCR, NASBA and LAMP are less affected by various inhibitory components of clinical samples [32,58]. This helps save the time and cost required for sample purification which is labor intensive and can take hours to complete.

The future of isothermal amplification

Time-saving multiplex NASBA (mNASBA) technologies are being developed to enable high throughput screening. An EOC-mNASBA assay for screening common lower respiratory tract pathogens including influenza A, influenza B, human parainfluenza viruses 1–4, respiratory syncytial virus, rubella virus and Coxsackie virus [59], and a real-time mNASBA detecting *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Legionella* spp. in respiratory specimens [60] have been reported. However, mNASBA was

shown to be less sensitive than the simplex NASBA reactions in both cases [59,61]. A microarray-based mNASBA procedure, mNASBA-on-microarray, which detects the expression of five genes and their single nucleotide polymorphism (SNP) parameters has been used for breast cancer diagnosis. This single-step process requires at least 10 pg of total RNA for the detection of the reference parameter RPS18. The LOD of the mNASBA-on-microarray procedure theoretically allows single-cell assays to be performed [62].

A multiplex LAMP (mLAMP) protocol using two sets of species-specific LAMP primers incorporated with a restriction endonuclease cleavage site for distinction between *B. bovis* and *B. bigemina* has been described [63]. Differential fluorescent-labeling of the LAMP amplification products can also be adapted for mLAMP screening [43]. There is much potential for further miniaturization of LAMP, as amplification of single DNA templates encapsulated in polyacrylamide microchambers as small as 1.5 mm $\times$ 17.5 mm have been demonstrated [64].

Isothermal nucleic acid amplification technologies are suitable for point of care (POC) applications, especially in low-resource settings. The other factor which facilitates its widespread adoption for clinical diagnosis is the signal detection method. Whereas incorporation of fluorescent markers increases the sensitivity of signal detection, a delicate and expensive fluorescence detector is essential. On the other hand, simple visual methods detectable by the naked eye are practical and affordable [65]. A breakthrough in developing new detection methods with a balance between cost and sensitivity will greatly enhance the applicability of isothermal amplification technologies for routine clinical diagnosis.

Microarray/chip-based technologies

Although PCR and other amplification methods now have multiplexing capability, they are costly and still not fast enough to meet clinical needs. The invention of microarray/chip-based technologies allows analysis of samples with a vast number of sequences in one test, which no amplification-based technologies can achieve.

Microarrays evolved from the work of E. M. Southern who first proposed a method to analyze nucleic acid fragments on nitrocellulose filters [66,67]. This technique, known as "Southern blotting," involves the transfer of gel-separated DNA fragments onto nitrocellulose filters, followed by hybridization with radio- or fluorescent-labeled probes. The DNA fragment(s) containing the labeled probe sequence can be identified through analysis using appropriate scanners. This idea gave rise to the dot blot method for analysis of multiple DNA targets which form an array of dots on nitrocellulose filters [68]. The reverse dot blot method was introduced for sequence typing of a single target hybridized with an array of probes immobilized on a nylon membrane [69]. Depending on the number of dots which can be accommodated, a large number of sequences can be tested. For pathogen screening, the expressed sequences of multiple pathogens can be immobilized on the filter to identify which pathogens are present in the tested samples. Thus a single reverse dot blot procedure can provide both genome analysis and diagnosis for multiple pathogens [70].

Improvements in constructing high-density dotted arrays greatly enhanced the throughput and efficiency of the test. The conventional nitrocellulose filter and nylon membrane are porous, causing diffusion and merging of droplets that not only limit the number of probes immobilized but also affect the positional accuracy of the dots and the array resolution. The problem was solved by using solid substrates such as glass and plastic slides.

Initially, UV was used to immobilize nucleic acids onto cellulose fabrics [71]. For immobilization of nucleic acids onto substrate surfaces, different types of molecular interactions, namely, adsorption, a biotin-avidin affinity pair, thiol-gold interactions and covalent linkages, have been employed (see Refs. [72–83] for details). The choice depends on the type of application, the substrate, the sample and the environment.

