

Advances in the application of molecular diagnostic techniques for the detection of infectious disease pathogens (Review)

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Abstract. Infectious diseases are a major global cause of morbidity and mortality, seriously affecting public health and socioeconomic stability. Since infectious diseases can be caused by a wide variety of pathogens with similar clinical manifestations and symptoms that are difficult to accurately distinguish, selecting the appropriate diagnostic techniques for the rapid identification of pathogens is crucial for clinical disease diagnosis and public health management. However, traditional diagnostic techniques have low detection rates, long detection times and limited automation, which means that they do not meet the requirements for rapid diagnosis. Recent years have seen continuous developments in molecular detection technology, which has a higher sensitivity and specificity, shorter detection time and increased automation, and performs an important role in the early and rapid detection of infectious disease pathogens. The present study summarizes recent progress in molecular diagnostic technologies such as PCR, isothermal amplification, gene chips and high-throughput sequencing for the detection of infectious disease pathogens, and compares the technical principles, advantages and disadvantages, applicability and costs of these diagnostic techniques.

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1. Introduction

Infectious diseases, which can be caused by a variety of bacteria, viruses, parasites and fungi, are one of the leading causes of morbidity and mortality worldwide (1,2). According to recent statistics, there are ~60 million deaths worldwide annually, at least 25% of which are caused by infectious diseases (3). The emergence of infectious diseases has created great challenges to public health and socioeconomic stability. Although the use of vaccines and antimicrobial and antiviral drugs can help combat infections to a certain extent, their efficacy can decrease with the emergence of new infectious agents and unknown drug-resistant pathogens (4,5). In addition, the development of vaccines and medicines requires lengthy clinical trials, which does not allow for the rapid and effective control of infectious diseases (6). In the absence of specific vaccines and drugs, the selection of appropriate detection techniques for the rapid and accurate identification of pathogens is the most effective means of dealing with infectious diseases, which can improve the efficiency of treatment for infectious diseases and reduce their spread, leading to a rapid response to serious public health events (7).

Traditional diagnostic techniques include microbial culture, hemagglutination inhibition tests and enzyme-linked immunosorbent assays (ELISAs). Of these, the culture of pathogenic microorganisms is the most time-consuming and their identification is mainly based on morphological characteristics, with low specificity and sensitivity. In addition, immunological methods such as hemagglutination inhibition assays and ELISAs, used for the detection of pathogen-specific antibodies or antigens, are simple to perform; however, they have disadvantages such as high false-positives, high cost and poor thermal stability (8,9). With the continuous development

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of genetic and genomic research, molecular diagnostic techniques focused on nucleic acid detection have provided new methods for the diagnosis of infectious diseases, with a short turnaround time and high sensitivity. Molecular diagnostic techniques can not only detect multiple pathogens, but can also analyze drug resistance genes of pathogens and pathogen homology analysis, and have gradually become an important tool in the early diagnosis of infectious diseases (10-12). At present, the commonly used molecular diagnostic techniques for infectious diseases include PCR, isothermal amplification reaction, gene chip technology and high-throughput sequencing technology.

2. PCR

Since 1985, PCR has become the most widely used nucleic acid amplification method for pathogen detection (13). With the development of PCR technology, molecular diagnostic techniques such as quantitative PCR (qPCR), digital PCR (dPCR) and high-resolution melting (HRM) based on the principle of conventional PCR (cPCR) have been widely used for the rapid and straightforward identification and drug resistance detection of known infectious disease pathogens (14-16). PCR performs an important role in the early diagnosis of infectious diseases.

qPCR. qPCR uses fluorescently labeled probes or double-stranded DNA-specific fluorescent dye to qualitatively and quantitatively analyze the fluorescence signal of amplification products in real time without the need to detect PCR products through complex electrophoresis steps (Fig. 1). This method is more automated and has a lower risk of contamination compared with cPCR (17). qPCR has been widely used for the early diagnosis and drug resistance detection of common clinical pathogens (18) and has the advantages of higher sensitivity, specificity, simplicity and rapidity compared with traditional diagnostic methods (19). A study comparing the detection rate of pneumococci using culture-based methods and direct detection by qPCR showed that the detection rate of conventional culture was 41.2%, while the positive colonization rate using qPCR was 43.7%, indicating a higher overall colonization rate of pneumococci using qPCR methods compared with conventional culture methods (20). Ingalagi *et al* (21) detected 200 subgingival plaque samples from patients with chronic periodontitis using qPCR and cell culture simultaneously, and results showed that the positive rate of qPCR and cell culture was 91.5 and 89.5%, respectively, suggesting that the qPCR method had a higher detection rate and clinical application value in the diagnosis of *Porphyromonas gingivalis* compared with the traditional bacterial culture method. Rolon Marrero *et al* (22) developed a qPCR assay for the simultaneous detection of *Helicobacter pylori* and genotypic markers of clarithromycin resistance directly from stool specimens by designing primers and TaqMan probes targeting the 23S rRNA gene of *Helicobacter pylori*. The assay can quickly, accurately and non-invasively diagnose *Helicobacter pylori*, and provide information on genotype susceptibility. This markedly shortens the detection time and helps reduce the use of invasive diagnostic processes, such as endoscopy and biopsy. Although qPCR is faster, more sensitive and more

specific than traditional diagnostic methods, it can only detect one pathogen in a single amplification reaction and is regarded as low-throughput; therefore, to meet the requirements for high-throughput detection, researchers developed multiplex qPCR (MqPCR). MqPCR can simultaneously detect multiple pathogenic infections in a single sample using various sets of primers and probes, which reduces detection time, labor and reagent costs, and sample consumption. MqPCR also has sensitivity and specificity rates comparable to those of qPCR. Bennett and Gunson (23) developed an MqPCR that could simultaneously detect adenovirus, astrovirus, rotavirus and sapovirus in stool samples; this had a reduced turnaround time and overall cost compared with qPCR. Recently, Jiang *et al* (24) developed an MqPCR assay capable of concurrently detecting nine respiratory pathogens with no cross-reactivity and a limit of detection (LoD) of 250-500 copies/ml (1.25-2.5 copies/reaction), which is a promising alternative for the early screening of acute respiratory tract infections; however, qPCR is the preferred method for the quantitative detection of common pathogens in general laboratories. Despite the low cost and mature nature of the technology (Table I), qPCR is prone to nucleic acid contamination, primer dimer formation, improper baseline setting and a number of other issues which can lead to false-positives. Furthermore, sample inhibitors, enzyme inactivation, insufficient enzyme concentration, a low template amount and/or an inappropriate annealing temperature may lead to false-negatives (25). In addition, qPCR is time-consuming, requires relatively complex and expensive instruments to achieve accurate ($\pm 0.5^{\circ}\text{C}$) and rapid ($>10^{\circ}\text{C/s}$) thermal cycles, and requires knowledgeable operators, making this technology difficult to utilize in areas and hospitals with limited access to precision instruments (Table II) (26-28).

