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Research Progress of Nucleic Acid Detection Technology Platforms for New Coronavirus SARS-CoV-2

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Abstract: The new coronavirus SARS-CoV-2 has spread to the whole world, seriously threatening human life and disrupting people’s lives. SARS-CoV-2 is a highly infectious virus with a long incubation period, encompassing asymptomatic infections. Therefore, accurate detection of SARS-CoV-2 is essential for the prevention and control of the epidemic. Currently, nucleic acid detection has played an important role in the prevention and control of SARS-CoV-2. A variety of nucleic acid detection technologies for SARS-CoV-2 have been developed, and some technologies have been converted into available kits for clinical detection. However, these technologies have different principles and different detection platforms. How to choose the appropriate nucleic acid detection technology for SARS-CoV-2 perplexes epidemic prevention and control. Based on the latest research progresses of nucleic acid technologies for SARS-CoV-2, this paper introduced the principles and technology platforms of these detection technologies, thoroughly compared the advantages and disadvantages of each technique and clarified the scope of applications, providing a reference for selecting the appropriate nucleic acid detection technology for SARS-CoV-2. Furthermore, this paper provided a prospect for developing technologies of detecting pathogens similar to SARS-CoV-2.

Key Words: SARS-CoV-2; Nucleic acid detection; Technical principle; Review

1 Introduction

Since a cluster of unexplained pneumonia infections occurred at the end of December 2019[1–3], the new coronavirus has spread rapidly around the world. According to statistics from the World Health Organization, as of May 1, 2020, the number of people infected with the new coronavirus has exceeded 3 million, and the epidemic has affected 215 countries and regions[4], causing a great threat to human health and life worldwide.

The new coronavirus is a positive-strand RNA virus with a 79.6% sequence similarity to SARS-CoV[5]. On February 11, 2020, the International Committee for Classification of Virology temporarily named the new coronavirus SARS-CoV-2. The diseases it causes are collectively referred to as COVID-19 (Coronavirus disease 2019)[6]. Although patients infected with SARS-CoV-2 show clinical symptoms similar to SARS-CoV and MERS-CoV infections, such as fever, dry cough, dyspnea, and bilateral ground-glass density shadows, and the virus has a long incubation period as well as asymptomatic infected persons[7], the difference is that the upper respiratory tract symptoms of most SARS-CoV-2 infected patients are not very obvious[8]. In addition, the binding force between S protein on the surface of
SARS-CoV-2 and the human receptor angiotensin-converting enzyme 2 (ACE2) is much higher than that of SARS-CoV[9], which means that SARS-CoV-2 is more infectious than SARS-Cov, providing the molecular basis for SARS-CoV-2 infection. Due to the influence of various factors, there were huge difficulties in the early detection of the epidemic and its prevention and control work. Consequently, the number of SARS-CoV-2 infections has increased exponentially.

In the absence of specific drugs and vaccines, accurate and quick identifying SARS-CoV-2 infected persons and timely isolation is the most effective means of epidemic prevention and control[10]. Notably, nucleic acid testing has played a vital role in the prevention and control of this epidemic, especially for the diagnosis of asymptomatic infections and the judgment of discharged patients during the recovery period. SARS-CoV-2 nucleic acid testing has become the main standard[11]. Therefore, the development of convenient and accurate SARS-CoV-2 nucleic acid detection techniques is particularly important for epidemic prevention and control.

Since the outbreak of the epidemic, a variety of SARS-CoV-2 nucleic acid detection techniques have been developed. These techniques have different principles and detection platforms, which have their respective advantages, disadvantages and scope of application. This article introduced and compared the existing SARS-CoV-2 nucleic acid detection technologies according to different detection platforms, providing a reference for the clinical selection of appropriate SARS-CoV-2 nucleic acid detection technology according to local conditions. We also put forward a prospect for establishment of a safer, faster and more accurate technology platform for pathogen nucleic acid detection in the future.

