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## ORIGINAL ARTICLE

# *Neisseria Gonorrhoeae* Based on Recombinase Polymerase Amplification Technology Establishment of Detection Method

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**SUMMARY**

***Background:*** **Recombinase polymerase amplification (RPA) is a novel nucleic acid isothermal amplification technique that can achieve rapid detection of the target, under 37 to 42°C conditions, within 30 minutes. It has the advantage of extreme sensitivity, strong specificity, and low instrument dependency and is particularly suitable for real-time detection in the field. It can be widely used in fields such as in vitro diagnostics, biosafety, and agriculture. This study was based on RPA technology, targeting the gyrA gene of *Neisseria gonorrhoeae* (*N. gonorrhoeae*), to establish a quick, accurate, and easy to operate method for detecting *N. gonorrhoeae* and to evaluate its specificity, sensitivity, and clinical, practical value.**

***Methods:*** **Specific primers and probes suitable for RPA and qPCR methods based on the specific conserved region of the gyrA gene of *N. gonorrhoeae* on GenBank (no. U08817.1) were designed An RPA method was developed and *N. gonorrhoeae* ATCC49226 and a number of clinical isolates were used as study subjects to validate the specificity and sensitivity of the RPA method for the detection of *N. gonorrhoeae*. A real-time fluorescence quantitative polymerase chain reaction (qPCR) method, with *N. gonorrhoeae* ATCC49226 as the research object, was established to verify the sensitivity of qPCR method for detecting *N. gonorrhoeae*. Finally, clinical samples were tested by using RPA and qPCR methods as performance validation experiments to determine the clinical utility of the RPA technique in detecting *N. gonorrhoeae*.**

***Results:*** **The established RPA detection method showed excellent specificity, with a specific amplification curve for *N. gonorrhoeae* alone, no cross-reactivity with other bacteria, and excellent reproducibility. The detection results could be obtained within 30 minutes, under the condition of 39°C, which was significantly lower than the detection time of traditional methods. The sensitivity of the RPA method for detecting pathogenic bacteria samples was $4 \times 10^2$ CFU/mL, which is consistent with the detection limit of qPCR methods. RPA and qPCR methods were used to detect 121 clinical isolates, out of which 30 strains of *N. gonorrhoeae* showed a specific amplification curve, while the remaining 91 strains of non-*N. gonorrhoeae* did not. Both methods had 100% accuracy and specificity in detecting *N. gonorrhoeae*.**

***Conclusions:*** **The RPA method developed in this study has the characteristics of being quick, accurate, and easy to operate, which was of great value for the rapid detection of *N. gonorrhoeae* in clinical samples.**

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**KEYWORDS**

*N. gonorrhoeae*, gyrA gene, real-time fluorescence recombinase polymerase amplification

## INTRODUCTION

Gonorrhea (gonococcal urethritis) is a sexually transmitted disease caused by *N. gonorrhoeae*, with a significant incidence worldwide [1]. According to statistics from the World Health Organization, in 2021, approximately 67.6 million people worldwide were infected with gonorrhea, with over 2 million new cases of infection [2]. Gonorrhea poses a serious threat to personal and public health due to its elevated infectivity [3] and concealment [4]. Therefore, rapid and accurate detection and diagnosis of *N. gonorrhoeae* is essential for disease prevention. Traditional methods for detecting gonorrhea include bacterial culture, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) [5], but these methods have drawbacks, such as lengthy detection time, complex operation, and high equipment requirements [6-8].

In recent years, rapid detection methods based on real-time fluorescence recombinase polymerase amplification (RPA) technology have gradually attracted attention and have been widely applied in clinical testing [9]. RPA technology is a nucleic acid amplification method performed under isothermal conditions, which has the characteristics of being rapid and having elevated sensitivity and elevated specificity [10]. In the reaction system, primers specifically bind to the target DNA, and recombinase can unravel the double stranded DNA, allowing primers to bind to the target DNA to form a hybrid [11]. Then, polymerase transcribes RNA primers into cDNA at low temperatures, ultimately obtaining a large number of fluorescent labeled DNA products [12]. Compared with traditional gonorrhea detection methods, RPA is an amplification technique performed at low temperatures (38 - 40℃) without the need for complex temperature cycling reactions [13]. It can complete the amplification reaction in half an hour. RPA technology has significant advantages for clinical detection. It can not only shorten detection time, but also reduce operational difficulty and equipment requirements and reduce detection costs [14]. In addition, RPA technology can also be used for detection in complex clinical samples, such as urine, secretions, etc., and it has broad application prospects [15].

