IDENTIFICATION OF BACTERIAL INTESTINAL PATHOGENS BY A PCR-REVERSE DOT BLOT PROCEDURE

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ABSTRACT

Intestinal infections which are the important public health concern worldwide, are caused by the bacterial intestinal pathogens. The aim at our study is to develop a simultaneous, rapid, sensitive and specific diagnostic assay by using a combined PCR-Reverse dot blot method for the identification of pathogen strains, including Bacillus cereus, Clostridium botulinum, Clostridium perfringen, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli O157:H7, Salmonella spp., Shigella spp., Vibrio cholerae, Vibrio parahaemolyticus, Yersinia enterocolitica and Brucella spp.

Based on the 16S and 23S DNA regions, the two sets of universal primers and twelve specific probes were obtained for amplification and specific detection of those twelve bacterial species. The initial experimental results using bacterial cultures and 50 clinical samples confirmed the in silico hypothesis that was previously established in universal primers and probes design as well as identified with some basic conditions for PCR and Reverse Dot Blot hybridization reactions. Thus, this procedure being further tested for the other kinds of samples such as fecal samples or foods.

Keywords*: 16S RDNA; 23S rDNA; PCR; Reverse dot blot; bacterial intestinal pathogens.*

1. Introduction

Intestinal infections which are the important public health concern worldwide, are caused by the bacterial intestinal pathogens. This is one of the global problems which are published by the World Health Organization (WHO) annually with the very large number of cases of intestinal infections and food poisoning (WHO). The main cause are using of food, drinking water,…ect, which are contaminated with some bacteria such as *Escherichia coli, Shigella* spp., *Salmonella* spp., *Staphylococcus aureus, Bacillus cereus…*, ect (Chamberlain, 2009; Jin *et al*, 2005; Krzyszycha, Bielak, 2004; Lê Huy Chính *et al*, 2005).

Clinical symptoms of those infected usually vomiting and diarrhea are difficult to distinguish among various bacterial infections which were mentioned in several publications such as Krzyszycha R. *et al*. (2004), Kudaka J. *et al*. (2005), Xing J.M. *et al*. (2009). Therefore, determining exactly bacterial infection is a require very essential of clinical laboratories (Ramaswamy *et al*, 2007). These techniques are now being applied to hospitals mainly based on bacterial culture. Although this is the gold standard of identifying

bacterial infection but it took several days to obtain results and therefore this affected to the selection of appropriate treatments for patients promptly (Huang *et al*, 2004; Jin *et al*, 2005; Maity *et al*, 2008; Xing *et al*, 2009). So, obviously, a quick method, sensitivity and specificity to accurately determine the pathogenic bacteria is an essential demand. Recently, the application of molecular hybridization techniques such as PCR Reverse Dot Blot (PCR-RDB) has been used, including in multi-detection of pathogenic bacteria (Chiang *et al*, 2006; Fiss *et al*, 1992; Gui, Patel, 2011; Myers *et al*, 2006; Volokhov *et al*, 2002). Jin LQ *et al* (2005) used PCR-RDB to detect some intestinal bacteria such as *Samonella* sp., *Escherichia coli, Shigella sp., Listeria monocytogenes, Vibrio parahaemolyticus, Staphylococcus aureus, Proteus sp., Bacellus cereus, Vibrio cholera, Enterococcus faecalis, Yersina enterocolytica* and *Campylobacter jejuni* based on 16S rDNA region. Xing JM *et al* (2009) established a protocol using PCR-RDB based on 16 and 23S rDNA regions to simultaneously detection of a group of bacteria including *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 *S. aureus*). Obviously, PCR-RDB is proving its advantages when applied simultaneously to detect multiple bacterial infections in some studies as just listed above.