2 SARS-CoV-2 nucleic acid detection technique based on different platforms

2.1 SARS-CoV-2 nucleic acid detection technique based on high-throughput sequencing platform

High-throughput sequencing technology has become an important tool for nucleic acid detection in recent years due to its advantages in direct obtaining genome sequence with high throughput. Many sequencing platforms have emerged, such as 454 pyrosequencing[12], Solexa sequencing[13] and other next generations sequencing platforms, as well as three-generation sequencing platforms such as SMRT sequencing[14] and nanopore sequencing[15]. These platforms have been widely used in disease diagnosis[16], microbial community analysis[17], pathogen identification[18], etc. The pathogen leading to this epidemic was also discovered by analyzing the bronchoalveolar lavage fluid of the early admitted patient through the next-generation sequencing platform (Fig.1). Sequencing results showed that the sequence identity between SARS-CoV-2 and SL-CoVZC45 virus reached 89.1%[9]. Thanks to high-throughput sequencing technology, researchers obtained the complete sequence information of SARS-CoV-2 when the samples were obtained in the early stage of the epidemic, which provided the basis for the subsequent establishment of rapid detection technologies.

The biggest advantage of high-throughput sequencing platform is that it can discover unknown pathogens and obtain the full gene sequences. Therefore, it is very suitable for identifying pathogens in the early stage of an outbreak and providing sequence information for the further development of pathogen nucleic acid detection technologies. In addition, using high-throughput sequencing technology could track the gene mutation in real time, which plays an important role in pathogen traceability, vaccine development, and disease epidemic trend prediction.

However, when high-throughput sequencing platforms are used for pathogen detection, they are often difficult to be popularized because of the expensive sequencing instruments, long detection cycles, and high detection costs. Compared with other sequencing platforms, nanopore sequencing technology has a longer read length and perform direct RNA sequencing. Moreover, the time from sequencing to a result could be reduced from several days to several hours by nanopore sequencing, outputting sequencing results in real time. The instrument in nanopore sequencing is compact, and thus it has certain advantages in pathogen detection. Recently,
a joint team formed by Prof. Tiangang Liu of Wuhan University and others announced a targeted multiple respiratory pathogen detection method\textsuperscript{[20]}, which could detect a variety of respiratory viruses including SARS-CoV-2 within 6 to 10 h based on MinION nanopore sequencing platform. With the continuous advancement of sequencing technology, it is believed that a faster, easier, and more accurate sequencing technology will come out soon, which will further promote the application of sequencing platforms in pathogen detection. Although nanopore sequencing technology has reduced the single run cost of high-throughput sequencing from tens of thousands of RMB to less than 10,000 RMB, the cost of detection is still high for clinical applications. Therefore, the current sequencing platforms are difficult to achieve low-cost and rapid detection of SARS-CoV-2, and has not been widely used in the prevention and control of this epidemic. However, the positions of high-throughput sequencing technology in the research of SARS-CoV-2 mutation, traceability and mechanism are still irreplaceable.

\subsection*{2.2 SARS-CoV-2 nucleic acid detection technique based on real-time PCR platform}

Compared with high-throughput sequencing platform, real-time PCR platform could achieve sensitive and quantitative detection of target nucleic acids with known sequences by a compact real-time fluorescent quantitative PCR instrument. Because of its stability, reliability and easy operation, real-time PCR platform has become the most widely used nucleic acid detection platform. When the epidemic broke out, scientific research institutions and technology companies quickly developed SARS-CoV-2 nucleic acid detection techniques and kits based on the real-time PCR platform. Most of these techniques are based on fluorescent probes to detect the amplified target nucleic acid. The basic principle is shown in Fig.2. Firstly, the virus genome is changed from RNA sequence into DNA sequence by reverse transcription, and then PCR amplification is performed. During the amplification process, when the target-specific Taqman probe hybridizes with the target sequence, the labeled fluorophore in the Taqman probe will be cleaved by the exonuclease activity of DNA polymerase to generate a fluorescent signal. The signal is recorded by the instrument at each PCR cycle to form an amplification curve, and the target can be quantitatively detected according to the time when the amplification curve rises\textsuperscript{[21]}. Most of the currently used SARS-CoV-2 nucleic acid detection kits based on the real-time PCR platform target the gene sequences of open reading frame 1a/b (Opening reading frame 1ab, ORF1ab), nucleocapsid protein (Nucleocapsid protein, N) and Envelope protein (Envelope protein, E) to design primers and probes\textsuperscript{[22]}, and the detection sensitivity is 200–1000 copies/mL.