This study used real-time fluorescence RPA technology for rapid detection of *N. gonorrhoeae* with the goal of developing an efficient, accurate, and simple detection method to address the shortcomings of traditional detection methods.

## MATERIALS AND METHODS

### General information

All strains were sourced from clinical specimens in the laboratory and standard strains were purchased within the past 5 years (Table 1). The isolates used in this study were retrospectively confirmed at the species level by using VITEK MS.

Reagents: Blood plate, MacConkey agar medium, TM chocolate agar selection medium (Thermo Fisher Scientific Biotech Co., Ltd., China), Sabouraud agar medium (Chongqing Pangtong Medical Equipment Co., Ltd., China), DNA constant temperature rapid amplification kit (fluorescent type) Anpu Future bioTechnology Co., Ltd., yeast/bacterial genome DNA extraction kit Tiangen Biochemical Technology (Beijing) Co., Ltd., GoTaq ® QPCR Master Mix Plomag (Beijing) bioTechnology Co., Ltd.

Instruments: Electrophoresis apparatus (BIORAD, USA), Metal bath (IKA), desktop freeze centrifuge (ST8R), CFX96 real-time fluorescence quantitative PCR instrument (BIORAD, USA), chemiluminescence imaging system ChemiDoc XRS+ (BIORAD, USA).

### Research method

Based on the nucleic acid sequence of the *gyrA gene* of *N. gonorrhoeae* on GenBank (no. U08817.1), specific primers and probes for RPA and qPCR methods were designed using its specific conserved region (Table 2). All primers and probes were synthesized by Beijing Liuhe Huada Gene Technology Co.

The RPA technology in this study mainly relied on three enzymes: recombinase which can bind to single stranded nucleic acid (oligonucleotide primers), single stranded DNA binding protein (SSB), and strand displacement DNA polymerase.

### Genomic DNA extraction

Inoculated *N. gonorrhoeae* ATCC49226 was put onto TM chocolate agar selection medium, at 37°C for 24 hours. Then, a modest amount of pure colonies in the inoculation ring were picked, 0.5 mcf concentration bacterial solution and another 1.5 mL sterile EP tube were prepared, 1 mL of bacterial solution was added and centrifuged at 12,000 r/minute for 1 minute, the supernatant was isolated and removed, and lastly, DNA was extracted according to the instructions of the yeast/bacterial genomic DNA extraction kit and stored at -20°C for future use.

### Establishment of real-time fluorescence recombinase polymerase amplification (RPA) method

*N. gonorrhoeae* ATCC49226 DNA as template RPA reaction system (50 μL) included: RPA-F (10 μMol/L) 2.0 μL, RPA-R (10 μMol/L) 2.0 μL, Probe (10 μMol/L) 0.6 μL, A buffer 29.4 μL, DNA template 5 μL, and $ddH_2O$ 8.5 μL. They were mixed thoroughly and added to the reaction tube containing the freeze-dried enzyme preparation. The mixture was blown and sucked on until it fully dissolved, then 2.5 μL B buffer were added. The tube was tightly covered, centrifuged and vortexed instantaneously, then placed in the CFX96 real-time fluorescence quantitative PCR instrument at 39℃ for 40 cycles.

**Establishment of real-time fluorescence quantitative polymerase chain reaction (qPCR) method**

*N. gonorrhoeae* ATCC49226 DNA as template qPCR system (20 μL) included: GoTaq ® Probe qPCR Master Mix 10.0 μL, qPCR-F (10 μMol/L) 1.0 μL, qPCR-R (10 μMol/L) 1.0 μL, PCR probe (10 μMol/L) 1 μL, DNA template 5 μL, and ddH2O 2 μL. After the above-mentioned system was sufficiently shaken and mixed, it was centrifuged instantaneously and placed in the CFX96 real-time fluorescence quantitative PCR instrument. The reaction procedure was: pre-denaturation at 95℃ for 30 seconds; denaturation at 95℃ for 5 seconds, extension at 60℃ for 35 seconds, 40 cycles, and collected fluorescence signals at 60℃.