With the PCR-RDB principle, after amplification step (moderately selective with universal primers), the distinction of each PCR product is characterized by each probe which specified to each bacterium in Reverse Dot Blot (RDB) hybridization step. Thus, PCR-RDB is very suitable for simultaneous detection of multiple pathogenic bacteria; technique is so simple, quick, semi-automatic and is completely eliminate the contamination. In terms of selection the target gene sequences in order to detect the group of bacteria, the DNA sequence spreading from 16S to 23S rDNA is still the most appropriate choice of PCR-RDB. At first, this sequence is less variation, probably presenting more than one copy/bacterial genome which increases the sensitivity of the technique using this sequence as target. Also, 16S and 23S rDNA genes contain several conservative regions which interleaved with variable regions among bacterial genus and/or species that easily to design universal primers as well as specific probes (Eom *et al*, 2007; Woese, 1987; Lee *et al*, 2014; Böhme *et al*, 2014).

Therefore, the aim at our study is to develop a simultaneous, quick, sensitive and specific diagnostic assay by using a PCR-RDB method for the identification of pathogenic bacteria including *Bacillus cereus, Clostridium botulinum, Clostridium perfringen, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae, Vibrio parahaemolyticus, Yersinia enterocolitica* and *Brucella* spp based on the 16S and 23S DNA regions.

2. Materials and methods

Clinical samples

A total of 50 blood culture bottles from Trung Vuong, HoChiMinh city Hospital and Pediatric Thanh Hoa Hospital, Vietnam were collected and submitted to our laboratory for DNA detection. These samples were also submitted for culture detected bacteria at Microbiology laboratories at two Hospitals mentioned above.

Other isolates

To test for the specificity and cross-reactivity of the PCR-RDB assay, *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Salmonella* spp., *Shigella* spp and *E. coli* strains that we collected from Institute of Pasteur, HoChiMinh city, were used.

Preparation of clinical specimens and cell culture samples

Swab samples were collected, transported to the laboratory at 4-8^{o}C. The specimen (a bacterial culture or a clinical sample) was squeezed and shaken vigorously for 1 min in 1 ml stroke-physiological saline solution (SPSS). After centrifugation at 13,000 rpm for 5 min, the pellet was dissolved in 50 µl buffer (250 mg/l proteinase K, 10 mmol/l Tris–HCl pH 7.8, 5 mmol/l EDTA, 0.45% Tween-20), followed by incubation at 56°C for 60 min and 100°C for 10 min. Centrifugation at 13,000 rpm for 5 min, supernatant liquid was collected and stored at −20°C later used for our novel PCR-RDB assay. Nucleic acids were extracted from each specimen using phenol/chloroform, according to Chomczynski & Sacchi method (1987) (Ge *et al*, 2002) with some modifications: 100 µl of each collected supernatant liquid was added by 900 µl Trizol, pH8. DNA was then precipitated by the same volume of Isopropanol and the helper seeDNA. DNA pellet was dissolved in TE 1X (Tris-EDTA) and stored at -20^{o}C until use.

PCR-RDB

Primers and probes (Table 1) used in this study were designed basing on 16S and 23S rDNA gene sequences published on Genbank and synthesized by Integrated DNA Technology (IDT, USA).

PCR was performed with a Mxpro-Mx3005P (Stratagene). The total volume of the PCR was 50 µl containing 20 ng of DNA, 100 nmol/L of each primers, 400 µmol/L of each dNTP, 1.5 Units of hot-start *Taq* polymerase and 3 mmol/L $MgCl_2$, adding DW to 50 µl. The cycling conditions were an initial denaturation at 95°C for 5 min, 40 cycles of 95°C for 30 s, and 56°C for 30 s and 72°C for 30 s.

Table 1. Primers, probes used in this study

NAME	FUNCTION	SEQUENCE (5'-3')	GENE/ORGANISM	SIZE OF PCR PRODUCT (BP)
Primer 16SF	Forward primer	CGCTGGCGGCAGGCCTAACACATGC	16S rRNA/11 bacteria	500
Primer 16SR	Reverse primer	GCGGCTGCTGGCACGGAGTTAGCC	16S rRNA/11 bacteria	
Primer 23SF	Forward primer	ACCGATAGTGAACCAGTACCGTGAG	23S rRNA/*E. coli* O157:H7	
Primer 23SR	Reverse primer	TTAAATGATGGCTGCTTCTAAGCC	23S rRNA/*E. coli* O157:H7	640
Probe BC	Probe	TGCTAGTTGAATAAGCTGGCACCTTGACG	16S rRNA/*B. cereus*	
Probe CB	Probe	TATAAGAGAATCGCATGATTTTCTTATCCAAAGATTTAT	16S rRNA/*C. botulium*	
Probe CP	Probe	ATGGCATCATCATTCAACCATTGGAGCAATCCGCTATGAGATGGACCC	16S rRNA/*C. perfringens*	
Probe STA	Probe	ACATATGTGTAAGTAACTGTGCACATCTTGACGGTA	16S rRNA/*S. aureus*	
Probe LIS	Probe	TGTTGTTAGAGAAGAACAAGGATAAGAGTAACTGCT	16S rRNA/*L. monocytogenes*	

