

Rapid detection of intestinal pathogens in fecal samples by an improved reverse dot blot method

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Abstract

AIM: To develop a new, rapid and accurate reverse dot blot (RDB) method for the detection of intestinal pathogens in fecal samples.

METHODS: The 12 intestinal pathogens tested were *Salmonella* spp., *Brucella* spp., *Escherichia coli* O157:H7, *Clostridium botulinum*, *Bacillus cereus*, *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, *Listeria monocytogenes* and *Staphylococcus aureus*. The two universal primers were designed to amplify two variable regions of bacterial 16S and 23S rDNA genes from all of the 12 bacterial species tested. Five hundred and forty fecal samples from the diarrhea patients were detected using the improved RDB assay.

RESULTS: The methods could identify the 12 intestinal pathogens specifically, and the detection limit was as low as 103 CFUs. The consistent detection rate of the improved RDB assay compared with the traditional culture method was up to 88.75%.

CONCLUSION: The hybridization results indicated that the improved RDB assay developed was a reliable method for the detection of intestinal pathogen in fecal samples.

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Key words: Immunoblotting; Intestinal pathogens; Feces

INTRODUCTION

Foodborne infections are an important public health concern worldwide. The World Health Organization and the US Centers for Disease Control and Prevention (CDC)^[1,2] report every year a large number of people affected by diseases caused by intestinal pathogens that have contaminated food. The main clinical manifestations of infection with intestinal pathogens are nausea, vomiting and diarrhea. The clinical syndromes caused by the different intestinal pathogens are usually not distinguishable^[3,4]. Therefore, identification of intestinal pathogens is heavily dependent on help from clinical laboratories. Most intestinal pathogens are difficult to incubate under the same conditions^[5]. Furthermore, the current assays for identifying pathogens are performed mainly using cultivation of cells. Although the cultivation has been a standard method of pathogenic identification, the whole procedure takes around 5 d, or even longer, to obtain final results. The time-consuming procedure not only makes the methods difficult for high-throughput use, but also involves specialized techniques and expertise^[6]. As a result, the patients would probably lose the optimal chance of therapy, and centers for disease control would not be able to take effective measures rapidly. Consequently, considerable effort should be devoted to establish rapid, sensitive and specific assays for identifying intestinal pathogens.

At present, many methods have been developed to detect intestinal pathogens, such as mass spectrometric analysis^[7], fluorescence polarization^[8,9], real-time fluorescence quantitative PCR^[10,11], microarray^[12,13], and sequencing. Most of these techniques are accurate, but time-consuming, labor-intensive, and hard to adapt to

high-throughput screening. They are only amenable to analysis by those who are well-trained and well-equipped, which is not suitable for small hospitals. Nevertheless, the reverse dot blot (RDB) method can be used to detect many pathogens simultaneously. As bacterial 16S and 23S rDNA genes, bacterial live fossils, have great significance in taxonomy^[14]. These two genes have been less changeable than others in the course of evolution. We combined flow-through hybridization technology with RDB assay to develop a rapid RDB method that can simultaneously detect 10 intestinal pathogens according to the bacterial 16S and 23S rDNA genes. Compared with the conventional passive hybridization process that required hours or even overnight hybridization, the flow-through hybridization takes only several minutes to complete, by directing the flow of the target molecules toward the immobilized probes.

MATERIALS AND METHODS

Bacterial strains and clinical samples

Bacterial reference strains were obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China (Table 1), and chosen from a wide range of genera or species. All the strains selected were cultured for 24-36 h according to conventional methods^[15,16]. All fecal samples were collected from 540 patients who had diarrhea from May 2006 to July 2007, at the Central Hospital in Huzhou, China. The clinical samples were isolated and identified by conventional methods and, except for the coagulase-negative *Staphylococcus aureus*, by the appropriate API test system.

Design of primers and pathogen-specific oligonucleotide probes^[17]

The primers were designed using Primer 5.0 software on conservative regions based on the *Escherichia coli* (*E. coli*) 16S rDNA and 23S rDNA (GenBank accession number U00096). All oligonucleotide probes were designed from variable regions between two pairs of primers of each pathogen available in the GenBank database (GenBank/EMBL/DDBJ). Multiple-sequence alignments were carried out by using the ClustalW program. By comparison of the sequences of the 16S and 23S rDNA regions of the target species, regions with interspecies variations could be identified and were used to develop species-specific probes.