**Specific validation of RPA method**

Using the established RPA method, a total of six bacterial strains, including some standard strains and clinically isolated strains of Table 1, were selected as the research subjects. A positive control and a negative control (from the built-in reagent kit) were set up to verify the specificity of RPA method for detecting *N. gonorrhoeae*.

**Sensitivity experiment of RPA method**

The bacterial concentrations of the test sample were prepared as $4 \times 10^5$ CFU/mL, $4 \times 10^4$ CFU/mL, $4 \times 10^3$ CFU/mL, $4 \times 10^2$ CFU/mL, and $4 \times 10^1$ CFU/mL by standard plate count method. DNA extraction kits were used to extract DNA from the tested bacterial samples, and the extracted genomic DNA was used as a template for RPA amplification. A negative control (from the built-in reagent kit) was set up, and detection was performed by using the established RPA method to determine the sensitivity of the RPA method for detecting *N. gonorrhoeae*.

**Sensitivity experiment of qPCR method**

Five concentrations of bacterial sample DNA were prepared as the research object, and a negative control (from the built-in reagent kit) was set up. The established qPCR method was used for detection to determine the sensitivity of the qPCR method for detecting *N. gonorrhoeae*.

**Clinical validation experiment of RPA and qPCR methods**

A total of 121 clinically isolated strains from Table 1 were selected for the study. All bacterial DNA was extracted by using the kit extraction method, and the extracted genomic DNA was used as a template. The RPA and qPCR methods were developed to simultaneously detect and validate the performance of clinical samples.

## RESULTS

**Selection of primers for RPA method**

Four sets of primers (F1R1, F1R2, F2R1, and F2R2) were simultaneously amplified. After screening, the fluorescence curve of the F1/R2 primer combination was optimal (Figure 1), and F1/R2 was ultimately determined as the primer for RPA method.

**The specificity test of RPA method**

Only *N. gonorrhoeae* ATCC49226 and positive control wells showed specific amplification curves, while the other five non-*N. gonorrhoeae* genomes and negative controls did not have specific amplification curves (Figure 2). The repeated experiments yielded consistent results, confirming that the RPA method had good specificity for detecting *N. gonorrhoeae* ATCC49226.

**Sensitivity test of RPA method**

According to the fluorescence reaction curve, specific amplification curves were observed in bacterial suspensions at 4 concentrations: $4 \times 10^5$ CFU/mL, $4 \times 10^4$ CFU/mL, $4 \times 10^3$ CFU/mL, and $4 \times 10^2$ CFU/mL. The bacterial suspension with a concentration of $4 \times 10^1$ CFU/mL and the negative control showed no specific amplification curve (Figure 3). The experiments were repeated, and the results were consistent, confirming that the minimum detection limit for detecting *N. gonorrhoeae* ATCC49226 through RPA method was $4 \times 10^2$ CFU/mL.

**Sensitivity test of qPCR method**

According to the fluorescence reaction curve, specific amplification curves were observed in bacterial suspensions at 4 concentrations: $4 \times 10^5$ CFU/mL, $4 \times 10^4$ CFU/mL, $4 \times 10^3$ CFU/mL, and $4 \times 10^2$ CFU/mL. The bacterial suspension with a concentration of $4 \times 10^1$ CFU/mL and the negative control showed no specific amplification curves (Figure 4). The experiment was repeated, and the results were consistent. In conclusion, the minimum detection limit for detecting *N. gonorrhoeae* ATCC49226 using RPA and qPCR methods confirmed to be the same.

**Clinical sample performance validation of RPA and qPCR methods**

A total of 121 bacterial strains were tested using established RPA and qPCR methods. Thirty strains of *N. gonorrhoeae* had specific amplification curves, while the remaining 91 strains of non-*N. gonorrhoeae* had no specific amplification curve in genomes (Table 3), and the repeated experiments yielded consistent results. The stable and reliable results of the real-time fluorescence RPA method developed in this study for the detection of *N. gonorrhoeae* could provide strong evidence for its adoption as a routine clinical test.