NAME	FUNCTION	SEQUENCE (5'-3')	GENE/ORGANISM	SIZE OF PCR PRODUCT (BP)
Probe SAL	Probe	GGTGTTGTGGTTAATAACCGCAGCAATTGA	16S rRNA/*Salmonella* sp.	
Probe SHI	Probe	GGGAGTAAAGTTAATACCTTTGCTCATTGA	16S rRNA/*Shigella* sp.	
Probe VPA	Probe	AAACGAGTTATCTGAACCTTCGGGGAACGATAACGG	16S rRNA/*V. parahaemolyticus*	
Probe VCH	Probe	CAGCACAGAGGAACTTGTTCCTTGGGTGGCGAG	16S rRNA/*V. cholerae*	
Probe YEN	Probe	CATAAAGGTTAATAACCTTTGTGATTGACGT-3	16S rRNA/*Y. enterocolitica*	
Probe BRU	Probe	CGTACCATTTGCTACGGAATAACTCAGGGAAACTTGTG	16S rRNA/*Brucella* sp.	
Probe ECO	Probe	TCACCCCATAAAAGAGGCTCCCACTGC-3	23S rRNA/ *E. coli* O157:H7	

RDB was done with some basic conditions such as: PCR product denaturation in 30 minutes, hybridization in 3 hrs at 40^{o}C, following membrane washing in 20 minutes and signal development after 30-45 minutes incubation of staining buffer.

3. Results and Discussion

Specificity of primers, probes *in silico*

BLAST searches were performed to check the specificity of the DNA sequences of the primers, probes showing suitable results (data not shown). In addition, we also checked some other physical characteristics of primers, probes using the analyzer tool integrated with IDT website http://www.idtdna.com/analyzer/Applications/OligoAnalyzer). Most of the characteristics of primers, probes used in this study were suitable (table 2), including the parameters of lengths, percentages of GC, melting temperatures and energy (kcal/mol) calculated by self- hetero-dimer and hairpin-loop structures of primers and/or probes.

Table 2. Theoretical characterization of primers, probes

NAME	LENGTH	Tm (°C)	%GC	HAIRPIN (ΔG)	SELF DIMER (ΔG)	HETERO DIMER (ΔG)	
Primer 16SF	25	68.4	68	-2.91	-12.47	-6.75	
Primer 16SR	24	68.5	70.8	-1.62	-7.81		
Primer 23SF	25	59.8	52	0.67	-3.65	-5.02	

NAME	LENGTH	Tm (°C)	%GC	HAIRPIN (ΔG)	SELF DIMER (ΔG)	HETERO DIMER (ΔG)	
Primer 23SR	24	55.6	41.7	-2.29	-7.81		
Probe BC		62.1					
Probe CB		57.1					
Probe CP		68.6					
Probe STA		61.5					
Probe LIS		60					
Probe SAL		61.2					
Probe SHI		56.9					
Probe VPA		63					
Probe VCH		67.3					
Probe YEN		64.3					
Probe BRU		63.5					
Probe ECO		66.7					

With probes, we focused on the melting temperature (Tm) characteristic especially, because of the purposing simultaneous detection of 12 bacteria by PCR-RDB. For that, these Tm(s) must be adjusted how easily to combined all of them onto a membrane in the same hybridization conditions. As the results, Tm(s) of 12 probes were not so much different (Table 2), range from 56.9^{o} to 68.6^{o}C, that could be easily solved in hybridization procedure.