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{Fig2}
\caption{Principle of RT-PCR (Taqman probe technology)}
\end{figure}

Since real-time PCR platform has the advantages of high sensitivity, good specificity, simple operation, etc., and large medical institutions are commonly equipped with related instruments, it is currently the main technology platform used for SARS-CoV-2 nucleic acid detection. However, nucleic acid extraction and amplification detection are usually carried out separately in the conventional real-time PCR platform, requiring highly specialized laboratories and trained operators. In addition, real-time PCR platform relies on a fluorescent quantitative PCR instrument with 2–4 h of detection time, not suitable for on-site screening and testing in resource-limited areas. Since the detection sensitivity is not sufficient for samples with virus load as low as limited by the kinds of fluorescent labels, the number of target copies, false-negative results often occur. Moreover, gets that could be detected is limited in one reaction, making it difficult to achieve multiplex combination screening for pathogens.

\subsection*{2.3 SARS-CoV-2 nucleic acid detection technique based on digital platform}

Except the factors of sample storage and extraction, the main reason for weak-positive or false-negative results in SARS-CoV-2 nucleic acid detection based on real-time fluorescent PCR is the insufficient sensitivity of the detection technique itself. When the viral concentration is as low as the order of a single copy, a detection technique with single-molecule detection sensitivity is required. Digital nucleic acid detection platform is a platform that can directly count target copies\textsuperscript{[23]}. Digital platform uses a microdroplet
generation system or a micro-reaction unit array chip to divide the nucleic acid amplification detection system (usually PCR) into tens of thousands of uniform small reaction units (Fig.3), each reaction unit has only one or zero target nucleic acid molecule. After the amplification reaction, a negative result or positive result of each reaction unit is counted. According to the Poisson distribution, the initial copies of the target nucleic acid could be obtained. Therefore, nucleic acid detection techniques based on digital platform could accurately quantitate the target nucleic acid with initial concentration as low as a single copy, which solve the false-negative problem of SARS-CoV-2 detection due to the low viral load of samples.

Digital platform that has been used for SARS-CoV-2 detection is mainly the chip-based droplet digital PCR (ddPCR) technology. After RNA template is added into the reaction system, the system undergoes a one-step reverse transcription PCR reaction. Amplification products of ORF1ab gene, N gene of SARS-CoV-2 and internal control gene are reported in each reaction unit. Finally, it could detect SARS-CoV-2 with high sensitivity and specificity by calculating the number of positive microdroplets within 2.5 h. A recent study has compared the detection results of 323 patients with new coronavirus infection by ddPCR and RT-PCR (the target genes of both methods are ORF1ab gene and N gene), and found the consistency of the two methods in 95 samples with high viral load was 100%. However, inconsistent test results were found in samples with low viral load. Among 67 samples that were single-gene positive results by RT-PCR, 41 samples were positive for double genes by ddPCR; among 161 samples that were negative by RT-PCR, 4 samples were positive by ddPCR. The above samples with false-negative results by RT-PCR showed that the absolute molecules of the virus were 11.1–123.2 copies by ddPCR, suggesting that ddPCR technology was more sensitive for SARS-CoV-2 detection.

Although digital platform is superior to other platforms in detection sensitivity and quantitative performance, the instruments and equipment of the digital detection platform are more expensive, and the detection throughput is limited, which is not suitable for testing a large number of samples. In addition, limited by the fluorescence detection channel, it is difficult for digital platform to perform multiplex detection of multiple targets.

### 2.4 SARS-CoV-2 nucleic acid detection technique based on nucleic acid mass spectrometry platform

The nucleic acid mass spectrometry platform uses matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) to analyze nucleic acid fragments\(^{23,26}\), and the principle of mass spectrometry for SARS-CoV-2 detection is shown in Fig.4. Firstly, nucleic acid samples are prepared and reverse transcription amplification are performed to enrich the target sequences, and then gene-specific primers will be added to perform a single base extension reaction. After the extension reaction, the product is purified and further co-crystallized with the matrix for detection on the mass spectrometer. Finally, the product molecules are excited and ionized by the laser into single-charged ions flying in the electric field. Since the flight time is inversely proportional to the ion mass, the molecular weight can be directly obtained by detecting the flight time of the nucleic acid molecule in the vacuum tube of the mass spectrometer, thereby the gene information are obtained by analyzing the mass spectra.