**Table 1. Information of the 122 isolates used for validation of the designed real-time RPA assay for detecting *N. gonorrhoeae*.**

| No. | Bacterial strain | Quantity (plant) | Source |
|---|---|---|---|
| 1 | *N. Gonorrhoeae* ATCC49226 | 1 | Laboratory standard strains |
| 2 | *E. Coli* ATCC25922 | 1 | Laboratory standard strains |
| 3 | *N. gonorrhoeae* | 30 | Clinical specimen separation |
| 4 | *N. meningitidis* | 12 | Clinical specimen separation |
| 5 | *N. sicca* | 12 | Clinical specimen separation |
| 6 | *N. subflava* | 12 | Clinical specimen separation |
| 7 | *N. flavescens* | 12 | Clinical specimen separation |
| 8 | *N. mucosa* | 10 | Clinical specimen separation |
| 9 | *S. aureus* | 6 | Clinical specimen separation |
| 10 | *Ps. aeruginosa* | 6 | Clinical specimen separation |
| 11 | *S. constella* | 6 | Clinical specimen separation |
| 12 | *E. coli* | 6 | Clinical specimen separation |
| 13 | *K. Pneumoniae* | 4 | Clinical specimen separation |
| 14 | *Aci. baumannii* | 4 | Clinical specimen separation |

**Table 2. Sequences of primers and probes.**

| Assay | Name | Sequence (5´-3´) and modification | Length (bp) |
|---|---|---|---|
| RPA | gyrA-F1 | CAAAAGCTATCTCGACTACGCCATGAGCGT | 30 |
| | gyrA-F2 | GCGTCATTGTCGGGCGCGCGCTGCCGGAC | 29 |
| | gyrA-R1 | GCCGCCTACAAAAAATCGGCGCGCATCGTC | 30 |
| | gyrA-R2 | GGCGACGTCATCGGTAAATACCACCCCCACG | 31 |
| | gyrA-P1 | ATTTTTCAGCTCGTGCATCGCGTACAGTACGC[FAM-dT]G[THF][BHQ1-dT]CCGGTGCACCGGCTT[C3Spacer] | 81 |
| qPCR | gyrA-F | TGCGCAAAAGCTATCTCGAC | 20 |
| | gyrA-R | TTGTAGGCGGCATTCCAGTT | 20 |
| | gyrA-P | FAM-TCGTGCATCGCGTACAGTACGCGCC-BHQ1 | 34 |

## DISCUSSION

In this study, we chose the gyrA gene of *N. gonorrhoeae* as the target gene for RPA detection (GenBank no. U08817.1), which was widely present and highly conserved in *N. gonorrhoeae*. Subsequently, RPA and qPCR specific primers and probes were designed. Finally, we optimized the conditions of the RPA reaction system, including temperature, time, and reactant concentration, and by systematically adjusting these conditions, we ultimately determined the most appropriate RPA reaction conditions for the detection of *N. gonorrhoeae*: a reaction temperature of 39°C and a reaction time of 30 minutes. To verify the specificity and sensitivity of the RPA method, we performed a series of validation experiments in the laboratory and compared it with the qPCR method. We collected 122 strains to be tested, including clinical isolates (120 strains) and laboratory standard strains (2 strains). These strains were stored under low temperature conditions of -80°C to maintain their activity and stability. First, we extracted the genomic DNA of the strains from these samples, the optimal primer combination F1/ R2 was then screened by RPA and product electrophoresis results. Then, we used this combination of primers for the subsequent RPA experiments in this study.

We used the RPA method to detect six strains of bacteria to be tested, and only *N. gonorrhoeae* ATCC49226 and a positive control showed a specific amplification curve. The remaining five bacterial strains and negative controls did not show specific amplification curves, indicated that the RPA method has excellent specificity for detecting *N. gonorrhoeae* ATCC49226. Secondly, by established RPA and qPCR methods, the sensitivity

**Table 3. Validation results of clinical sample performance using RPA and qPCR methods.**

| Bacterial strain | Source | Quantity (plant) | RPA-positive (plant) | qPCR-positive (plant) |
|---|---|---|---|---|
| ***N. gonorrhoeae*** | **Clinical Specimen separation** | **30** | **30** | **30** |
| ***N. meningitidis*** | **Clinical Specimen separation** | **12** | **0** | **0** |
| ***N. sicca*** | **Clinical Specimen separation** | **12** | **0** | **0** |
| ***N. subflava*** | **Clinical Specimen separation** | **12** | **0** | **0** |
| ***N. flavescens*** | **Clinical Specimen separation** | **12** | **0** | **0** |
| ***N. mucosa*** | **Clinical Specimen separation** | **10** | **0** | **0** |
| ***S. aureus*** | **Clinical Specimen separation** | **6** | **0** | **0** |
| ***Ps. aeruginosa*** | **Clinical Specimen separation** | **6** | **0** | **0** |
| ***S. constella*** | **Clinical Specimen separation** | **6** | **0** | **0** |
| ***E. coli*** | **Clinical Specimen separation** | **6** | **0** | **0** |
| ***K. Pneumoniae*** | **Clinical Specimen separation** | **4** | **0** | **0** |
| ***Aci. baumannii*** | **Clinical Specimen separation** | **4** | **0** | **0** |
| ***E. Coli* ATCC25922** | **Laboratory Standard strains** | **1** | **0** | **0** |