Specificity of primers, probes *in vitro*

The specificity of primers was 100% since and the assay was able to detect DNAs from all strains mentioned in materials and methods (Data not shown). The first RDB experiment using PCR products have been mentioned above, was conducted with some major conditions such as hybridization temperature at 40^{0}C, 3 hrs for hybridization,

using 20 µl of each PCR product of each Dot and membrane washing within 20 seconds. As the results, some hybridization signals were not good: for example, beside the specific signal between STA probe and PCR product from *Staphylococcus aureus* (Fig. 1, arrow noted C1), two other unexpected signals were also obtained (Fig. 1, arrows noted B1 and B5). It means that between CP and LIS probes and PCR product from *Staphylococcus aureus* still have crossing hybridization even the signals were faint.

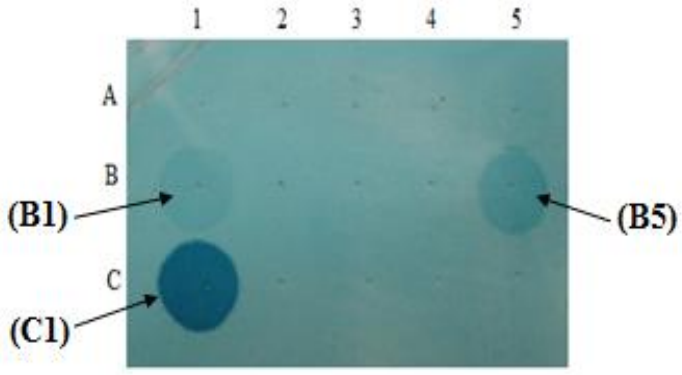


Figure 1. PCR-RDB results between PCR product from *Staphylococcus aureus* and 12 probes.

The specific check, *in silico*, has also been conducted soon after. As the results, between LIS (specificity for *Listeria monocytogenes*) and CP (specificity for *Clostridium perfringens*) probe sequences also showed high variable compared with some representative 16S rDNA sequences from *Staphylococcus aureus* (Fig. 2 and 3).

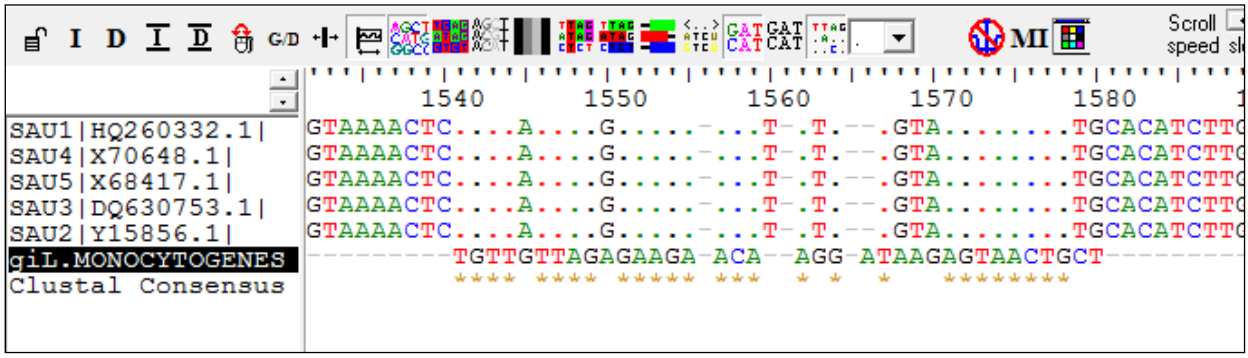


Figure 2. Alignment among LIS (specificity for *Listeria monocytogenes*) sequence and representative 16S rDNA sequences from *Staphylococcus aureus* by Bioedit software.