dPCR. dPCR performs absolute quantification of target genes in samples by dividing the amplification reaction into thousands of independent sections using microplates, capillaries, oil emulsions or microarrays, amplifying each target gene in separate compartments, distinguishing the generated droplets as negative or positive based on the setting of the fluorescence threshold, and calculating the target gene content through the ratio of negative and positive droplets (Fig. 2) (29). This partitioned amplification reduces template competition, increases the sensitivity of the reaction and allows dPCR to detect low levels of pathogens, minor mutations and rare allele targets (30-33). Therefore, it is particularly suitable for quantifying viruses with sequence diversity and samples with low microorganism content (34), such as BK polyomavirus, human rhinovirus (HRV) and human immunodeficiency virus (HIV). Studies have shown that dPCR can accurately monitor HRV serum viral load with sequence diversity (35) and latent HIV reservoirs (36), and accurately quantify small amounts of human papillomavirus (HPV) in the blood circulation (37) and *Mycobacterium tuberculosis* (MTB) in whole blood samples from infants not exhibiting early respiratory symptoms (38). In addition, dPCR can accurately quantify minor mutations in DNA; for example, it can quantify the frequency of drug resistance gene mutations in influenza viruses and identify mutations in the hepatitis C virus core protein gene. The method is able to detect $<0.1\%$ of rare variants in the wild-type viral background, which is difficult to achieve through the use

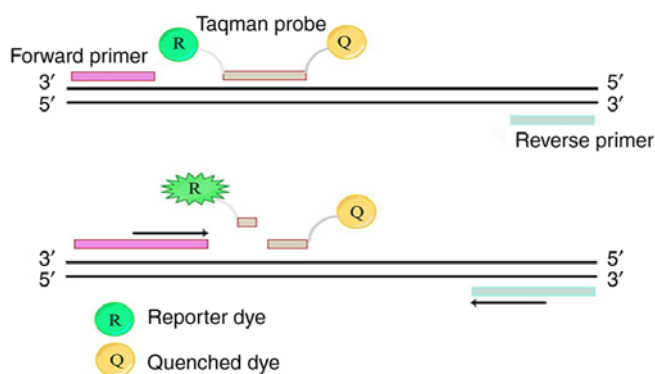


Figure 1. Principle of quantitative PCR. This figure was drawn using Figdraw software online (<https://www.figdraw.com/static/index.html>). The qPCR amplification system contained a pair of primers and a specific fluorescent probe, which labeled with a reporter fluorophore and a quenched fluorophore at each end. When the probe is intact, the fluorescence signal emitted by the reporter group is absorbed by the quenched group. When PCR amplification is performed, the probe is digested and degraded by the Taq enzyme so that the reporter and quenched groups are separated, and the reporter group gives a fluorescent signal.

of other molecular diagnostic techniques (39). Compared with qPCR, the main advantage of dPCR is that it achieves absolute quantification without relying on a standard curve. As a result, dPCR is particularly well suited for quantitative monitoring of low pathogen content during disease incubation or following the administration of medication (Table II). However, the accurate quantification of dPCR relies on the correct threshold setting to distinguish between positive and negative droplets, while the discrimination itself is influenced by a number of factors, such as the quality and quantity of the sample, the melting temperature, and primer and probe length (40). In addition, the instruments and reagents of dPCR technology are expensive, resulting in high detection costs, while the exposed nature of the droplet preparation system leads to an increased risk of contamination and makes it easy to cause false-positives (41) (Table I) (42-44).

HRM. HRM is a novel molecular diagnostic technique developed by Gundry *et al* (45), based on the principle that different double-stranded DNA molecules have different melting temperatures. The technique uses fluorescent dyes or probes to monitor changes in the shape of the melting curve to rapidly and accurately detect and identify various pathogens (Fig. 3) (46). With the advantages of rapidity, and high sensitivity and specificity, HRM is often used for species identification, genotyping and drug resistance gene detection of known pathogens (47). Wen *et al* (48) established a multi-channel real-time fluorescence PCR melting curve analysis, which showed high sensitivity and specificity in the detection of a number of clinically invasive fungi. This method can accurately and rapidly identify *Candida spp.*, *Cryptococcus spp.* and *Aspergillus spp.*, and further genotype *Candida spp.* to facilitate early clinical diagnosis and precise treatment. Banowary *et al* (49) optimized MqPCR and HRM curve analysis to simultaneously detect and distinguish jejunum bacterium from *Escherichia coli*, with a sensitivity and specificity of 100 and 92%, respectively, and the results showed that this technique could accurately and rapidly differentiate

the genotype *Campylobacter jejuni* from *Escherichia coli*. In another study, Tong *et al* (50) developed and evaluated an qPCR HRM diagnostic assay to detect the H275Y mutation, which is responsible for oseltamivir resistance in the H1N1 influenza virus, with a total run time of 62 min. In conclusion, HRM is a rapid, high-throughput and low-cost method for species identification and genotyping. Compared with TaqMan probe-based genotyping and traditional mutation analysis, HRM is simple and does not require the use of probes, so the detection time and cost are lower and the success rate is higher (Table I) (51). HRM is suitable for mutation detection and large-scale analysis of single nucleotide polymorphisms, and its high specificity and sensitivity have been demonstrated in practical applications spanning basic research to clinical diagnosis. In addition, following PCR amplification, the samples are directly subjected to HRM analysis, which completes the closed tube operation and reduces the risk of contamination (Table II) (52). However, HRM has high requirements for the uniformity of instrument temperature and accuracy of primer design, and poor primer design may lead to false-positives (53).