Clin-ToF I time-of-flight mass spectrometer and Clin-ToF II time-of-flight mass spectrometer have been selected in the second batch of medical equipment catalogs issued by the China Medical Equipment Association for the prevention and control of new coronary pneumonia epidemics, indicating that the nucleic acid mass spectrometer platform could also be used for SARS-CoV-2 detection.

The advantage of nucleic acid mass spectrometry is that it can distinguish oligonucleotide fragments with a single base difference, achieving low-cost and high-throughput multiplex detection by quality labels instead of fluorescent labelings. However, utilizing mass spectrometry platform to detect target
nucleic acids requires multiple steps such as sample pre-processing, PCR product preparation, product purification and mass spectrometry analysis, especially needing strict requirements for sample processing. Moreover, different matrix components of MALDI would also directly affect MS signal intensity.

### 2.5 SARS-CoV-2 nucleic acid detection technique based on paper-based analysis platform

To reduce the reliance on instruments and equipment, a paper-based analysis platform was developed for nucleic acid detection. It is a kind of detection platform that completes the whole biochemical analysis on paper[27], and the common paper-based analysis platform is test strip technology[28]. Usually, a paper-based analysis platform is coupled with nucleic acid isothermal amplification technology to further reduce the dependence on instruments and equipment. Commonly used formats are test strips based on loop-mediated isothermal amplification (LAMP)[29], recombinase polymerase amplification (RPA)[30], etc. Taking LAMP reaction as an example, the principle of common test strip detection is shown in Fig.5. At a constant temperature of 63 °C (60–65 °C), AMV reverse transcriptase transcribes RNA into cDNA, followed by the amplification with 3 pairs of LAMP primers (F3-B3, FIP-BIP and LF-LB) and Bst polymerase possessing strand displacement activity, and a large number of labeled target LAMP products could be generated. Then the products are added to the sample pad in the test strip, and move under the action of the buffer to interact with the modified colloidal gold at the conjugate pad. Since the colloidal gold at the conjugate pad is modified with...
antibodies and streptavidin, immunoaffinity interaction will occur when the target is present, and there will be colored bands in the test line and the control line on the test strip. When there is no target, the colored band only appears at the position of the control line. Therefore, the negative or positive result could be directly judged by the naked eyes.

The SHERLOCK technology based on Cas13 and RPA amplification was used for the detection of SARS-CoV-2. This method designed a nucleic acid test strip that specifically targeted the spike protein (S) gene and ORF1ab gene of SARS-CoV-2. It could achieve the detection of SARS-CoV-2 within 1 h, and the sensitivity was 10–100 copies/μL. Besides, a strategy based on test strips that coupled Cas12 with RT-LAMP (reverse transcription LAMP) to establish a visual detection method for SARS-CoV-2 was reported recently. As low as 10 copies/μL of extracted RNA could be detected within 45 min by the reported method, providing an idea for the rapid detection of SARS-CoV-2 on site.

The SARS-CoV-2 detection technique based on the paper-based analysis platform commonly depends on a constant temperature reaction, thus it does not require sophisticated instrument and the amplification speed is fast. After adding the amplification product to the test strip or inserting the test strip into the reaction system after the amplification, colored bands could display on the test strip, and the presence or absence of the target virus could be judged directly with the naked eyes by the color bands without relying on professional analysis equipment. Therefore, the detection techniques for SARS-CoV-2 on the paper-based analysis platform have the advantages of simple operation, fast detection, and low instrument requirements, meeting the needs of on-site detection and particularly suitable for pathogen detection in areas with limited medical resources, helping to quickly screen and detect pathogens on site.

CapitalBio Technology Corporation developed a nucleic acid detection kit for six respiratory virus based on isothermal amplification technology in a microfluidic chip. After simple nucleic acid extraction, extracted RNA could be loaded into the microfluidic chip with mixed reagents for nucleic acid amplification and analyzed by a microfluidic nucleic acid analyzer. The detection of 6 kinds of respiratory viruses including SARS-CoV-2 could be completed within 1.5 h. It could detect 15 copies of SARS-CoV-2 molecules per reaction, providing an efficient detection tool for the prevention and control of the epidemic.