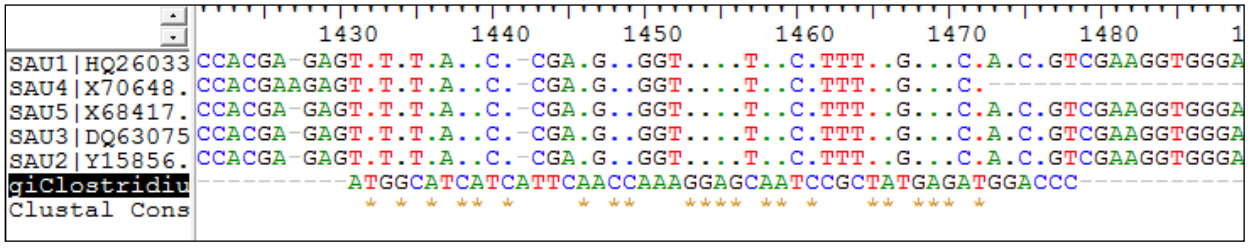


Figure 3. Alignment among CP (specificity for *Clostridium perfringens*) sequence and representative 16S rDNA sequences from *Staphylococcus aureus* by Bioedit software.

Therefore, the hybridization was modified by some major conditions such as hybridization temperature at 42^0C and also washing within 30 seconds. As the results, the hybridization signals must be better: crossing hybridization signals were less (Fig. 4).

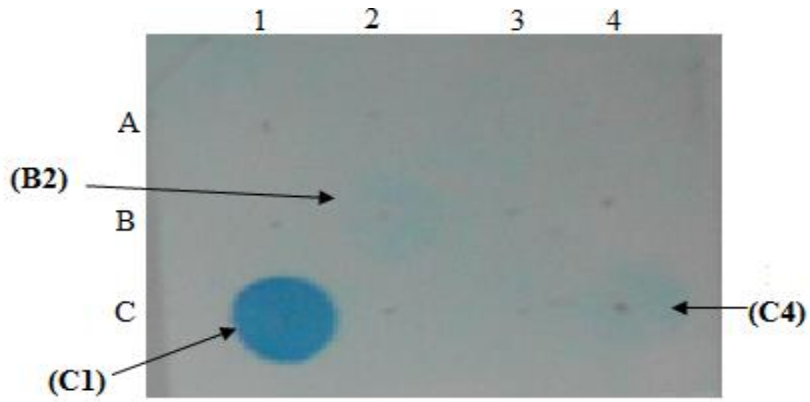


Figure 4. PCR-RDB results between PCR product from *Staphylococcus aureus* and 12 probes in modified hybridization conditions.

Increase the hybidization temperature to 45^0C and other conditions given the same previous experiments, we got the results as expected (Fig. 5, membrane noted 2). Figure 5 also showed other PCR-RDB results from PCR products of *Bacillus cereus (1), Listeria monocytogenes (3), Salmonella* sp. (4), and *Shigella* sp. (5) with 12 probes positioned on membrane.

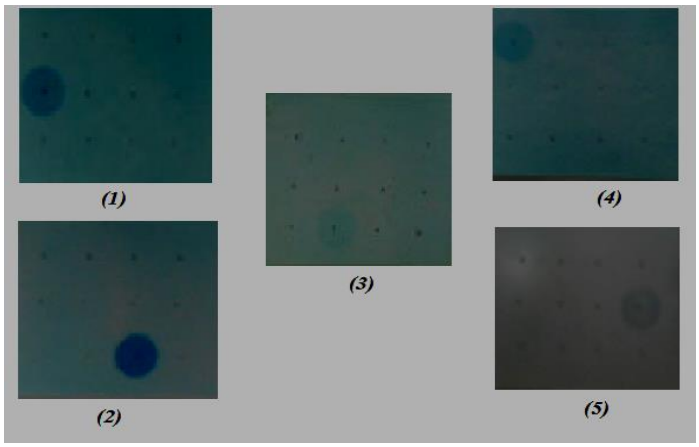
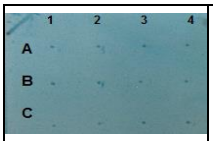


Figure 5. PCR-RDB results of PCR products from *Bacillus cereus* (1), *Staphylococcus aureus* (2) *Listeria monocytogenes* (3), *Salmonella* sp. (4) and *Shigella* sp. (5) and 12 probes that were fixed in each dot on each membrane.

PCR-RDB on clinical samples

All samples have been tested by the two assays: our novel PCR-RDB (with the matrix of 12 probes fixed on each membrane shown on table 3) and culture detected bacteria.