3. Isothermal amplification

Isothermal amplification is a simple nucleic acid amplification technique performed at constant temperature, which does not require a thermal cycler and is therefore simple in terms of temperature control (54). The amplified products can often be monitored by turbidity, color change, lateral flow test strips and fluorescence curves, making it suitable for resource-poor areas and primary healthcare units. To date, loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA) and nucleic acid sequence-based amplification (NASBA) have been used for rapid field detection of infectious disease pathogens (55). However, the target product of the isothermal amplification reaction is short and susceptible to contamination by exogenous genetic material, which can result in false-positives. In addition, isothermal amplification primer design is complex, and to date, there is no dedicated primer design software, which may limit the widespread use of isothermal amplification for the diagnosis of clinical pathogens to some extent (56).

LAMP. Invented in 2000 by Notomi *et al* (57), LAMP is an isothermal nucleic acid amplification technique performed at 60-65°C; its reaction mixture includes *Bacillus stearothermophilus* DNA polymerase, dNTPs, two outer and two inner primers, and reaction buffer containing magnesium ions. LAMP exponentially amplifies DNA by recognizing six or eight specific sequences on target DNA with three or four pairs of primers in the presence of DNA polymerase with a high strand displacement activity (Fig. 4), amplifying DNA up to 10⁹-10¹⁰ times within 15-60 min (58), and is particularly suitable for rapid on-site diagnosis of infectious diseases. To date, with the continuous development of isothermal technology, a variety of novel LAMP technologies have been derived from basic LAMP. These novel LAMP technologies are quicker, more specific and more sensitive than basic LAMP, as well as being better suited for the rapid detection and diagnosis of infectious disease pathogens in resource-poor areas and less well-established facilities (Table II). Vo *et al* (59) developed

Table I. Summary of sample characteristics, detection limits, and costs of common molecular diagnostic techniques.

Method	Sample type/s	Sample preparation	Sample volume, μ l	Limit of detection	Analysis time	Cost	(Refs.)
Quantitative PCR	Blood, urine, feces, secretions and other body fluids	Nucleic acid extraction and purification	200-300	100-500 copies/ml	2 h	Low (<15\$ per sample)	(24,26)
Digital PCR	Blood, urine, feces, secretions, tissue samples and paraffin-embedded samples	Nucleic acid extraction, purification and microtitration	200-300	100-500 copies/ml	2 h	High (>\$70 per sample)	(42,43)
High-resolution melting	Blood, urine, stool, secretions and biopsy specimens	Nucleic acid extraction and purification	200-300	10-20 ng/ μ l	1 h	Low (<15\$ per sample)	(50-53)
Loop-mediated isothermal amplification	Blood, urine, feces, secretions and other body fluids	Treatment with weak alkali or heat lysis (without extraction and purification)	200-300	10 ¹ -10 ² copies/ μ l	15-60 min	Low (<15\$ per sample)	(58-60,63)
Recombinase polymerase amplification	Blood, urine, feces, secretions, plant material, culture media, etc.	Treatment with weak alkali or heat lysis (without extraction and purification)	200-300	10 ¹ -10 ² copies/ μ l	5-30 min	Low (<15\$ per sample)	(70,71)
Nucleic acid sequence-based amplification	Blood, urine, feces, secretions and foods of animal origin	RNA extraction	200-300	10 ² copies/ml	1.5-2 h	Medium (\$15-\$70 per sample)	(74,75,77)
Solid-phase chip	Blood, urine, feces, secretions, tissue samples, etc.	Nucleic acid extraction and purification, PCR amplification and fluorescent labeling	200-300	10 ³ copies/ml	4-6 h	Medium (\$15-\$70 per sample)	(82,87,88)
Liquid-phase chip	Blood, urine, feces, secretions, tissue samples, etc.	Nucleic acid extraction and purification, PCR amplification and bead hybridization	200-300	0.1 pg/ml	35-60 min	Medium (\$15-\$70 per sample)	(89,91)
Next-generation sequencing	Blood, urine, feces, respiratory secretions, cerebrospinal fluid, tissue samples, etc.	Nucleic acid extraction and purification, end modification, addition of connectors, magnetic bead purification, PCR amplification	200-300	10-20 ng/ μ l	24-48 h	High (>\$70 per sample)	(99-101, 105)
Third-generation sequencing	Blood, urine, feces, respiratory secretions, cerebrospinal fluid, tissue samples, etc.	Nucleic acid extraction, purification and construction of a genomic library	200-300	10-20 ng/ μ l	10 min-6 h	High (>\$70 per sample)	(107,110, 114)

Table II. Summary of the application characteristics of common molecular diagnostic techniques.

Method	Principle	Applicability	Advantages	Limitations	False negatives and false positives (Refs.)
Quantitative PCR	Real-time monitoring of PCR progression was performed using fluorescence signal accumulation.	Routine quantitative detection of common pathogens such as novel coronavirus, HBV, human papillomavirus and others in the laboratory.	Good specificity, high sensitivity, high degree of automation, low cost	Numerous interference factors, time-consuming, instruments are expensive	False-positives: Nucleic acid contamination, non-specific amplification. False-negatives: Inhibitors, enzyme inactivation, too little template (17,25, 27,28)
Digital PCR	Amplification reactions were performed for individual nucleic acid molecules in a separate space.	Absolute quantification of low-content pathogens such as HBV, HIV and MTB, etc.	Absolute quantification, low sample requirement, high tolerability	High cost, limited throughput, complex operation	False-positive: Nucleic acid contamination (29,34, 41,44)
High-resolution melting	The different melting temperatures of mononucleotides lead to different melting curves.	Genotyping of pathogens such as <i>Escherichia coli</i> , <i>Staphylococcus aureus</i> , adenoviruses, etc.	Rapid, high throughput, short time, low cost	High requirement for uniformity of temperature and primer design	False-positives: Non-specific amplification (46-50, 52,53)
Loop-mediated isothermal amplification	Isothermal amplification was performed using four specific primers in the presence of <i>Bacillus stearothermophilus</i> DNA polymerase.	Primary field screening of pathogens such as MTB, <i>Plasmodium</i> and Leishmaniasis in low-resource areas	Low instrument requirements, fast reaction speed, low cost, high sample tolerance	Complex primer design, non-specific amplification, low throughput	False-positive: Cross-contamination (57,58, 63)
Recombinase polymerase amplification	A recombinase-primer complex is formed when a recombinase and a primer combine to initiate DNA synthesis.	Rapid on-site detection of pathogens such as novel coronavirus, adenovirus, MTB, etc.	Simple equipment, short assay time, high sample tolerance	No specialized primer design software, non-specific amplification, poor quantitative separation rate	False-positive: Cross-contamination (64-68, 71,72)
Nucleic acid sequence-based amplification	RNA was cycled and amplified by T7 RNA polymerase, RNase H and avian myeloblast virus reverse transcriptase.	Rapid detection of RNA viruses such as influenza virus, HBV and HIV should be carried out in primary hospitals.	High selectivity for RNA molecules, wide range of detection samples	Higher cost, complex reaction components, ineffective for detecting DNA pathogens	False-positive: Non-specific amplification (73,77, 78)
Solid-phase chip	Nucleic acid hybridization combined with fluorescence detection	Large-scale screening is required to determine the pathogen composition of mixed infections.	High throughput, high efficiency, fast integration	High cost, low sensitivity	False-negative: Highly variable pathogens. (80,85, 87,88)

Table II. Continued.