By eliminating the diffusion effect and adjusting the hydrophobicity of both the droplets and surfaces, the dot size can be precisely controlled. Using advanced automatic spotting machines, both the dot size and the array resolution have adequately reached the micrometer scale and thus the term "microarray" was derived [73]. Two major spotting technologies, contact-mode [73] and inkjet-mode [84,85], are commercially available. Selection between these two depends on cost, throughput and resolution considerations. The highest density of a microarray, to date, was achieved by combining the solid-phase synthesis and photolithography technologies, producing a fine resolution of more than 400 000 probes synthesized on an area of 1.28 cm^2 [86,87]. The photolithography technique was well established in microelectronics, where the term "chip" originated [88]. Therefore, it is reasonable to interpret the name GeneChip (an Affymetrix product) as a combination of microarray and microelectronic technologies. Generically, a microarray processed with microelectronic fabrication was referred to as a "chip." However, confusion gradually arose as the names "chip" and "microarray" were often used interchangeably. In addition to DNA chips, different kinds of biologic species, such as proteins, cells and tissues can be attached to chips, which allows many novel experiments to be performed. The invention of high density DNA chips enabled a parallel analysis of a target sample with many thousands of sequences in one test, a feature which other amplification-based technologies cannot attain.

Clinical applications of microarrays

Microarray-based methods have been applied to clinical

diagnostic studies. For example, the tumor suppressor p53, a transcription factor, plays key roles in multiple cellular pathways. A microarray-based re-sequencing test, the p53 GeneChip assay, was first used for detecting p53 mutations in cancer [89]. In addition, a targeted oligonucleotide array was developed to interrogate 179 clinically relevant genomic loci. These multiplex arrays were shown to deliver results that were consistent with those from bacterial artificial chromosome arrays [90]. High density microarrays were also used to identify signatures for distal metastasis of lymph-node-negative primary breast cancer [91] and for screening clinical samples for viruses [92–94]. A multi-pathogen identification microarray capable of identifying 18 pathogenic prokaryotes, eukaryotes and viruses was demonstrated to be more sensitive than PCR [95].

The high-throughput capability of chips has also benefited scientific research in genome-wide SNP analysis [96,97], comparative genome sequencing [98], comparative genomic hybridization [99,100], ChIp-Chip [101], DNA methylation [102] and expression analysis [103,104].

Laboratory-on-a-chip (LOAC) system

Incorporation of microelectronics with microarray technologies has become increasingly important in the development of new diagnostics. Integration of electronic circuits can add new functionalities, such as electrically accelerating the hybridization process [105,106], embedding sensors [107,108] and performing electrical detections [109,110]. Compatibility with standard microelectronic fabrication and miniaturization of DNA chips provide a cost-effective solution for mass production. The fabrication technology, specifically microelectromechanical systems technology [111], is capable of creating precise geometrical structures of sub-micrometer scale on solid substrates. The structures utilized include nanowires, microfluidic channels, stripes, wells, etc. [112,113]. Thus, in conjunction with basic mechanical automations and software, the chip can handle various tasks previously dependent on human operations. The chip can also integrate laboratory procedures, such as microfluidic systems, exchange of aqueous solutions, temperature control in a reaction chamber, providing optimum conditions for hybridization, post-hybridization detection and data analysis. The idea of developing an all-in-one, fully automatic system for completing an entire analysis protocol led to the creation of the laboratory-on-a-chip (LOAC) system. LOAC greatly simplifies the high-throughput analysis since human involvement is minimal. The automation also provides operational convenience and ensures repeatable results. It should also be able to offer very rapid multiple detections with high sensitivity and specificity, thus reducing the cost. Therefore, LOAC systems are recognized as a promising technology for delivering POC diagnostic capabilities that could revolutionize medicine [114]. Currently, commercial exploitation has been slow, but is gaining pace. STMicroelectronics and Toshiba have developed LOAC systems, namely the “In-Check system” and “Genelyzer,” respectively. Nanogen Inc., acquired by Epoch Biosciences in 2004, launched the NanoChip workstation and the NanoChip microarray. However, these systems were mainly adopted for research purposes and have not yet achieved the expected success in commercialization.

