# Application of PCR Reverse Dot Blot Hybridization Technology for Detecting Pathogenic Bacteria in Medical Masks

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**Abstract:** To establish a rapid method utilizing PCR reverse dot blot hybridization technology for detecting pathogenic bacteria in masks. Methods: Specific genes were selected: nuc for Staphylococcus aureus, uidA for Escherichia coli, eta for Pseudomonas aeruginosa, and sly for Streptococcus hemolyticus. The bacterial 16S rDNA gene was used as a positive control. Primers and probes were designed and labeled accordingly. Probes were chemically immobilized onto a nylon membrane, and PCR products were hybridized with these fixed probes. Biotin-labeled primers bound to streptavidin, which was conjugated with horseradish peroxidase; upon reaction with a chromogenic substrate, a blue color developed. Results: The established method specifically detected pathogenic bacteria with a sensitivity ranging from 4 to 10 cfu per sample. Conclusion: The developed detection method is rapid, highly specific, and sensitive, making it suitable for the quick detection of pathogenic bacteria in masks.

## 1 Introduction

One of the key microbiological indicators in medical mask testing includes microbial limits, pathogenic bacteria detection, sterility testing, and bacterial filtration efficiency [1]. Pathogenic bacteria detection focuses on Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Streptococcus hemolyticus, as these pathogens have high pathogenicity and directly impact user health. Traditional detection methods are time-consuming and involve complex procedures, hindering rapid responses to microbial contamination [2,3]. Therefore, it is necessary to establish a rapid, sensitive, and highly specific method for the detection and identification of pathogenic bacteria[4].

Currently, molecular biology techniques are increasingly applied to pathogen detection [5]. Enzyme-linked immunoassay (ELISA) takes surface antigens or secreted toxins of target pathogens as the main detection targets. Based on the combination of antigens and antibodies, it can detect bacteria and spores, and has good sensitivity and specificity for the detection of a variety of foodborne pathogens. However, this technique has high selectivity for reagents, and it is difficult to analyze multiple components at the same time, and cross-react to compounds with similar structures. Multiple fluorescent PCR technology is to add fluorescent molecules into the PCR reaction and judge the detection results by fluorescence. This method has good specificity and can detect multiple pathogens at the same time. However, this technology has high equipment requirements, and the use of multiple primers will lead to competition among different primers [6,7,8]. Among them, PCR reverse dot blot hybridization technology, known for its speed, sensitivity, and efficiency, has been used in detecting pathogens in food and pharmaceuticals. In this study, PCR reverse dot hybridization technique was used to detect pathogenic bacteria, which is highly accurate and specific based on the principle of nucleic acid base complementary pairing. This study is simple and efficient to detect multiple pathogens at the same time. This method does not require high-end equipment and reagents and has lowcost. This study applies PCR reverse dot blot hybridization technology to the detection of pathogenic bacteria in medical masks, providing a reliable molecular biology approach for the rapid and accurate detection of pathogens in masks [9].

## 2 Materials and Methods

### 2.1 Experimental Materials

#### *2.1.1 Bacterial Strains*

*Staphylococcus aureus* (ATCC 6538), Escherichia coli (ATCC 8739), and *Pseudomonas aeruginosa* (ATCC 9027) were purchased from Guangdong Huankai Microbial Technology Co., Ltd. *Streptococcus hemolyticus* [CMCC(B)32210] was obtained from the China Center of Industrial Culture Collection.

#### *2.1.2 Consumables and Reagents*

Nylon membranes were purchased from PALL

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Corporation. Streptavidin-horseradish peroxidase (SA-HRP) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were obtained from Sangon Biotech (Shanghai) Co., Ltd. 3,3',5,5'-Tetramethylbenzidine (TMB) chromogenic solution was acquired from Beyotime Biotechnology Co., Ltd. Tryptic Soy Agar (TSA) was sourced from Merck (Germany). KAPA HiFi HotStart ReadMix was procured from KAPA Biosystems (USA). Solutions A, B, and C were prepared in-house; their compositions are detailed in Table 1.