Table 3. Matrix of 12 probes fixed on each membrane

	**1**	**2**	**3**	**4**
A	**SAL** (*Salmonella* spp.)	**BRU** (*Brucella* spp.)	**ECO** (*E.coli O157:*H7)	**CB** (*C. botulinum*)
B	**BC** (*B. cereus*)	**CP** (*C. perfringens*)	**VPA** (*V. parahaemolyticus*)	**SHI** (*Shigella* spp.)
C	**VCH** (*V. cholerae*)	**LIS** (*L. monocytogenes*)	**STA** (*S. aureus*)	**YEN** (*Y. enterocolitica*)

Of the 50 samples collected from Trung Vuong Hospital of HoChiMinh city and Pediatric Hospital of Thanh Hoa province, 14 samples were infected with *Staphylococcus aureus* (Fig. 6, noted C3 on each membrane), and 1 sample was infected with *Shigella* sp. by PCR-RDB test (Fig. 6, noted B4).

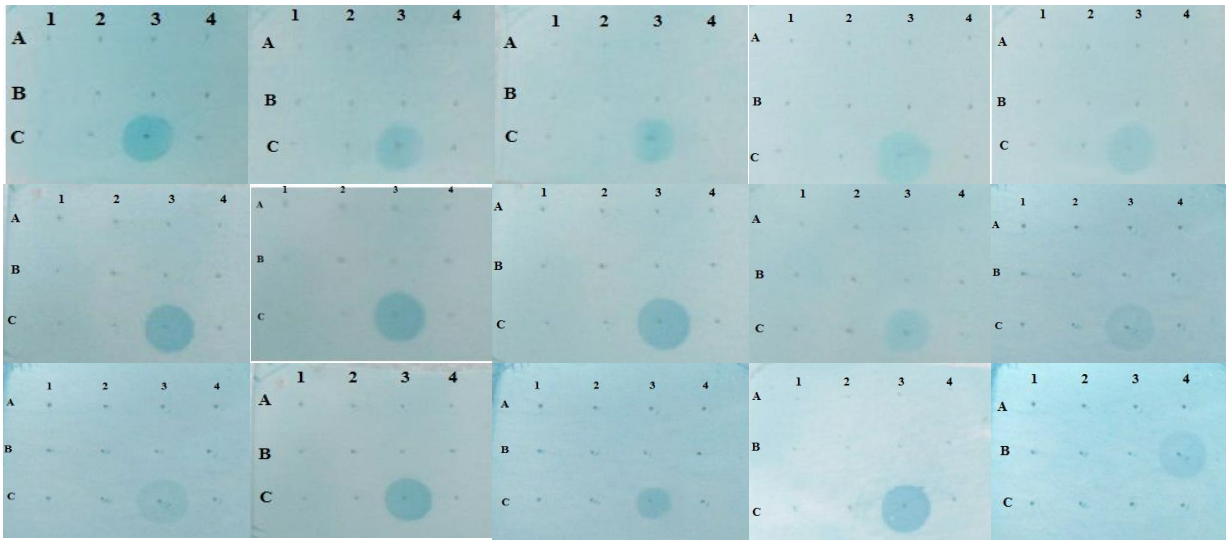


Figure 6. PCR-RDB results between PCR products from clinical samples and 12 probes that were fixed in each dot on each membrane.

The 35 other samples that infected by other bacteria showing on table 4 were not included in 12 bacteria that were PCR-RDB targets of this study (Table 4), therefore the RDB results were negative. Especially, two samples that infected by *Escherichia coli* (samples number 48 and 49, table 4) were also not detected by ECO probe. We clarified later those samples infected by not *Escherichia coli* O157:H7. So, the results obtained were completely consistent with the specificities by the two tests: PCR-RDB and bacterial culture, no false-positive PCR-RDB results compared to culture detected bacteria (Table 4).