The SARS-CoV-2 detection technology based on the microfluidic chip platform integrates the multi-step reaction of sample detection on a small chip, greatly reducing the demand for detection reagents and samples. Moreover, the reaction speed is fast, the degree of automation is high, and the nucleic acid detection throughput could be improved through the well-designed microfluidic chip. However, most of the current commercial microfluidic chip platforms are still not compatible with nucleic acid extraction, and the goal of fully automated nucleic acid detection has not yet been achieved. Moreover, the design and manufacturing cost of a microfluidic chip is relatively high, needing further optimization to reduce the cost of detection.

2.6 SARS-CoV-2 nucleic acid detection technique based on microfluidic platform

Conventional biochemical detection technologies include multiple steps of sample preparation, reagent processing, biochemical reaction and product detection, while the microfluidic platform could integrate multi-step or all operations into a small platform. There were reports of microfluidic platforms developed on paper-based platforms, which integrated nucleic acid extraction and detection operations on a paper-based chip, through origami technique or paper-based valve design to manipulate the progress of the reaction. However, paper-based chip technique needs many manual operations with poor flexibility. Thus it is still in the laboratory research stage and has not been used for the detection of SARS-CoV-2. Currently, the relatively mature microfluidic platforms are based on microfluidic chips made of materials with good optical transparency, electrical insulation and reactive inertness (Quartz, polydimethylsiloxane, etc.) with a size of only a few square centimeters. After being further processed, a microfluidic chip uses microchannels, micropumps, microinjection valves and micro outlets to form an automatic control system that allows a small amount of liquid to flow, achieving the purpose of analyzing samples with high efficiency and accuracy. Owing to the small size and lightweight of the equipment supporting the microfluidic platform, it is particularly suitable for the detection in areas with limited medical resources, helping to quickly screen and detect pathogens on site.

2.7 SARS-CoV-2 nucleic acid detection technique based on integrated automation platform

In different nucleic acid detection technology platforms, nucleic acid extraction operation plays an important role in the accurate detection of pathogens. Conventional nucleic acid extraction includes manual extraction and automatic instrument extraction, the extraction product usually needs to
be manually added to the detection system in the practical detection process. When dealing with highly infectious virus samples, manual operation undoubtedly increases the infection risk for operators. To overcome the shortcomings of separate sample extraction steps, an integrated automated nucleic acid detection platform came into being. This is a closed automated nucleic acid detection system that integrates sample extraction, nucleic acid detection and result output, which avoid excessive errors and biosafety issues caused by the manual operation as well as the risk of cross-contamination during sample testing.

The first integrated automated analysis platform is the GeneXpert® Systems, whose core is a real-time fluorescent quantitative PCR instrument that could realize fully automated sample preparation and detection. The collected original samples are directly added into the enclosed cassettes to be loaded into the detection instrument, then the instrument will automatically complete a series of operations such as sample lysis, nucleic acid extraction, and quantitative PCR detection, and output the analysis results after the reaction, achieving the effect "Sample in-Answer out". Recently, a new product named Xpert® Xpress SARS-CoV-2 based on the GeneXpert® System was developed, which could enable the qualitative detection of samples within 45 min, greatly improving the detection efficiency of SARS-CoV-2. However, GeneXpert platform is mainly based on PCR[33], which requires thermal cycling and critical temperature control with expensive instruments. The platform needs high-cost reagents and consumables, not suitable for clinical applications. Therefore, it is necessary to develop an integrated automated platform with nucleic acid amplification and detection under constant temperature conditions to reduce the cost of clinical sample detection. Simultaneous amplification and testing (SAT) is a new technology for RNA detection. This technology introduces the T7 promoter into cDNA through reverse transcription and generates double-strand DNA, which will be catalyzed by T7 RNA polymerase and generate multiple RNA molecules containing the target sequence, and then enters the next cycle of RNA reverse transcription and transcription. The molecular beacon that bind to the target RNA reports the detection signal in real time during the entire process. The above reactions are all performed at 42 °C, achieving efficient amplification and detection of RNA targets under constant temperature conditions[39]. At the end of March 2020, Rendu Biotechnology Corporation successfully developed a fully automatic nucleic acid detection platform for SARS-CoV-2 based on an automatic SAT nucleic acid analysis system (AutoSAT), which realized the closed automatic detection (sample in-result out) of SARS-CoV-2. In addition, it could be used for processing a single sample or batch samples. The first sample took 90 min from sample injection to report output, and up to 700 samples could be tested in batches within 24 h.