**Table 1**. Compositions of Solutions A, B, and C

| Solution | Composition |
|---|---|
| A | 100 mL of 20× SSC, 10 mL of 10% SDS, add distilled water to a final volume of 1,000 mL |
| B | 25 mL of 20× SSC, 10 mL of 10% SDS, add distilled water to a final volume of 1,000 mL |
| C | 100 mL of 1 M sodium citrate, add distilled water to a final volume of 1,000 mL |

### *2.1.3 Primers and Probes*

The sequences of the primers and probes are detailed in Table 2.

**Table 2.** Primer and Probe Information

| Target Bacterium | Primer/Probe | Sequence (5'-3') | Marker |
|---|---|---|---|
| Staphylococcus aureus | nuc-F | AGCG ATTGATGG TGAT ACGG T | 5'Biotin |
| | nuc-R | ATGC ACTT GCTT CAGG ACCA | 5'Biotin |
| | nuc-Probe | TTGG TTGA TACA CCTG AAAC AAAG | 5'NH2 C6 |
| Escherichia coli | uidA-F | CGG TGA TAT CGT CCA CCC AG | 5'Biotin |
| | uidA-R | GAT GTC ACD CCG TAT GTT ATT G | 5'Biotin |
| | uidA-Probe | GGC GGG ATA GTC TGC CAG TTC AGT T | 5'NH2 C6 |
| Pseudomonas aeruginosa | eta-F | CAC CGC CAA CTG GAG GA | 5'Biotin |
| | eta-R | CGC CGA AGA CGA TGC TTT | 5'Biotin |
| | eta-Probe | TAT GTG TTC GTC GGC TAC CAC GGC A | 5'NH2 C6 |
| Streptococcus hemolyticus | sly-F | GCT ACT AGT GTA GCT GAA ACA A | 5'Biotin |
| | sly-R | AGC AAC AAG TAG TAC AGC AGC A | 5'Biotin |
| | sly-Probe | AAA CAA CTC AAG TTG CTC CTG GAG G | 5'NH2 C6 |
| 16SrDNA | 16S-F | GCC TAA YAC ATG CAA GTM GA | 5'Biotin |
| | 16S-R | TGG CAC GKA GTT AGC CG | 5'Biotin |
| | 16S-Probe | TTA CTC ACC CGT SCG CCR CT | 5'NH2 C6 |

## 2.2 Experimental Methods

### *2.2.1 Primer and Probe Preparation*

Specific genes were selected for each bacterium: *nuc* for *Staphylococcus aureus*, *uidA* for *Escherichia coli*, *eta* for *Pseudomonas aeruginosa*, and *sly* for *Streptococcus hemolyticus*. Primers and probes were designed accordingly, with biotin labels attached to the 5' ends of the primers and amino modifications applied to the 5' ends of the probes. Additionally, the universal 16S rDNA gene present in bacteria was chosen as a positive control. Primers and probes for this control were designed and labeled following the same principles as above.

### *2.2.2 Bacterial PCR Amplification*

Bacterial PCR was performed according to the conditions outlined in Tables 3 and 4.

**Table 3**. Colony PCR Reaction System

| Component | Volume per Reaction |
|---|---|
| Template (Bacterial Culture) | 5 μL |
| Primer F (1 μM) | 10 μL |
| Primer R (1 μM) | 10 μL |
| 2KAPA HiFi HotStart | 25 μL |
| ReadMix | 50 μL |
| Total Volume | |

**Table 4.** Bacterial PCR Reaction Program

| Step | Temperature (°C) | Time (min) |
|---|---|---|
| 1 | 95 | 3:00 |
| 2 | 95 | 0:30 |
| 3 | 55 | 0:30 |
| 4 | 72 | 0:30 |
| 5 | go to step 2 | 44 cycles |
| 6 | 72 | 5:00 |
| 7 | 4 | ∞ |

#### *2.2.3 PCR Reverse Dot Blot Hybridization*

##### *2.2.3.1 Membrane Strip Preparation*

Nylon membranes were cut to an appropriate size. After activation with 16% EDC for 30 minutes, probes were spotted onto the designated positions on the membrane.