Table 4. Summarize of two identified bacterial tests results from 50 clinical samples

No	Bacterial culture	PCR		RDB (positive probe)
		16S primer pairs	23S primer pairs	
1	*Pseudomonas aeruginosa*	+	∞	-
2	*Pseudomonas aeruginosa*	+	∞	
3	*Pseudomonas aeruginosa*	+	∞	
4	*Pseudomonas aeruginosa*	+	∞	
5	*Pseudomonas aeruginosa*	+	∞	
6	*Pseudomonas aeruginosa*	+	∞	
7	*Pseudomonas aeruginosa*	+	∞	
8	*Stenotrophomonas maltophilia*	+	∞	-
9	*Stenotrophomonas maltophilia*	+	∞	
10	*Stenotrophomonas maltophilia*	+	∞	
11	*Stenotrophomonas maltophilia*	+	∞	
12	*Stenotrophomonas maltophilia*	+	∞	
13	*Staphylococcus aureus*	+	∞	**+ (SAU)**
14	*Staphylococcus aureus*	+	∞	**+ (SAU)**
15	*Staphylococcus aureus*	+	∞	**+ (SAU)**
16	*Staphylococcus aureus*	+	∞	**+ (SAU)**
17	*Staphylococcus aureus*	+	∞	**+ (SAU)**
18	*Staphylococcus aureus*	+	∞	**+ (SAU)**
19	*Staphylococcus aureus*	+	∞	**+ (SAU)**
20	*Staphylococcus aureus*	+	∞	**+ (SAU)**
21	*Staphylococcus aureus*	+	∞	**+ (SAU)**
22	*Staphylococcus aureus*	+	∞	**+ (SAU)**
23	*Staphylococcus aureus*	+	∞	**+ (SAU)**
24	*Staphylococcus aureus*	+	∞	**+ (SAU)**
25	*Staphylococcus aureus*	+	∞	**+ (SAU)**
26	*Staphylococcus aureus*	+	∞	**+ (SAU)**
27	*Shigella sonnei*	+	∞	**+ (SHI)**
28	*Streptococcus mitis*	+	∞	-
29	*Streptococcus mitis*	+	∞	
30	*Streptococcus mitis*	+	∞	
31	*Streptococcus mitis*	+	∞	
32	*Streptococcus mitis*	+	∞	

No	Bacterial culture	PCR		RDB (positive probe)
		16S primer pairs	23S primer pairs	
33	*Streptococcus mitis*	+	∞	
34	*Streptococcus mitis*	+	∞	
35	*Streptococcus mitis*	+	∞	
36	*Streptococcus mitis*	+	∞	
37	*Streptococcus mitis*	+	∞	
38	*Streptococcus mitis*	+	∞	
39	*Streptococcus mitis*	+	∞	
40	*Streptococcus mitis*	+	∞	
41	*Streptococcus pneumoniae*	+	∞	-
42	*Streptococcus pneumoniae*	+	∞	
43	*Haemophilus influenzae*	+	∞	-
44	*Haemophilus influenzae*	+	∞	
45	*Haemophilus influenzae*	+	∞	
46	*Acinetobacter baumannii*	+	∞	-
47	*Acinetobacter baumannii*	+	∞	
48	*Escherichia coli*	∞	+	-
49	*Escherichia coli*	∞	+	
50	*Klebsiella pneumoniae*	+	∞	-

Some of the PCR products were also confirmed by DNA sequencing (Macrogen, Korea) showing 100% specificity (data not shown).

In the future, the continued testing PCR-RDB using other kinds of more appropriate samples such as fecal sample or foods will be conducted. Also, the additional kinds of positive control, negative control as well as blank and colour control for PCR-RDB technique will also be our future progress.

4. Conclusion

Two universal pair primers and 12 specific probes based on 16S and 23S rDNA target genes to detect 12 pathogenic intestinal bacteria including *Bacillus cereus, Clostridium botulinum, Clostridium perfringen, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli O157:H7, Salmonella* spp., *Shigella* spp., *Vibrio cholerae, Vibrio parahaemolyticus, Yersinia enterocolitica* and *Brucella* spp. showed suitable function, theoretically as well as experimentally. The initial experimental results using bacterial cultures and 50 clinical samples have informed us some basic conditions of PCR-RDB.

The procedure thus being further tested for other kinds of samples such as fecal samples or foods.

Acknowledgments

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