The SARS-CoV-2 detection technology based on an integrated automated platform integrates sample extraction, nucleic acid detection, and output into a single system, achieving the goal of "sample in-result out" for detecting pathogen nucleic acids automatically. This platform does not require professional operators and strict laboratory facilities, the instrument is portable, and the detection efficiency is high with good repeatability. Hence, it is especially suitable for rapid screening of pathogens on site. However, the current integrated automation platform is still in the development stage, the costs of instruments and consumables are high, and most instruments have low detection throughput in a single run. Therefore, there are great potentials before clinical promotion.

In summary, the above technologies have both advantages and disadvantages in nucleic acid detection for SARS-CoV-2 (Table 1). Therefore, when choosing a technique to detect

| Technology platforms | Advantages | Disadvantages | Suitable applications |
|----------------------|------------|---------------|-----------------------|
| High throughput sequencing platform | High sensitivity, high throughput, quantitative, perfect accuracy, whole genome sequencing | Sophisticated instruments, open-tube and tedious operation, high cost, complicated data analysis | High-throughput pathogen detection, identifying new pathogens, nucleic acid sequence traceability, mutation and mechanism research |
| Real-time PCR platform | High sensitivity, high specificity, quantitative, closed-tube operation | Sophisticated instruments, complicated optimization of methods, low throughput, strict division of laboratories | Low-throughput clinical sample testing |
| Digital platform | Absolute quantitation with single-molecule resolution, high specificity, perfect accuracy | Sophisticated instruments, low cost, low throughput | Quantitative testing of clinical samples, especially suitable for samples with low viral load |
| Mass spectrometry for nucleic acid analysis | Quantitative, high throughput, high specificity, low cost | Sophisticated instruments, open-tube and tedious operation | High-throughput pathogen detection, multi-omics joint analysis |
| Paper-based analysis platform | Portable instruments, low cost, easy operation, rapid reaction, convenient readout | Low sensitivity, difficult to quantify, low throughput, open-tube operation | Rapid on-site screening of pathogens, especially suitable for areas with limited experimental equipment and emergency situations |
| Microfluidic platform | Less manual operation, rapid reaction, portable instruments, mid-throughput assay | Difficult to quantify, Complex chip design, expensive manufacturing cost | Rapid on-site screening of pathogens, especially suitable for areas with limited experimental equipment |
| Integrated automation platform | Sample in-answer out, high sensitivity, high specificity, perfect accuracy, portable instruments | Few existing platforms, high-priced instruments and consumables, low throughput | Expected to be widely used in detecting clinical samples, especially in the rapid screening of pathogens on site |
SARS-CoV-2, the technology platform needs to be selected according to actual conditions. For example, in emergency situations with limited resources, paper-based analysis platform and microfluidic platform are suitable for rapid screening of SARS-CoV-2 on site. When detecting a sample with low viral load or the routine assay shows weak-positive result, a digital platform is preferred for accurate detection of the testing sample.

3 Conclusions and prospect

Although the number of confirmed cases of COVID-19 worldwide is still increasing, nucleic acid detection techniques for SARS-CoV-2 are also evolving. According to the nucleic acid detection technology platform, this article introduced the main detection techniques of SARS-CoV-2. Since different technology platforms have both advantages and disadvantages in nucleic acid detection, the detection technology needs to be selected according to actual conditions in practical applications. It is found that even POCT (Point-of-care testing) technologies, which have the advantages of fast detection, low cost, and independence of large equipment, also expose poor safety, insufficient sensitivity and other issues in detecting SARS-CoV-2. To deal with the malignant viruses like SARS-CoV-2, which has the characteristics of strong infectivity and long incubation period, the subsequent direction of developing the nucleic acid detection technology platform needs to pay attention to two points. The detection method should have good specificity, high sensitivity, and fast response; the equipment needs less manual operation, high throughput, low-cost manufacturing and to be more portable. However, the existing integrated automation platforms for SARS-CoV-2 detection have low throughput, and most platforms could only increase the throughput by running multiple instruments in parallel, which undoubtedly increases the cost of the detection. Therefore, the development of a high-throughput, full automation platform for pathogen detection is the future trend and challenge.

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