Method	Principle	Applicability	Advantages	Limitations	False negatives and false positives (Refs.)
Liquid-phase chip	Nucleic acid hybridization combined with fluorescence detection and flow cytometry	Large-scale screening for influenza virus, respiratory syncytial virus and novel coronavirus was carried out at entry and exit ports.	High throughput, high sensitivity, fast reaction and small amount of sample	Cross-reactivity	Positive results: (89-91) Cross-reactions
Next-generation sequencing	Sequencing while synthesizing; fluorescence signal	Detection of emerging and rare pathogens such as novel coronavirus, Epstein-Barr virus, <i>Chlamydia psittaci</i> , etc.	Sequence analysis of unknown mutations or nucleic acid fragments	Short sequence read length, high cost, long sequencing run time and complicated process	False-positive: Background nucleic acid and exogenous nucleic acid contamination. (96,99, 102, 105) False-negative: Low pathogen concentration, nucleic acid degradation,
Third-generation sequencing	Single-molecule fluorescence sequencing; nanopore sequencing single-molecule sequencing based on electrical signals	Real-time field analysis and epidemic surveillance of 2019 novel coronaviruses, Ebola virus, adenoviruses and other viruses	Long read length, real-time sequence information, fast running speed and portable sequencing device	Lower throughput, higher error rate and higher cost	False-positives: Interference of background nucleic acid, contamination by exogenous nucleic acid and mixing of colonized bacteria (105, 106, 114)

HIV, human immunodeficiency virus; HBV, Hepatitis B virus; MTB, *Mycobacterium tuberculosis*.

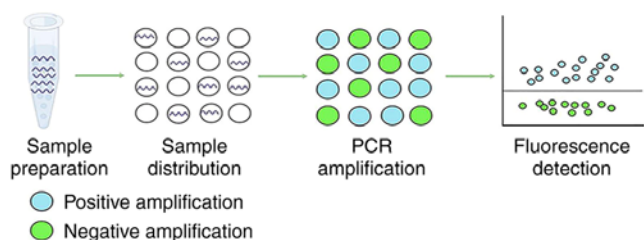


Figure 2. Principle of digital PCR. This figure was drawn using Figdraw software online (<https://www.figdraw.com/static/index.html>). The process of digital PCR mainly includes sample preparation, sample distribution, PCR amplification, and fluorescence signal detection and analysis. The EP tube contains nucleic acid extracted from the sample to be tested. First, the sample to be tested was microdropped to make ~20,000 small droplets of water in oil, and then the microdrop system was amplified. Finally, the fluorescence signal of each microdrop was detected, and the data was analyzed.

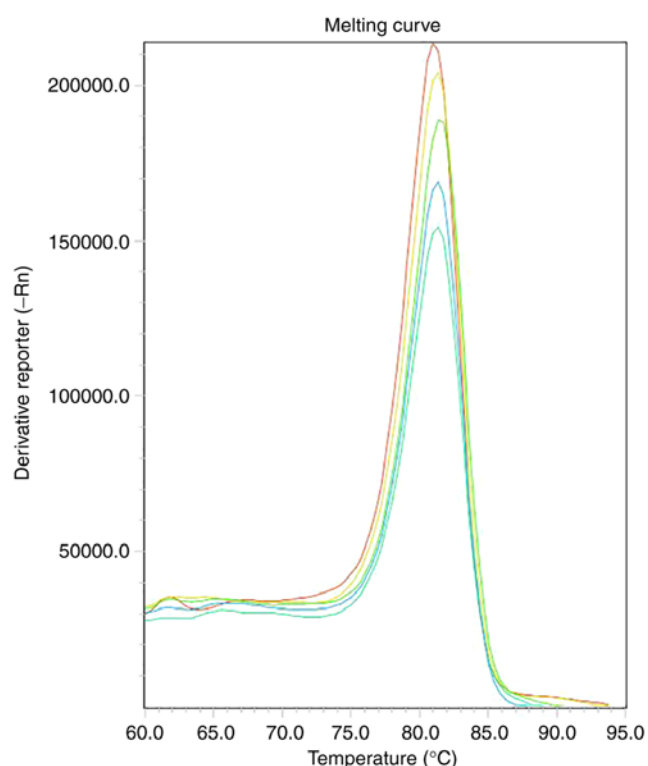


Figure 3. Schematic representation of high-resolution melting. Based on the principle that different shapes of melting curves are formed by different melting temperatures of single nucleotides, the specific dsDNA fluorescent dye is used to generate different shapes of melting curves to detect the samples. The different colored lines represent the high-resolution melting curves of different samples.

a colorimetric LAMP that can detect HPV DNA in oral rinse samples and can easily distinguish between two high-risk HPV subtypes, HPV-16 and HPV-18, enabling the rapid detection of HPV subtypes in primary hospitals. Chen *et al* (60) produced a novel reverse-transcription LAMP assay for the rapid detection of major pathogens in upper respiratory tract infections based on real-time monitoring with SYBR Green I. Experimental results showed that the LoD of this method was 10^2 copies/ml RNA, which is similar to that of qPCR. No cross-reactivity was observed in the 10 studied viruses, making it suitable for the rapid diagnosis of upper respiratory