##### *2.2.3.2 Hybridization*

Denatured single-stranded PCR products, membrane strips, and preheated Solution A were mixed in a single tube and hybridized at 51°C for 2 hours in a hybridization instrument. After hybridization, the membrane strips were removed and washed in preheated Solution B (51°C) with shaking for 5 minutes.

##### *2.2.3.3 Color Development*

The membrane strips were incubated with 10 mL of Solution A containing SA-HRP at room temperature with shaking for 30 minutes. After incubation, the strips were washed twice with Solution A and once with Solution C. TMB substrate solution was added for color development in the dark for 5–30 minutes, and the reaction was terminated with distilled water.

#### *2.2.4 Detection Limit*

To determine the detection limit of this method, samples with varying bacterial concentrations were repeatedly tested. The bacterial concentration at which at least 90% of the samples tested positive was considered the detection limit. Under these conditions, the relative limit of detection (RLOD) was calculated by comparing the reference method with the method under validation. The acceptance criterion was RLOD ≤ 1.5.

$RLOD=\ln[n_{ref}/ (n_{ref}-y_{ref})]/\ln[n_{val}/ (nval-y_{val})]$

In the formula:

$y_{ref}$——Number of positive results detected by the reference method;

$n_{ref}$——Number of repeated tests conducted using the reference method;

$y_{val}$——Number of confirmed positive results detected by the validation method;

$n_{val}$——Number of repeated tests conducted using the validation method.

The reference method refers to the traditional culture method, which is detailed as follows:

1) Detection of *Staphylococcus aureus*

A bacterial suspension was prepared and inoculated into 20 tubes of SCDLP liquid medium for enrichment. After enrichment, streaking was performed on blood agar plates and incubated at 36°C for 48 hours. Typical colonies were selected and subjected to Gram staining. Colonies identified as Gram-positive (*G+*), grape-like cocci without spores or capsules were further tested using the mannitol fermentation assay. A colony was inoculated into 10 mL of mannitol broth and incubated at 36°C for 24 hours; a color change from red to yellow indicated a positive result. Additionally, colonies from the blood agar plate were transferred to nutrient broth and incubated at 36°C for 24 hours, followed by a coagulase test.

2) Detection of *Escherichia coli*

A bacterial suspension was prepared and inoculated into 20 tubes of lactose bile fermentation broth. Tubes producing acid and gas were selected, and streaking was performed on eosin methylene blue (EMB) agar plates. Typical colonies were selected for Gram staining and microscopic examination. Colonies identified as Gram-negative (*G-*) non-spore-forming bacilli were further tested in lactose fermentation broth.

3) Detection of *Pseudomonas aeruginosa*

A bacterial suspension was prepared and inoculated into 20 tubes of SCDLP liquid medium. The culture developed a thin bacterial film on the surface, with the medium appearing yellow-green or blue-green. Samples from the bacterial film were streaked onto cetrimide agar plates and incubated. Typical colonies were selected for Gram staining. Colonies identified as *G-*, were further tested for oxidase activity, pyocyanin production, nitrate reduction with gas production, and gelatin liquefaction.

4) Detection of *Hemolytic Streptococcus*

A bacterial suspension was prepared and inoculated into 20 tubes of glucose broth. The culture was streaked onto blood agar plates, and typical colonies were selected for Gram staining. Colonies identified as *G+*, chain-forming cocci were subjected to the streptokinase test and bacitracin sensitivity test.

Validation Method:

A bacterial suspension was prepared and inoculated into 20 tubes of the corresponding liquid culture medium. After incubation, PCR amplification and hybridization were performed.

#### *2.2.5 Reproducibility*

Reproducibility refers to the precision of measurements obtained under identical conditions by the same experimenter. Precision is assessed by repeatedly testing aliquots of the same homogenized bacterial culture and is expressed as the standard deviation of the results.

Since the experimental results were displayed as blue circular dots, ImageJ software was used to analyze the dots quantitatively. The gray value of the dot was calculated based on the intensity of the color bands to assess result precision. The gray value was calculated as follows:

1. The nylon membrane with blue dots was uploaded to a computer, and the image was converted to grayscale.
2. The grayscale image with dots was uploaded to ImageJ software and converted to an 8-bit format.
3. The entire image background was normalized to eliminate background interference.
4. Measurement parameters were set, and the unit was adjusted to "single pixel."
5. The target dots were selected for measurement, and the results were analyzed.