Challenges

Microarray/chip technologies are not well suited for use in routine clinical diagnosis. An ideal clinical diagnostic tool is expected to deliver early and accurate diagnosis which depends on the sensitivity and specificity of the system, respectively.

Sensitivity is defined as the lowest detectable concentration or copy number of the target. High sensitivity is desirable in diagnostics, especially during an outbreak of infectious diseases, so that infected individuals can be identified and treated at an early stage. The sensitivity required for diagnosing influenza is typically ~10^3 copies/µl [115–117], while the sensitivity for the current microarray and chip technology is ~50 fM, which corresponds to ~10^4 copies/µl [118] which does not meet the clinical need. Although an amplification step can be included to increase copy number before sample analysis, the diagnostic time will be prolonged as well. The low sensitivity of microarrays is attributed to the hybridization mechanism and the detection methods. The hybridization process is a slow thermodynamic process which takes hours to days to complete. The targets require a number of collisions before they find the correct orientations to hybridize with the complementary probes. On a microarray, a hybridization event is restricted to the surface, which further lowers the efficiency. Optimizations of the hybridization conditions have been reported, including probe concentration [119], buffer condition [120,121], temperature, probe spacer [122], rotation of the hybridization chamber [123] and incorporation of the photovoltaic effect [124]. To accelerate the hybridization process, Nanogen scientists incorporated hydrogel permeation coating onto every spot in their DNA chips [106,125,126]. The hydrogel confined the probes and metal electrodes located below, and when electrically connected, nucleic acids were driven to the spots by electrophoresis. This process increases the DNA concentration on the spots, thus enhancing hybridization. However, proper choice of buffer is crucial because of the pH change during electrophoresis [106]. By using a pulse with a rise/fall time, the hybridization efficiency was enhanced [127–129]. However, the success of this method requires a significant amount of nucleic acids distributed close to the surface, and clinical samples usually contain only small amounts of DNA. Other methods using electrokinetics have also been reported [130,131]. Although these methods have all been demonstrated to enhance hybridization efficiency, they have not been properly utilized in clinical applications

due to reliability and consistency issues and a lack of clinical samples for trial performance. A better solution toward increasing the hybridization efficiency has yet to be devised. Detection methods employing fluorescent, electrochemical [132], electrical [24,25], optical [133], nano-particle [134] and quantum-dot [135] technology have been reported. However, the most sensitive detection method is not necessarily favorable for clinical applications. Other factors including cost and ease of operation, time for developing signals and integration to the entire system need to be considered.

Specificity is reflected by the accuracy in detecting the causative agents of the disease. Design of specific probes is the most critical step and requires substantial understanding of the genetic makeup of disease organisms. Moreover, there are a vast number of different probes immobilized on the chip. Random binding and non-specific hybridization resulting in false-positives is therefore unavoidable. An appropriately stringent washing procedure can be implemented to enhance the specificity.

Although the incorporation of microelectronics fabrication processes has relieved the financial pressure arising from the mass production of DNA chips, expenses for immobilizing the probes on the chip, equipping an advanced optical system for detection and a precise fluidic system are still high, imposing major difficulties in adopting microarray/chip-based technology for clinical diagnosis. However, it has great potential to become a mainstream diagnostic tool since it can provide a powerful parallel analysis of multiple pathogens in one test that no other technologies can achieve. The introduction of the LOAC system has greatly improved the miniaturization process and simplified this complex technology. However, its clinical application in fighting infectious diseases is still far off until various issues, such as increasing the sensitivity and specificity of the system, reducing the cost and increasing the speed of hybridization, are resolved.