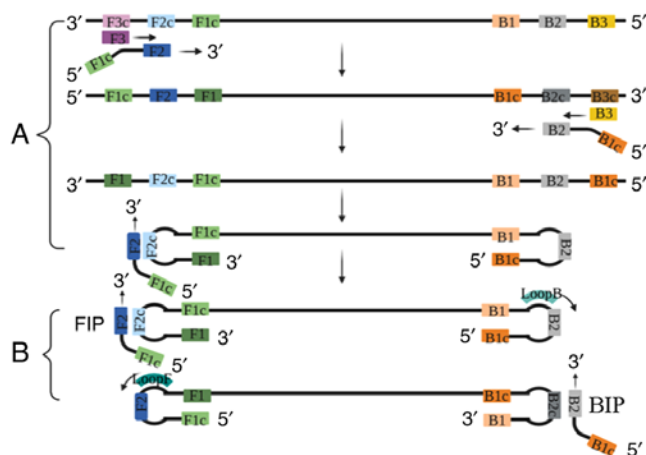


Figure 4. Principle of loop-mediated isothermal amplification. This figure was drawn using biorender software online (<https://app.biorender.com/>). (A) The initial phase. The F2 sequence of FIP binds to F2c, and F3 binds to F3c and extends to displace the complete complementary single strand. F1c on FIP and F1 on this single strand are complementary structures that self-base pair to form a ring structure. BIP and B3 successively initiate strand synthesis, similar to FIP and F3. (B) The amplification cycle phase. Using the stem-loop structure as a template, FIP binds to the F2c region of the stem-loop to initiate strand displacement synthesis, and B2 on the BIP primer binds to B2 to initiate a new round of amplification. Two loop primers, LooF and LooB also combined with the stem-loop structure to initiate strand displacement synthesis, respectively, and the cycle began again.

tract infections in primary hospitals and laboratories. In another study, Kim *et al* (61) designed two sets of specific primers and probes to develop a multiplex LAMP assay for the differential detection of MTB and non-tuberculous mycobacteria (NTM), with an analytical sensitivity of 100% for MTB and 98.4% (121/123) for NTM, and 100% for detection specificity compared with acid-fast staining and culture methods. This multiplex LAMP assay has a higher sensitivity and specificity than traditional LAMP assays for MTB and NTM, and is expected to serve as a new tool for the rapid detection and differentiation of MTB from NTM. Previously, Phillips *et al* (62) combined LAMP technology with lateral flow immunoassay (LFA) and a computer or cellphone to develop a rapid, low-cost, real-time, autonomous microfluidic analysis device that can detect HIV-1 viral particles in as low as 3×10^5 cells/ml or 2.3×10^7 copies/ml HIV in whole blood. This integrated device enables the early detection of HIV viral load from the whole blood of HIV-infected patients in clinical laboratories within primary care facilities. These novel LAMP technologies combine thermostatic amplification with visual detection, eliminating the need for amplifiers and detectors and further reducing the detection costs (Table I). However, complex primers and high false-positive rates may limit their application to a certain extent.

RPA. RPA is a sensitive, specific and rapid isothermal amplification method that was first reported in 2006 (63). RPA, whose core components include DNA polymerases and DNA-binding proteins and recombinases, expands target DNA from as low as 1-10 copies of target DNA in a single reaction to detectable levels within 30 min at 37-42°C (Fig. 5). This method is advantageous since it does not require amplification apparatus, has a short detection time and high

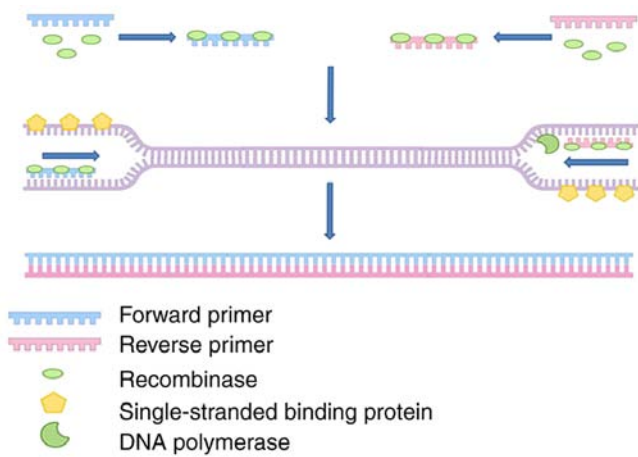


Figure 5. Principle of recombinase polymerase amplification. This figure was drawn using Figdraw software online (<https://www.figdraw.com/static/index.html>). The recombinase combines with the primer to form a primer-recombinase complex, which searches for homologous sequences in the double-stranded DNA for strand exchange, followed by DNA synthesis under the action of DNA polymerase, and the single-strand binding protein stabilizes the DNA single strand.

sample tolerance, and has been successfully combined with different assays for rapid field detection of infectious disease pathogens. The most commonly used endpoint detection method for RPA is LFA (64–67). Qi *et al.* (68) successfully established a universal typing method for the detection of human adenovirus by combining RPA technology with lateral flow test strips, which allows for detection within 25 min using only basic constant temperature equipment. This method has good detection performance and is suitable for the rapid detection of human adenovirus in resource-limited areas. Mayran *et al.* (69) combined rapid DNA extraction with isothermal RPA and monitored the results by LFA, developing a new RPA-LFA screening method to detect Hepatitis B virus (HBV) DNA in pregnant women with hypervolemia. Achieving a sensitivity and specificity of 98.6 and 88.2%, respectively, this new RPA-LFA method allowed for the rapid detection of HBV. To further optimize and improve the throughput of RPA-LFA, Li *et al.* (70) developed a seven-fold assay using different markers, which further reduced detection time and cost, making it suitable for the rapid field detection of infectious diseases (Table I) (71). To promote the application of RPA for in-field detection of infectious diseases, it is necessary to develop sample preparation methods and portable or fully automatic RPA diagnostic equipment that are also suitable for field detection in areas with poor medical infrastructure (Table II) (72).

NASBA. NASBA is an efficient, isothermal amplification method developed by Compton in 1991 (73). The amplification reaction was performed at 41°C, and the 100- to 250-bp nucleic acid target sequence was amplified $\sim 10^{12}$ times within 90 min (74). The NASBA reaction mixture involves three enzymes, T7 RNA polymerase, RNase H and avian myeloblast virus reverse transcriptase, which selectively and rapidly amplify RNA in the presence of background DNA, with good sensitivity, making NASBA most suited for the analysis of RNA samples (Fig. 6).