## 3 Results and Analysis

### 3.1 Establishment of a Pathogenic Bacteria Detection Method Based on PCR Reverse Dot Blot Hybridization

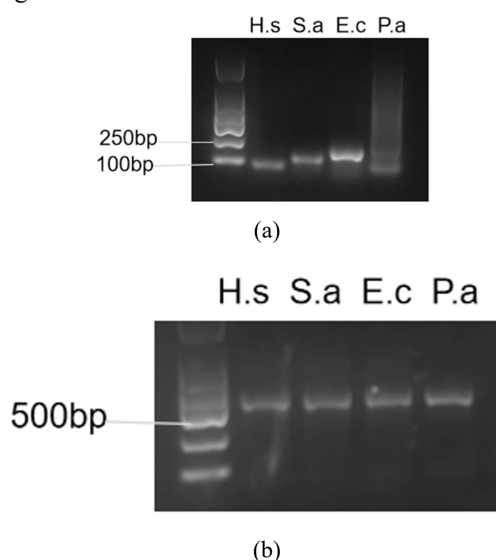
The following species-specific genes were selected for the identification of pathogenic bacteria:

- *Staphylococcus aureus*: Thermonuclease (*nuc*)
- *Streptococcus β-hemolyticus*: Streptolysin (*sly*)
- *Pseudomonas aeruginosa*: Exotoxin A (*eta*)
- *Escherichia coli*: β-glucuronidase (*uidA*)

These genes are highly conserved within species and specific between genera, making them suitable for pathogenic bacteria identification.

Additionally, the conserved bacterial gene 16S rDNA was selected as a quality control gene.

Primers and probes were designed for the four species-specific genes and the control gene. PCR products were analyzed by gel electrophoresis to verify the specificity of the primers for the target genes and the universality of the control gene. The results are shown in the figure below:




(a)

(b)

**Figure 1.** Primers and probes designed for the four species-specific genes and the control gene

Figure 1 presents the PCR products of 16S rDNA, while Figure B displays the PCR products of the four species-specific genes (nuc, sly, eta, and uidA).

A carboxyl-rich nylon membrane was selected and activated using 16% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). After activation, the amino-modified (5′ Aminolinker C6) species-specific probes were covalently linked to the membrane surface, creating probe strips.

For bacterial culture PCR, primers were biotin-labeled (5′ Biotin) to generate biotin-tagged PCR products. The membrane strip and PCR products were then added to a hybridization solution for hybridization, facilitated by base pairing complementarity between the probe and the PCR product. After hybridization, unbound PCR products were washed away, leaving the hybridized membrane.

To detect successful hybridization, streptavidin was incubated with the membrane to bind to the biotin-labeled PCR products. Horseradish peroxidase (HRP), conjugated to streptavidin, catalyzed the oxidation of TMB (3,3′,5,5′-tetramethylbenzidine), producing a visible blue signal. The appearance of blue spots confirmed successful hybridization, indicating the presence of the corresponding pathogenic bacteria.

The detection results for the four pathogens are shown in Figure 2 below:

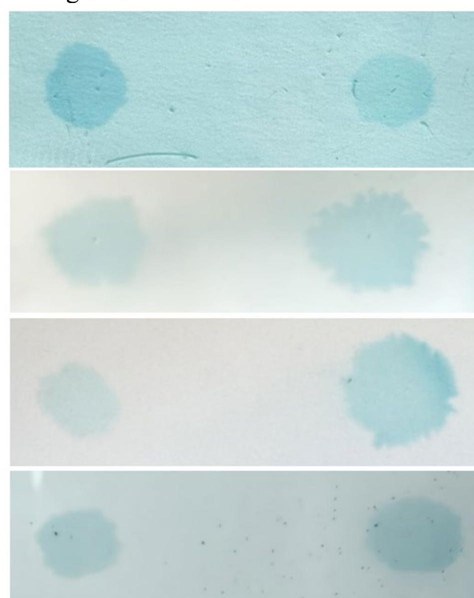


**Figure 2**. Detection results of *Staphylococcus aureus, Escherichia coli, Streptococcus β-hemolyticus*, and *Pseudomonas aeruginosa*.