The reverse primer was labeled with biotin at the 5' end, and the hybridization probes were labeled with amino group at the 5' end. In order to judge the validity of the hybridization process, we designed a color control probe for the hybridization control, which was labeled with a biotin group at the 5' end and an amino group at the 3' end. The color control probe can bind only with the chromogen but not with the targeting molecule. All oligonucleotide primers (Table 2) and probes (Table 3) were synthesized commercially at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Table 1 Standard strains used in the present study

Genus or species	Standard strain ATCC accession no.
<i>S. aureus</i>	26001, 26111, 26113
<i>V. cholerae</i>	16025, 16026, 16028
<i>Shigella</i> spp.	51081, 51207, 51335
<i>E. coli</i> O157:H7	44752, 43889, 43859
<i>V. parahaemolyticus</i>	20502, 20506, 20507
<i>Salmonella</i> spp.	50001, 50004, 50013
<i>Y. enterocolitica</i>	52207, 52211, 52215
<i>L. monocytogenes</i>	54003, 54005, 54006
<i>Brucella</i> spp.	23456
<i>C. botulinum</i>	64201, 64203
<i>B. cereus</i>	63301, 6051, 63509
<i>C. perfringens</i>	64711, 13048

Table 2 Universal primers used in the present study

Primer name	Sequence (5'→3')	PCR product size (bp)
16SF	CGCTGGCGGCAGGCCTAACACATGC	500
16SR	Biotin-GCGGCTGCTGGCACGGAGT-TAGCC	
23SF	ACCGATAGTGAACAGTACCGTGAG	640
23SR	Biotin-TTAAATGATGGCTGCTTCTA-AGCC	

DNA isolation and PCR amplification

Processing of the fecal samples and subsequent bacterial DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen) and the genomic DNA of bacterial reference strains was extracted using the QIAamp DNA Mini Kit (Qiagen). Five microliters of the DNA was amplified by PCR in 50 µL of 1 × PCR buffer that contained 200 µmol/L of each dNTP, 2 U *Taq* DNA polymerase (Takara), 0.06 µmol/L forward primers (23S-F and 16S-F) and 0.3 µmol/L reverse primers (16S-R and 23S-R). In order to prevent contamination, we replaced dTTP with dUTP and added 0.5 U uracil-DNA glycosylase (UDG) to the PCR system. The amplification was performed by using an Applied Biosystems 9600 thermal cycler (Perkin-Elmer) under the following conditions: incubation at 50°C for 3 min, before an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s. A final extension was performed at 72°C for 5 min.

Membrane preparation and subsequent immobilization of oligonucleotides

Biodyne C membranes (Pall Co.) were rinsed briefly with 0.1 mol/L HCl, and then treated for 15 min with freshly prepared 20% EDC (w/v) [N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride; Sigma-Aldrich, commercial grade] in deionized water, rinsed with deionized water, and the amino-modified oligonucleotide probes dissolved in 0.5 mol/L sodium bicarbonate buffer (pH 8.4) were dotted at the given positions on the membrane (Figure 1). The amino group of the probe may bind with the carboxyl group of the membrane. The dots were rinsed with Tris-buffered saline/0.1%