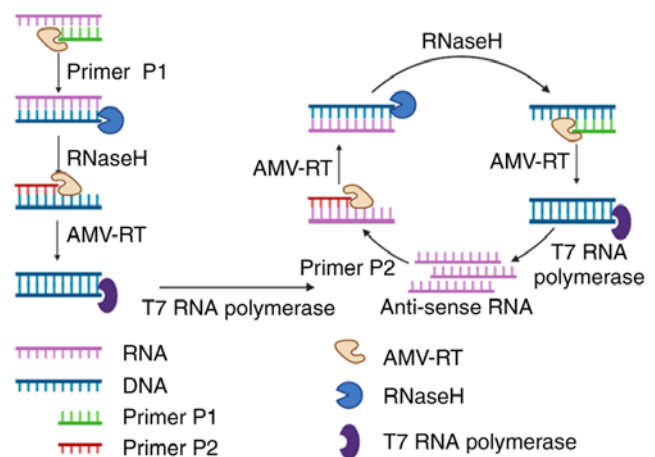


Figure 6. Principle of nucleic acid sequence-based amplification. This figure was drawn using biorender software online (<https://app.biorender.com/>). Nucleic acid sequence-based amplification uses three enzymes (RNaseH, AMV-RT and T7 RNA polymerase) and two primers (primer P1 and primer P2). After RNA extraction of the sample, it enters the cyclic amplification process through primers (primer P1 and primer P2) and two successive reverse transcriptase reactions and the product was directly single-stranded anti-sense RNA (anti-sense RNA).

During the COVID-19 pandemic, Kia *et al.* (75) developed a reverse transcription-NASBA (RT-NASBA) assay for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA using molecular beacon probes based on nucleocapsid and RNA-dependent RNA polymerase genes, with an LoD of 200 copies/ml (Table I). Compared with the Sansure RT-qPCR US Food and Drug Administration (FDA)-approved kit (Sansure Biotech, Inc.), its clinical sensitivity was 97.64%, which renders it a simpler and faster detection method for SARS-CoV-2. Yrad *et al.* (76) developed an NASBA-based LFA device that can detect dengue virus RNA at concentrations of as low as 0.01 μM within 20 min, with an LoD of 1.2×10^4 PFU/ml in the sera of patients with a dengue virus infection. This method has broad applications and will be especially useful for dengue virus detection in resource-limited areas. However, it should be noted that the NASBA amplification efficiency is low when the nucleotide sequence is >250 or <120 bp (Table II) (77). In addition, due to the low temperature requirements of the NASBA reaction, it is easy for primer dimerization and non-specific amplification to occur, which markedly increases the false-positive rate (78).

4. Gene chip technology

Gene chip technology mainly includes traditional solid- and liquid-phase chips; it is based on the principle of nucleic acid molecular hybridization. Gene sequences in samples are detected by hybridization between target sequences and probes fixed on different materials. Multiple pathogens can be simultaneously detected and identified, and clinicians can be quickly provided with multiple pathogen information (79). However, this technology requires a large amount of known pathogen genetic information as a basis and can only be used for the intentional screening of known pathogen genomes; it cannot detect novel pathogens.

Solid-phase chip. Solid-phase microarrays use specific probes attached to solid supports to detect labeled target molecules in solution, and can detect and analyze a large number of pathogens simultaneously, which effectively shortens detection time (Fig. 7A). It is a high-throughput molecular diagnostic tool suitable for the detection of multiple infectious disease pathogens and drug resistance gene analysis (80). Nasrabadi *et al* (81) developed 16S and 23S rDNA-based probes able to simultaneously detect and identify eight food-borne bacterial pathogens. Ma *et al* (82) developed and evaluated a solid-phase chip for the simultaneous detection of 15 types of bacteria directly from respiratory tract specimens of patients with pneumonia, thus reducing detection time, facilitating the early administration of antimicrobial drugs and preventing bacterial resistance caused by empirical antibiotic treatment. Recent studies have shown that the CapitalBio DNA chip (CapitalBio Corporation) can detect the resistance of MTB to rifampicin and isoniazid, and the corresponding gene mutations within 6 h, which is quicker and more accurate than traditional bacterial culture and drug susceptibility tests (83-85). Currently, a number of automatic detection platforms based on solid-phase chips are entering the market. For example, FilmArray (BioFire Diagnostics) is an integrated platform that combines fully automated sample preparation, nucleic acid extraction, PCR amplification and automatic detection, which can detect >100 different nucleic acid targets at a time and can identify a variety of common respiratory pathogens from a single sample within 1 h (86). This technique is often used for the qualitative detection of common pathogens such as influenza virus, adenovirus, *Streptococcus pneumoniae* and other common respiratory tract pathogens, as well as common pathogens such as *Norovirus*, *Rotavirus*, *Salmonella*, *Shigella* and other common intestinal infection-causing pathogens (Table II). The technology is especially useful in a clinical setting for determining the pathogen composition of mixed infections and for large-scale screening of infectious diseases (87). However, solid-phase chip technology has certain limitations. First, the use of fluorescent oligonucleotides represents a significant cost (Table I). In addition, the reference sequence information of the pathogen is usually used to design the array, so it may lead to false-negatives for highly variable pathogens (88).

Liquid-phase chip. Liquid-phase suspension chip technology couples oligonucleotide probes to fluorescent microspheres with different proportions of color configurations and classifies them according to their internal colors using laser detection and flow cytometry to achieve high-resolution automated detection (Fig. 7B) (89). Liquid-phase chip technology is commonly used in gene expression analysis, microRNA analysis, single nucleotide polymorphism analysis, specific sequence analysis and microbial detection. For pathogen detection, liquid chip technology can simultaneously identify and genotype a variety of pathogens in a single complex sample, and the technology exhibits high sensitivity, high throughput and high automation (90); it is suitable for large-scale screening of infectious diseases at entry and exit ports (Table II) (91). Currently, its representative technology, xMAP[®] technology (Luminex Corporation), has been approved by the FDA for the multiplexed detection of pathogens such as viruses, bacteria, parasites and fungi, allowing for the rapid diagnosis of a single