(*The left blue spot represents 16S rDNA, while the right blue spot corresponds to the respective species-specific gene.*)

### 3.2 Detection Limit

Bacterial colonies of *Staphylococcus aureus, Escherichia coli, Streptococcus β-hemolyticus*, and *Pseudomonas aeruginosa* were collected from freshly cultured agar plates and suspended in PBS to a McFarland standard of 0.5, which corresponds to approximately $10^8$ cfu/mL. The bacterial suspensions were serially diluted in PBS to

final concentrations of $10^2$ cfu/mL and $10^1$ cfu/mL, which were used as the bacterial test solutions.

Detection Process:

- 1 mL of each test solution was inoculated into the respective liquid culture medium and incubated.
- The samples were then analyzed using both the traditional culture method and the new PCR reverse dot blot hybridization method.

Results:

- All test solutions with a bacterial concentration of $10^2$ cfu/mL were successfully detected.
- For test solutions with $10^1$ cfu/mL, the detection rate was 90% or higher.

Based on these findings, the detection limit of the newly developed method was determined to be $10^1$ cfu per reaction.

Using the results from both the traditional and new methods, the relative limit of detection (RLOD) was calculated to be 1, indicating comparable sensitivity.

Detailed data are presented in Table 5.

**Table 5.** Detection Limit Results

| Pathogenic Bacterium | Bacterial Load (cfu/sample) | Number of Positive Tubes | Number of Negative Tubes | RLOD |
|---|---|---|---|---|
| Staphylococcus aureus | 4cfu/sample | 18 | 2 | 1.0 |
| Streptococcus hemolyticus | 7cfu/sample | 19 | 1 | 1.0 |
| Escherichia coli | 10cfu/sample | 20 | 0 | 1.0 |
| Pseudomonas aeruginosa | 5cfu/sample | 19 | 1 | 1.0 |

### 3.3 Reproducibility

To evaluate the reproducibility of the newly developed method, the same bacterial culture tube was used for multiple hybridization tests.

After hybridization, the gray values of the hybridization spots were measured, and the standard deviation was calculated to assess the reproducibility of the method.

Specific values are presented in Table 6, and the gray value distribution is illustrated in Figure 3.

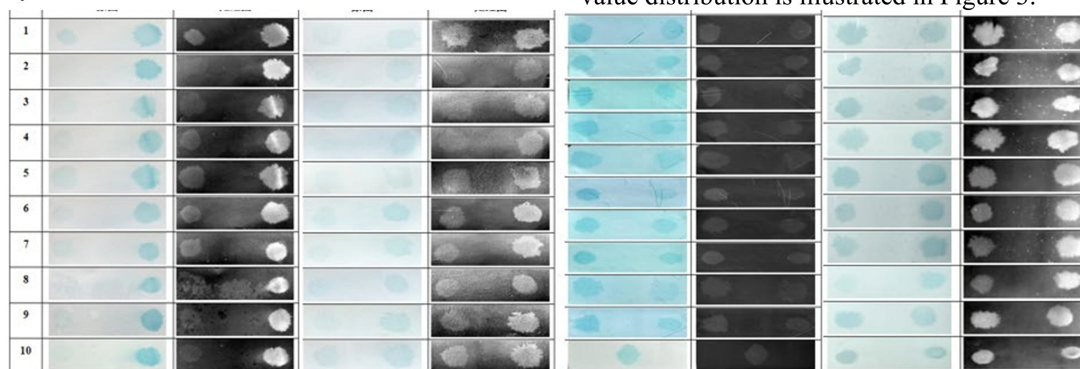


**Figure 3.** Repeated detection of the four pathogenic bacteria (from left to right: Streptococcus β-hemolyticus, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa).