Table 3 Oligonucleotide probes used in the present study

Probe	Sequence (5'→3')	Target
1	GGGAGTAAAGTTAATACCTTIGCTCATTGA	<i>Salmonella</i> spp.
2	CACACTGGAAC TGAGACACGGTCGAGACTCCTACG GGA	Bacterial universal probe
3	CGTACCATTGCTACGGAATAACTCAGGAAAAC TTGTG	<i>Brucella</i> spp.
4	TCACCCATAAAAAGAGGCTCCCACTGC	<i>E. coli</i> O157:H7
5	TATAAGAGAATCGCATGATTTTCTTATCCAAAGATTTAT	<i>C. botulinum</i>
6	IGCTAGTTGAATAAGCTGGCACCTTGACG	<i>B. cereus</i>
7	ATGGCATCATCATTCAACCATTGGAGCAATCCGCTATGAGATGGACCC	<i>C. perfringens</i>
8	GGTTTCAGGTTCTTTTTCACCTCCCTCGCCG	Common probe for <i>Shigella</i> and <i>Salmonella</i> spp.
9	AAACGAGTTATCTGAACCTTCGGGGAACGATAACGG	<i>V. parahaemolyticus</i>
10	GAAGGCCTTTTCGATAATGATACCGGCGCTCTGCTCTCCC	<i>Shigella</i> spp. and Enteroinvasive <i>E. coli</i>
11	GGTGTGTGGTAAATAACCGCAGCAATIGA	<i>Shigella</i> spp.
12	CTTCAATAATGCCAGCAGCTCCAACCCCGAAAATAGATA	<i>Salmonella typhi</i>
13	CATAAAGGTTAATAACCTTTGTGATTGACGT	<i>Y. enterocolitica</i>
14	GCGGCAGCGGGAAGTAGTTTACTACTTTGCCGG	<i>Yersinia</i> spp.
15	CAGCACAGAGGAAC TTGTTCCTTGGGTGGCGAG	<i>V. cholerae</i>
16	TGTTGTTAGAGAAGAACAAGGATAAGAGTAAC TGCT	<i>L. monocytogenes</i>
17	ACATATGTGTAAGTAAC TGTCACATCTTGACGGTA	<i>S. aureus</i>
P	TTTGGCTAACTCCGTGCCAGCAGCCGCG	Positive control
C	BIOTIN-CCGCTGTATCACAAGGGCTGGTACCTTT	Color control
N	TTTCCGCTGTATCACAAGGGCTGGTACC	Negative control
B	0.5 mol/L sodium bicarbonate buffer	Blank control

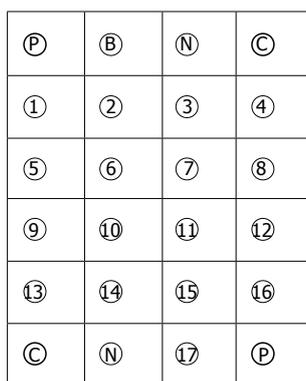


Figure 1 Layout of oligonucleotide probes. Their sequences are indicated in Table 3.

by pumping. All of the hybridization solution, washing solution, POD solution, and coloring solution flowed through the membrane automatically. The improved RDB method actively directed the flow of the targeting molecules toward the immobilized probes within the membrane fibers. The complementary molecules were hybridized and formed duplex DNA; at the same time, any unbound molecules were removed by passing through the membrane. This speeded up the interaction between the complementary molecules, reduced the hybridization time from hours down to minutes, and provided results hundreds of times faster than by using traditional passive hybridization methods.

Tween-20. Any remaining active groups were quenched with 0.1 mol/L NaOH for 10 min. Finally, filters were rinsed with deionized water and air-dried for storage, or were used immediately for hybridization.

RDB and flow-through hybridization

The improved RDB method was used according to the principle of flow-through hybridization, which was performed on the KaiPu DNA hybriMax Rapid Hybridization Machine (Hong Kong DNA Ltd., Hong Kong, China). Its detailed steps were as follows: (1) denature the PCR products (or omitted); (2) prehybridize the membrane (or omitted); (3) hybridize the target PCR products with the specific probes at 42°C for 15 min; (4) wash the unhybridized PCR products; (5) combine peroxidase (POD) with the biotin group on the PCR products or on the color control probe at 37°C for 5 min; (6) wash the membrane to eliminate the uncombined POD; and (7) color with 3,3',5,5'-tetramethylbenzidine (TMB) chromogen. We set positive and negative controls for all detections. The machine worked on the basis of the particular principle of flow-through hybridization; there was a negative pressure under the airproof hybridization membrane, which was produced

RESULTS

Dual PCR amplification from DNA from clinical fecal samples

The 16S and 23S rDNA from intestinal pathogens were amplified simultaneously directly from fecal samples using asymmetric PCR. All the fecal samples tested gave PCR products with bands of approximately 500 bp and 640 bp (Figure 2 shows partial PCR amplification results for intestinal pathogens from fecal samples).

Validation of the bacterial reference strains using the improved RDB method

The PCR products were used to hybridize with the oligonucleotide probes on the membrane, followed by signal acquisition using the TMB to generate the respective hybridization maps. The results are shown in Figure 3. A given isolate was easily identified as one of the target pathogens from the hybridization signals of the probe spot. The results were in close agreement with those predicted from the layout of the probes. For instance, in the hybridization map shown in Figure 3, array A, there were strong hybridization signals at the

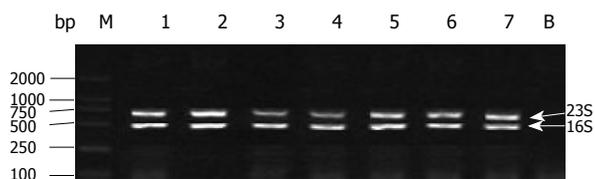


Figure 2 Partial dual PCR amplification results for intestinal pathogens from fecal samples. M: DNA marker 2000; B: Blank control.