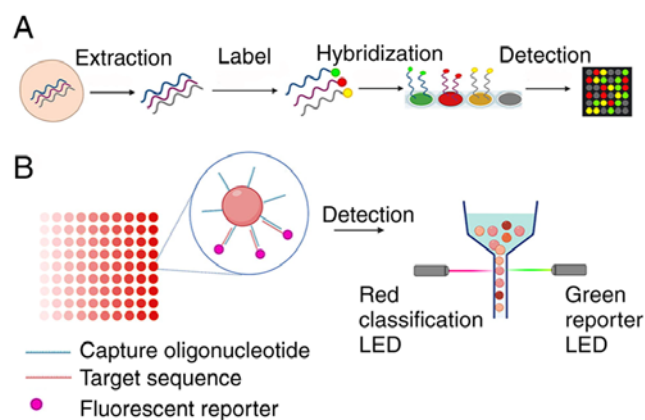


Figure 7. Principle of gene chip. (A) Solid-phase chip. (B) Liquid-phase chip. This figure was drawn using biorender software online (<https://app.biorender.com/>). (A) Solid-phase chip arranges a large number of nucleic acid molecules on an array carrier, hybridizes with the labeled nucleic acid to be tested in the sample and detects the strength of the hybridization signal to determine the number of nucleic acid molecules in the sample. (B) Liquid-phase chip couples oligonucleotide probes to fluorescent microspheres with different color configuration ratios and analyzes their internal color and fluorescence signals by flow cytometry and laser detection to achieve high-resolution automated detection.

sample with good detection performance. One of these systems, the xTAG[®] Respiratory Viral Panel (Luminex Corporation), is a commercial kit for detecting multiple respiratory virus nucleic acids in human nasopharyngeal samples simultaneously. In addition to respiratory viruses, microsphere-based multiplex nucleic acid detection has been successfully used for the detection of various bacteria, viruses and parasites in human fecal samples. One of these systems, the xTAG Gastrointestinal Pathogen Panel (Luminex Corporation), is a multiplex nucleic acid detection kit designed to rapidly detect various bacterial, viral and parasitic nucleic acids in human fecal samples, with an overall sensitivity and specificity of 96.3 and 99.8%, respectively (92).

5. High-throughput sequencing

Gene sequencing, including first-, second- and third-generation sequencing (TGS), is the most accurate method of identifying infectious pathogens; it has been successfully used for the diagnosis of known pathogens and the identification of unknown pathogens. First generation sequencing, represented by Sanger sequencing, is mainly used for targeted sequencing to reveal the sequences of several specific low-throughput sites, which is only suitable for small-scale analysis (93). With the completion of the Human Genome Project in the early 21st century and the rapid development of sequencing technology, high-throughput and low-cost next-generation sequencing (NGS) and TGS technologies have emerged (94,95).

NGS. NGS can read billions of nucleotide sequences in a single assay, it does not rely on cell culture and it retrieves all DNA (Fig. 8A); it can also comprehensively detect microbial species and sequences (96), which makes it suitable as a complementary means of pathogen detection when no clear etiological evidence is available from routine laboratory testing (Table II).

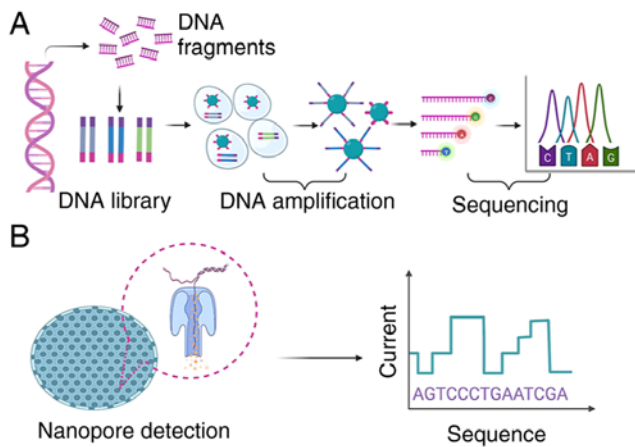


Figure 8. Principle of sequencing technologies. (A) Next-generation sequencing. (B) Third-generation Sequencing. This figure was drawn using biorender software online (<https://app.biorender.com/>). (A) Next-generation sequencing uses fluorescence with different colors to label four different dNTPs. When the complementary strand is synthesized by DNA polymerase, different fluorescence is released with each addition of dNTP. The sequence information of the DNA to be tested is obtained by analyzing the captured fluorescence signal. (B) Third-generation sequencing uses electrophoresis technology to drive a single nucleic acid molecule through a nanopore and identifies the nucleic acid sequence by detecting the change in current caused by different bases of nucleic acid passing through the nanopore.

For the identification and genotyping of known pathogens, NGS is significantly more sensitive than traditional methods. Zhang *et al* (97) used NGS technology to diagnose bacterial meningitis in patients. By comparing bacterial culture with the Alere BinaxNow[®] *Streptococcus pneumoniae* Antigen test (Abbott Rapid Diagnostics), it was found that the sensitivity and specificity of NGS for the diagnosis of bacterial meningitis was 70.3 and 93.9%, respectively. The positive and negative predictive values were 81.4 and 91.3%, respectively, which revealed high sensitivity and specificity for the identification of *Streptococcus pneumoniae*. A study found that in the detection of pulmonary infectious disease pathogens, the positive rates of bacterial culture and NGS (a measure of the sensitivity of detection between culture and NGS) were 17.54 and 42.11%, respectively. In addition, 94.49% of other pathogens associated with human infectious diseases were detected by NGS in samples from patients with pulmonary infection who tested negative for traditional pathogens, suggesting that NGS could detect and identify multiple pathogens simultaneously with higher accuracy (98). According to NGS results, not only pathogen identification and typing, but also drug resistance gene detection, virulence gene detection and host immune response analysis, can be performed (94), thus guiding clinical diagnosis, disease treatment, and vaccine research and development. In addition, NGS can detect pathogens and identify rare unknown pathogens from different types of biological samples that cannot be detected by conventional assays, such as *Chlamydia psittaci* in bronchoalveolar lavage fluid (99), *Naegleria flexneri* and *Brucella* in cerebrospinal fluid (100), and the Chikungunya and mumps viruses in the blood (101), which allows for the timely diagnosis of rare pathogens, and promotes early and accurate treatment. NGS is suitable for the rapid identification of emerging and re-emerging pathogens, whole-genome sequencing, genomic variation and

evolution, and epidemiological investigation and tracking. NGS is a powerful tool for tracking the source and chain of transmission of epidemics and for monitoring the evolution of pathogens (102); it allows comprehensive access to pathogen genome information in a short period of time, enabling researchers and healthcare providers to rapidly respond to infectious diseases. However, there are several challenges in the practical application of clinical pathogen diagnosis, such as high purity and high concentrations of nucleic acids for nucleic acid processing in the preparation stages, the need for PCR amplification, the inability to directly detect RNA, the short read length and the need to use special bioinformatics tools for complex data analysis (103). The biggest obstacle, however, is interpreting complex sequencing data for pathogen determination (104,105).