The future of molecular diagnostics

Clinical diagnostic tests are categorized as high complexity, moderate complexity or waived according to the United States Clinical Laboratory Improvement Amendments. However, there are no waived POC nucleic acid systems currently available. High complexity tests are limited to central laboratories, whereas moderate complexity tests can be performed near patients in a hospital. Developments in "fully automated sample-in/answer-out" testing systems affordable for use in clinical diagnosis will be vital. Fighting against infectious diseases requires a high penetration of technology into society. To prevent outbreaks on the front line the diagnostics should appear not just in hospitals or a few major disease control centers, but also serve general practitioners in public clinics and healthcare centers. In the penetration process, the cost of a diagnostic system is equivalently important to its technical performance.

Nucleic acid purification and extraction is the bottleneck for most diagnostic technique applications at the POC because it is a lengthy manual process. Most infectious disease applications require extraction and concentration of target nucleic acids from sample input volumes $> 500\ \mu l$ to reach a suitably low LOD. This requirement often cannot be accommodated in microfluidic devices. There are no commercially viable products currently available, possibly because of the challenges related to system complexity, manufacturability, and performance reproducibility [65].

What is the ideal diagnostic technology? Many promising candidates have been developed toward an ASSURED standard, such as protein chips, tissue chips and LOAC. Genomic, proteomic and metabolomic technologies are making their way into clinical diagnostics. Personalized medicine and proper diagnostics accompanied by personalized therapy represent the future of medicine. The ideal future diagnostic tool, therefore, should be multiplexed and gather information from the genome, the proteome and the metabolome, and also provide this information quickly and easily at an affordable cost. Many hurdles must be overcome before these techniques can be transformed from research tools into routine clinical practices.

Conclusions

Despite decades of developments toward their treatment and prevention, infectious diseases remain a major cause of death and asthenia and are responsible for worsening the living condition of millions of people around the world. However, the current allocated resources and the existing healthcare system are not adequate for future needs. Also, the ever-changing epidemiologies of infectious diseases present consistent challenges to traditional diagnostics. Rapid advances in molecular methods have led to the development of sensitive and specific nucleic acid-based diagnostic tests which strengthen our ability to diagnose, treat and control infectious diseases that cause public health problems. We have reviewed several popular and important nucleic acid-based diagnostic methods, many of which have the potential to be used in the clinic. However, there is still a long way to go until these methods can be routinely used in hospitals. It is likely that biomarkers will be found that are specific for classes of infectious agents and that will provide insights to guide clinical management, even in cases of chronic diseases. However, many hurdles still need to be cleared; for example hybridization, the bottleneck step in most procedures, is still slow and causes delays in diagnosis. We believe that some future nucleic acid technologies will break through the bottlenecks to achieving the perfect diagnostic systems. In particular, an accurate, fast, easy and cost-effective diagnostic for infectious diseases will offer advantages to both basic

scientists, clinical doctors and, most importantly, patients. Ideal future diagnostics should not only be "ASSURED," but should also be multiplexed. The more information we can get from a single rapid test, the more powerful will be our awareness and response in battling future pandemic outbreaks. Therefore, a more concerted worldwide effort should be made toward developing and promoting the ideal nucleic acid diagnostic technology.

Acknowledgements

Thanks to the contribution to this article by Alice Poon. This work was supported by grants from the National Key Technology R&D Program (No. 2008BAK41B01-5) and the National Science and Technology Major Project—Development New Genetically Modified Organism (No. 2009ZX08012-006B).

Abbreviations

World Health Organization (WHO); Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free and Delivered to those who need it (ASSURED); polymerase chain reaction (PCR); real-time polymerase chain reaction (RT-PCR); nucleic acid sequence-based amplification (NASBA); multiplex NASBA (mNASBA); single nucleotide polymorphism (SNP); loop-mediated isothermal amplification (LAMP); multiplex LAMP (mLAMP); enhanced real-time polymerase chain reaction (ERT-PCR); limit of detection (LOD); point of care (POC); electrochemiluminescent (ECL); enzyme-linked oligonucleotide capture (EOC); digoxigenin (DIG); polyethylenimine (PEI); laboratory-on-a-chip (LOAC); severe acute respiratory syndrome (SARS); SARS coronavirus (SARS-CoV); human immunodeficiency virus (HIV).

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