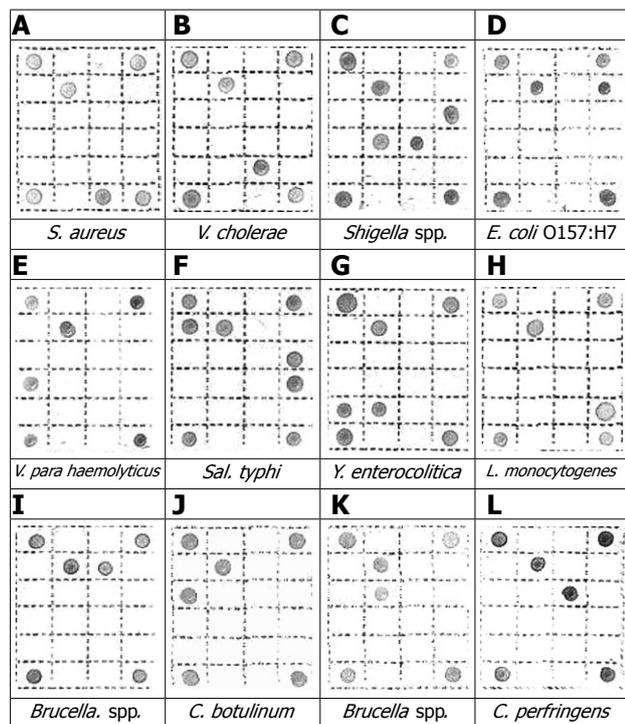


Figure 3 Typical hybridization profiles on the membrane from pure bacterial culture.

sites that corresponded to oligonucleotide probes 17, therefore, the pathogen was sequentially identified as *S. aureus*. Based on the results of multiple experiments, we regarded a hybridization signal as specific if the foreground signal at an oligonucleotide probe site was a stronger color than its background signal. It was easy to identify the specific hybridization signals directly from the hybridization maps by the naked eye. The strains were *Salmonella* spp., *Brucella* spp., *E. coli* O157:H7, *Clostridium botulinum*, *Bacillus cereus*, *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, *Listeria monocytogenes*, and *S. aureus*.

Detection limit of the improved RDB assay

Serial dilutions of a clinical isolate of *E. coli* O157:H7 were tested by using the improved RDB method. The data indicated that as few as 10³ CFUs could be detected consistently.

Detecting the intestinal pathogens from fecal samples directly

To evaluate the application of this assay, 540 fecal samples from patients with diarrhea were detected.

Table 4 Comparison between the improved RDB and culture

Intestinal pathogen	RDB (+)/ culture (+)	RDB (+)/ culture (-)	RDB (-)/ culture (+)
<i>S. aureus</i>	52	13	12
<i>V. cholerae</i>	4	3	5
<i>Shigella</i> spp.	51	3	3
<i>E. coli</i> O157:H7	28	2	2
<i>V. parahaemolyticus</i>	158	16	11
<i>Salmonella</i> spp.	36	5	3
<i>Y. enterocolitica</i>	3	4	4
<i>L. monocytogenes</i>	4	1	2
<i>B. cereus</i>	3	0	1
<i>C. perfringens</i>	2	0	1
<i>S. aureus</i> and <i>Shigella</i> spp.	2	2	0
<i>S. aureus</i> and <i>Salmonella</i> spp.	2	0	0
<i>Salmonella</i> spp. and <i>Shigella</i> spp.	3	0	0
<i>V. parahaemolyticus</i> and <i>V. cholerae</i>	1	0	0
<i>V. parahaemolyticus</i> and <i>Shigella</i> spp.	5	1	0
Total	354	50 ¹	44 ²

¹ was compared with ², $\chi^2 = 464, P > 0.05$. RDB: Reverse dot blot.