TGS. TGS, represented by the Oxford Nanopore Technologies (ONT) MinION (ONT), was launched in 2015 (106); it combines genetic engineering and computer-aided technology to determine the base sequence by detecting the changes in electrical current as DNA or RNA passes through the nanopore (Fig. 8B). The high-throughput, rapid DNA sequencing technology produces ultralong reads without the need for labeling, which simplifies the detection process and reduces costs (107). TGS has a number of advantages over NGS. NGS cannot directly detect RNA and has a short read length in the field of pathogen diagnosis. Furthermore, TGS can directly carry out RNA sequencing without a reverse transcription process, which shortens detection time. Keller *et al* (108) described direct RNA sequencing of five influenza A virus genomes, with 100% nucleotide coverage and 99% agreement between Nanopore and Illumina Inc.-based sequencing results by modifying the RNA sequencing method published by Oxford Nanopore Technologies, Ltd. Recently, TGS has been used for the RNA sequencing of SARS-CoV-2, where it can detect SARS-CoV-2 and other respiratory viruses simultaneously within 6-10 h (109), markedly reducing detection time compared with NGS. In addition, TGS sequencing has significantly increased read length capabilities, enabling complete gene sequencing and identifying novel full-length transcript variants and gene fusions that cannot be detected by NGS (110), thus improving the likelihood of detecting and identifying pathogenic species. At present, portable sequencing devices based on the Nanopore system have been used for real-time on-site analysis and out-of-hospital bedside detection of novel coronavirus, Ebola virus, adenovirus and a number of other viruses (111-113). They are suitable for epidemic surveillance and virus mutation monitoring in resource-limited situations and also for the identification of rare and difficult-to-culture bacteria (Table II) (105). However, the high error rate of TGS is the biggest obstacle to its application in the field of microbial detection. Nonetheless, with continued optimization of nanopore structures and base-calling algorithms, and with improvements in sequence acquisition speed and base recognition accuracy, TGS is expected to become an ideal tool for the detection of known pathogens and the discovery of rare and unknown pathogens (114).

6. Other molecular diagnostic techniques

Biosensing technology uses a combination of target biomarkers and ionic conductive materials to generate signals, which are detected and analyzed by sensors (optical, electrochemical or piezoelectric) and reading devices (115). Currently, the use of photoelectric biosensing of nucleic acids is increasing in popularity due to its sensitivity and speed for the early diagnosis and quantitative analysis of infectious diseases. Sheng *et al* (116) developed a label-free biosensor with an RNA aptamer for the sensitive, rapid quantitative detection of food pathogens without the isolation, purification and enrichment processes. A further study found that optical label-free biosensors can detect and quantify MTB, mycobacterial proteins and interferons quickly and efficiently, making them beneficial for the early detection of tuberculosis (117). Fluorescence *in situ* hybridization (FISH) is a molecular diagnostic technique used to detect and localize specific nucleic acid sequences in cells. To improve throughput, FISH can be used in combination with flow cytometry to detect target nucleic acid sequences in thousands of individual cells. Flow cytometry-based FISH (flow-FISH) uses fluorescent probes that target DNA or RNA to detect specific genes or pathogens; it can also be multiplexed so that multiple gene targets or pathogens can be measured simultaneously (118). Flow-FISH has been used for bacterial identification and detecting gene expression, for monitoring viral multiplication in infected cells, and for colony analysis and counting. Recently, the use of *in vivo* bacterial sorting technology assisted by flow-FISH has made it easier to isolate, classify and purify live bacteria based on target genes, and to study the role of target genes in the growth, substance metabolism, bacterial virulence and antibiotic resistance of bacteria (119). Mass spectrometry analysis of the molecular mass and charge of biomarkers will improve the quality of the model compared with the reference spectra and can be used for the identification of pathogenic microorganisms at the species and genus levels; with its high accuracy and signal acquisition speed, it is expected to become a routine tool for rapid clinical analysis of multiple pathogenic microorganisms in a single sample (120,121).

7. Conclusion

In conclusion, molecular diagnostic technology provides an improved choice for the diagnosis of infectious diseases compared to traditional diagnostic techniques, such as microbial culture, hemagglutination inhibition tests, and ELISA. The careful selection and combination of different molecular diagnostic technologies according to a user's needs can provide a timely and accurate diagnosis of infectious disease pathogens and facilitate precision treatment, to effectively control diseases. qPCR technology is mature, low-cost and suitable for the qualitative and quantitative analysis of common pathogens in standard laboratories. dPCR can be used for the absolute quantification of target genes in samples, and is particularly suitable for the analysis of samples containing low pathogen levels, and the detection of small mutations and rare allele targets. As a fast, high-throughput and cost-effective technique, HRM is often used for mutation detection and large-scale analysis of single nucleotide polymorphisms.

Isothermal PCR can be used for nucleic acid amplification at a constant temperature, which does not require a thermocycler, and is more suitable for the rapid detection of pathogens in resource-limited areas and primary medical units. Gene chip technology has the ability to detect and identify multiple pathogens simultaneously, which is particularly useful in clinical settings for the pathogenic composition determination of mixed infections. However, this technology can only screen for the genomes of known pathogens and cannot detect new, unknown pathogens, unlike gene sequencing technology, which can comprehensively detect the types and sequences of pathogens. The molecular diagnostic techniques outlined in the present review need further improvement. First, nucleic acid extraction and purification steps in molecular diagnostic techniques are cumbersome. Therefore, it is essential to streamline the existing nucleic acid extraction procedures or develop molecular techniques to avoid nucleic acid extraction. Second, most molecular diagnostic reagents require low-temperature transport and storage, which increases the cost of molecular diagnostics and hinders their application in remote or resource-limited areas, so ready-to-use, room temperature-stable reaction mixtures need to be studied to reduce costs and increase their applicability in these areas. Finally, molecular diagnostic techniques such as qPCR, dPCR and sequencing are instrument-dependent, meaning that rapid on-site detection of pathogens in resource-limited conditions can prove difficult. Continuous improvement of molecular diagnostic technology will help to create more high-throughput, automated and portable instruments with high sensitivity and specificity to aid in the rapid diagnosis and treatment of infectious diseases worldwide.

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Authors' contributions

YZD, YJZ, QQL and XJJ developed the manuscript concept and produced the initial draft. JC and HJZ provided valuable comments on this first draft. YZD, YJZ, QQL, XJJ, JC and HJZ critically revised the manuscript for intellectual content. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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