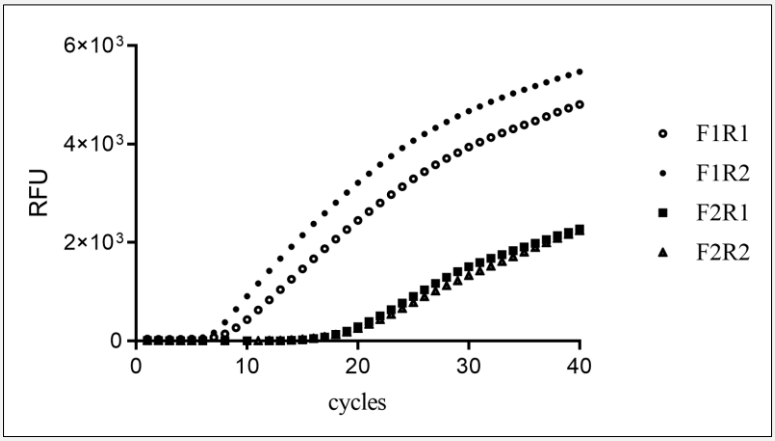


**Figure 1. Primer screening for RPA method.**

of the two methods for detecting *N. gonorrhoeae* ATCC49226 was compared. According to the fluorescence reaction curves, 4 concentrations of bacterial suspension were observed; $4 \times 10^5$ CFU/mL, $4 \times 10^4$ CFU/mL, $4 \times 10^3$ CFU/mL, and $4 \times 10^2$ CFU/mL had specific amplification curves. $4 \times 10^1$ CFU/mL and a negative control showed no specific amplification curves, which confirmed the sensitivity of RPA and qPCR methods for detecting *N. gonorrhoeae* ATCC49226, both of which were $4 \times 10^2$ CFU/mL. Finally, to verify the accuracy of the RPA technology, we compared it with the qPCR method. A total of 121 bacterial strains were detected by using the RPA method, and 30 strains of *N. gonorrhoeae* showed specific amplification curves. The remaining 91 non-gonococcal Neisseria strains had no specific amplification curves, which was identical with the expected result. The sensitivity and specificity of this experiment were both 100%. We used the same samples, experimental conditions, and fluorescence detection in each experiment. By comparing the results of the different experiments, we confirmed the repeatability and stability of this method. Through this