**Table 6.** Gray Values for the Detection of Four Pathogenic Bacteria

| Serial Number | Pathogenic Bacterium | Grayscale Value | Standard Deviation |
|---|---|---|---|
| 1 | Group B Streptococcus | 55.3 | 8.1 |
| 2 | | 62.2 | |
| 3 | | 58.3 | |
| 4 | | 64.3 | |
| 5 | | 59.9 | |
| 6 | | 57.4 | |
| 7 | | 59.7 | |
| 8 | | 62.6 | |
| 9 | | 83.9 | |
| 10 | | 66.9 | |
| 1 | Escherichia coli | 28.3 | 5.0 |
| 2 | | 36.9 | |
| 3 | | 42.1 | |
| 4 | | 43.2 | |
| 5 | | 28.9 | |
| 6 | | 38.8 | |
| 7 | | 33.1 | |
| 8 | | 36.6 | |
| 9 | | 38.3 | |
| 10 | | 38.3 | |
| 1 | Staphylococcus aureus | 73.0 | 5.6 |
| 2 | | 69.5 | |
| 3 | | 70.7 | |
| 4 | | 69.6 | |
| 5 | | 63.7 | |
| 6 | | 71.6 | |
| 7 | | 57.2 | |
| 8 | | 67.7 | |
| 9 | | 59.9 | |

| | | | |
|---|---|---|---|
| 10 | | 60.4 | |
| 1 | Pseudomonas aeruginosa | 65.3 | 16.6 |
| 2 | | 56.5 | |
| 3 | | 68.4 | |
| 4 | | 58.8 | |
| 5 | | 85.8 | |
| 6 | | 91.6 | |
| 7 | | 91.8 | |
| 8 | | 47.1 | |
| 9 | | 49.1 | |
| 10 | | 62.4 | |

## 4 Discussion and Conclusion

As an essential tool for preventing disease transmission, face masks play a crucial role during outbreaks of infectious diseases. One of the key quality indicators of face masks is the absence of pathogenic bacteria. However, traditional methods for detecting pathogens are time-consuming, labor-intensive, and highly dependent on operator expertise, which significantly limits the efficiency of mask inspections. This issue becomes especially critical during public health emergencies, where rapid and reliable testing is essential.

In traditional pathogen detection methods, the required testing times for each bacterium are as follows:

- *Staphylococcus aureus*: 3 days
- *Escherichia coli*: 3 days
- *Streptococcus β-hemolyticus*: 4 days
- *Pseudomonas aeruginosa*: 3 days

Additionally, each pathogen requires a different cultivation method, leading to a substantial workload for laboratory personnel. Although the enzyme-linked immunoassay or multi-fluorescent PCR technology also shorten the detection time to a certain extent, the enzyme-linked immunoassay cannot detect multiple pathogens at the same time, and the multi-fluorescent PCR technology has high equipment requirements, and there are difficulties in the design of primers and probes.

In this study, a detection method based on PCR reverse dot hybridization technique was designed and established, which could detect 4 kinds of pathogenic bacteria simultaneously. In this method, the bacterial solution was obtained by increasing the bacterial culture for 24h. The specific gene was amplified by PCR. The PCR product is hybridized with the probe, and the results can be viewed directly by the naked eye through TMB color rendering. The whole test process takes only 2 days, which saves time at least 30% compared with traditional culture methods. The detection protocol is unified for all four pathogens, which can be carried out at the same time, streamlining the process. Unlike traditional culture-based methods [10], the results are visually detectable, making the method less reliant on specialized equipment and operator experience, significantly reducing the burden on laboratory staff [11].

Another important advantage of this new method is that it is based on the principle of nucleic acid hybridization, offering high specificity, excellent accuracy, and strong reproducibility. The detection limit and repeatability of this method are tested. The detection limit can be as low as 4-10 cfu/ tube, and the repeatability is excellent.

However, it is important to note certain limitations of this study. Due to the lack of actual mask samples, the evaluation was conducted using artificially prepared bacterial suspensions as simulated test samples. Further validation using real-world samples is necessary to confirm its effectiveness in practical applications [12].

In summary, we have developed a pathogenic bacteria detection method based on PCR reverse dot blot hybridization, which is rapid, highly sensitive, highly specific, and easy to operate. Given its efficiency and reliability, this method holds great potential for application in pathogen detection.

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