Table 4 compares the results obtained for the intestinal pathogens by a conventional culture and the improved RDB assay. By the improved RDB assay, 404 (74.81%, 404/540) samples were found to be positive for intestinal pathogens, and mixed pathogens were detected from 16 samples. By the culture method, 399 (73.89%, 399/540) samples were found to be positive for pathogens, and mixed pathogens were detected from 13 samples. A total of 354 samples that were RDB positive were also culture positive. Additionally, 50 samples were detected by RDB but were not found by culture. Forty-four of the culture-positive samples were RDB negative. The data indicated that 354 (88.75%, 354/399) specimens identified using RDB were the same as those identified by conventional methods, $\chi^2 = 464, P > 0.05$.

DISCUSSION

With the development of more aggressive therapeutic regimens, especially for the treatment of intestinal pathogens, the incidence of foodborne infections has increased. The early initiation of antibacterial treatment is critical in reducing the high mortality rate in patients with infection. Early and accurate identification of the pathogen is the most important and critical step in providing adequate antibacterial therapy in time. The conventional method of identification of intestinal pathogens used in clinical microbiology is based on phenotypic features and physiological tests, and is therefore time-consuming. Instead, molecular genotyping methods could provide a rapid and specific means of identification of intestinal pathogens. At present, diagnostic DNA microarrays are applied for the identification of viruses^[18-21], bacteria^[22-26], and mechanisms of resistance to certain antibiotics^[27-29].

However, the conventional hybridization methods are conducted on two-dimensional surfaces, which require several hours to complete the molecular hybridization

process, and large volumes of sample and reagent. In this study, we described the successful application of the improved RDB method to detect intestinal pathogens. It is a simple, rapid, semiautomatic, reliable, and contamination-proof approach to screen pathogens from fecal samples. We developed a commercially prepared intestinal pathogens detection kit equipped with the KaiPu DNA HybriMax Rapid Hybridization Machine. The machine was designed based on the particular principle of flow-through hybridization. There is a negative pressure under the airproof hybridization membrane that is produced by pumping, so the improved method actively directs the flow of the target molecules toward the immobilized probes within the membrane fibers, which enables rapid hybridization to occur. The dominant characteristic of the improved RDB method is that all of the PCR products, washing buffer, binding solution, and coloring solution flow through the hybrid membrane quickly and directly, with the help of negative pressure, which is semi-automated and is essentially different from the traditional method. The complementary molecules are hybridized and form duplex DNA; at the same time, any unbound molecules are removed through the membrane. This speeds up the interaction between the complementary molecules, reduces the hybridization time from hours down to minutes, and provides results hundreds of times faster than the traditional passive hybridization methods^[30].

For the present study, we designed and optimized not only the specific probes for the specific target pathogens, but also the color control probe for the hybridization operation to reach 100% specificity. The color control probe can bind only with the chromogen, but it cannot bind with the target molecule, which helps to judge the validity of hybridization. Instead of using dTTP, we used dUTP and UDG in the PCR system to prevent PCR products from causing contamination. In addition, the improved RDB method is clean, versatile, and less expensive than traditional hybridization. The improved RDB assay directs all of the PCR products and solution to directly flow through the hybrid membrane, which increases the diffusivity and local reaction concentration of the nucleic acid molecule, which occurs in three-dimensional volumes.

We detected 540 fecal samples from patients with diarrhea using the improved RDB assay and culture in parallel. The consistent detection rate of the improved RDB assay compared with the traditional culture method was up to 88.75%. However, the reason that 10 samples were detected by RDB but were not found by culture is that the PCR can amplify DNA fragments even from dead strains, or that the domain bacterial colony grew too rapidly to separate it from the target intestinal pathogens. Otherwise, there is a large amount of unknown substance to disturb the PCR, so that five of the culture-positive samples were RDB negative. However, the data indicated that there was no significant difference between the improved RDB assay and culture to detect the intestinal pathogens from fecal samples. All of these findings indicate that the method is sensitive, specific, and ensures quality in clinical tests.

COMMENTS

Background

Early and accurate identification of the pathogen is the most important and critical step for clinical diagnosis and antimicrobial therapy. Many molecular methods have been applied to pathogen diagnosis.

Innovations and breakthroughs

A new, rapid and accurate reverse dot blot (RDB) method was developed for the detection of intestinal pathogens in fecal samples collected from human subjects, by using the flow-through hybridization principle. The above assay is rapid, accurate stable, reliable and convenient.

Applications

The new technique may play an important role in detection of food poisoning, environmental monitoring, and clinical diagnosis of infectious disease.

Peer review

This is a well performed and interesting study that examined rapid detection methods for intestinal pathogens.

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