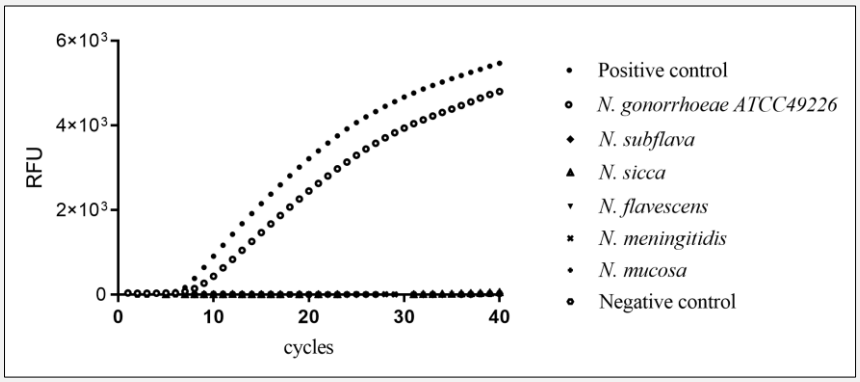


**Figure 2. Specific validation of RPA method.**

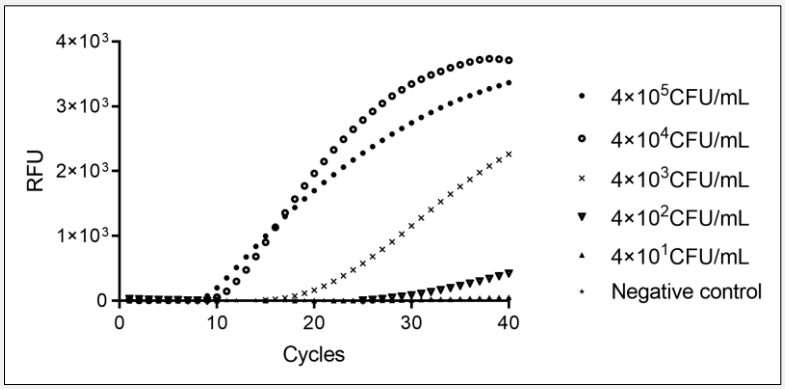


**Figure 3. Sensitivity detection of RPA method.**

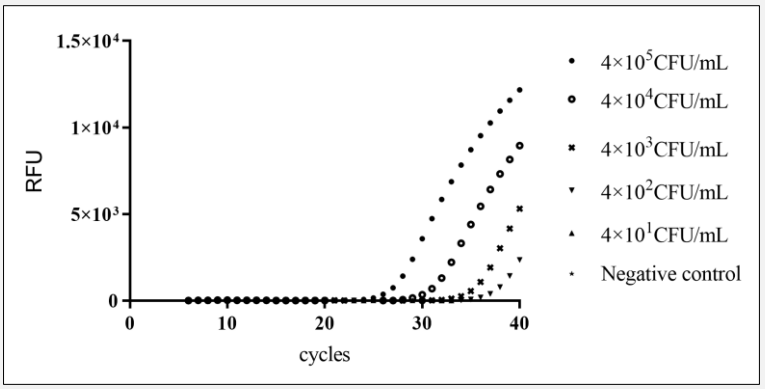


**Figure 4. Sensitivity detection of qPCR method.**

study, we successfully applied RPA technology to achieve rapid detection of *N. gonorrhoeae* and achieved excellent detection results. This technology had various advantages, such as speed, sensitivity, strong specificity, and simple operation [16].

In this research, in order to avoid the formation of secondary structures that might affect amplification by primers, RPA primers with lengths of 28 - 35 bp and amplification products of 150 - 300 bp were designed. In addition, the probe sequence did not overlap with the primer recognition site, avoided palindrome sequences, internal secondary structures, and continuous repeating bases. Through reasonable design, no nonspecific curves were observed in all experiments of this study. In addition, we have noted further reports on the elimination of false positive signals in primer dimers and primer probe dimers. The biosensor developed by Fan [17] reduces the production of non-specific RPA amplification by designing enzyme cleavage sites on primers, introducing SDA, and using molecular beacons (MB) to detect the final product. Yang [18] developed a convenient, economical, and effective biosensor for detecting *N. gonorrhoeae* by using a leak proof probe based on recombinant polymerase amplification mediated lateral flow bands, with no false positive results. A study by Chen [19] reported a ring-mediated isothermal amplification method for biosensors based on polymer nanoparticles, which was used to identify *N. gonorrhoeae* in clinical samples. The specificity of the determination was 100 percent, and the detection time and accuracy of the results were similar to those of the present study. Combining future research directions with biosensors is certainly a welcome option.

There were still some shortcomings in this study. First of all, this study mainly focused on the principles, methods, and applications of RPA technology, and research on the biological characteristics of *N. gonorrhoeae* is still relatively limited. Future research could improve assay performance and robustness, as well as explore the implementation of RPA for detecting antimicrobial resistance in *N. gonorrhoeae*. Secondly, although this method performed adequately in specificity, additional research can increase the variety of microorganisms and compare it with additional target bacteria to verify its detection range. Finally, due to the strong variability of *N. gonorrhoeae* [20], we have only studied the *gyrA gene* in extremely conserved regions; this study did not consider that mutations in *gyrA* could lead to false negative results. Subsequent research needs to consider the adaptability and feasibility of other variant strains.

In summary, this study achieved rapid detection of *N. gonorrhoeae* through RPA technology and achieved excellent research results and it provided a quick and convenient method for the detection and screening of *N. gonorrhoeae*. We hope that in future research, RPA technology can be further improved, the biological characteristics of *N. gonorrhoeae* can be explored in-depth, and more accurate and effective detection methods can be discovered, providing more powerful support for the prevention and control of *N. gonorrhoeae*.

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**Declaration of Interest:**

The authors declare that they have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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