Co-detection of five species of water-borne bacteria by multiplex PCR☆

Hongying Fan[1], Qingping Wu[2,*], Xiaoxia Kou[2]

[1]*The Microbiology Department of Southern Medical University, Guangzhou, Guangdong* 510515, *China;* [2]*Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology, Guangzhou, Guangdong* 510070, *China*

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Abstract

Objective. A multiplex polymerase chain reaction (multiplex PCR) assay was developed to detect simultaneously *Salmonella sp.*, *Shigella sp.*, *Pseudomonas aeruginosa*, *Eterohaemorrhagic Escherichia* (*EHEC*) and *Vibrio prahaemolyticus* in a tube. *Methods*. The five pairs of primers were designed and composed according to the virulence-associated, high-conservative and specific genes of these pathogens, optimized the system and condition of multiplex PCR. *Results*. The detection sensitivity of multiplex PCR were 10^1 cfu, 10^2 cfu, 10^2 cfu, 10^2 cfu and 10^1 cfu of one assey for *EHEC*, *Shigella sp.*, *Vibrio prahaemolyticus*, *Pseudomonas aeruginosa* and *Salmonella sp.*, respectively. The manual polluted water and 100 natural water samples were examined by multiplex PCR, and the results were clear, specific and coincident. It took six to eight hours to detect a sample. *Conclusion*. This multiplex PCR method would be a routine and practical protocol for detecting and identifying pathogenic microorganism from food, clinical or environmental samples. Not only was this method more sensitive, specific and efficient, but also the processing was rapid and simple. It could provide the experiment proof for the bacterial pathogens detection in water quickly and accurately. [Life Science Journal. 2008; 5(4): 47 – 54] (ISSN: 1097 – 8135).

Keywords: water-borne; bacterial pathogens; multiplex PCR; detection; sensitivity

1 Introduction

Food and water contaminated with infectious and toxigenic micro-organisms has been a major public health concern throughout the world. It's the precondition for control and prevention of pathogenic bacterials from spreading. Routine microbiological monitoring of water for pathogenic bacteria is required, as a measure to prevent the spread of water-borne diseases. In particular, water-borne infections like typhoid fever, cholera, dysentery and traveller's diarrhea, caused by different types of bacterial pathogens pose a major public health hazard, especially in developing countries. The spectrum of water-borne infections is also expanding, and many infectious diseases once believed to be conquered are on the rise[1]. Regular monitoring of water-borne pathogens is required to protect public health. However, the lack of accurate and cost-effective diagnostic tests is a major obstacle in the prevention and control of infections and outbreaks transmitted by water-borne pathogens.

Traditionally, detection and enumeration of bacterial pathogens have been largely based on the use of selective culture and standard biochemical methods. Such methods suffer from a number of drawbacks. First, pathogenic bacteria which normally occur in low numbers tend to incur large errors in sampling and enumeration. Second, culture-based methods are time-consuming, tedious, invariably monospecific, and low throughput. Third, many pathogenic organisms in the environment, although viable, are either difficult to culture or non-culturable, but can still cause illnesses[2]. Due to these difficulties, examination of water samples for pathogens like *Shigella*, *Salmonella*, *Vibrio prahaemolyticus*, *Eterohaemorrhagic Escherichia (EHEC)*, ect. are normally not performed during routine

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*Corresponding author. Email: wuqp203@yahoo.com.cn

microbiological assessment of water quality.

A number of immunological protocols have recently been developed for the specific detection of pathogenic bacteria like *Salmonella*, *EHEC O*157, *Vibrio parahaemolyticus*, and *Pseudomonas aeruginosa*. Some of these tests, however, are known to show variable sensitivities. They either depended on factors such as specificity of the antibodies, interference components in the medium used for culture enrichment, or the relative levels of gene expression of the target antigens. Recently, the use of nucleic acid probes and PCR have provided highly sensitive detection methods for specific pathogens in environmental samples[3,4]. Improved detection of pathogenic *E. coli* by immuno-capture PCR, and the sensitive detection of *Salmonella* by real-time PCR have also been developed; but these procedures are all monospecific and are either laborious or very expensive for routine use in water testing laboratories.

More recently, the use of the multiplex polymerase chain reaction (multiplex PCR) has provided rapid and highly sensitive methods for the specific detection of pathogenic microorganisms in the aquatic environment. To date, most multiplex PCR assays for pathogen detection have focused on only one, two or three different types of organisms[5, 6]. In this study, we reported for the development of an multiplex PCR method that permits the simultaneous detection of five different types of water-borne pathogens, *Shigell*a, *Salmonella*, *Vibrio prahaemolyticus*, *Pseudomonas aeruginosa* and *EHEC* in a single tube. The application and efficacy of this method for microbiological assessment of water quality at various sites around Guangzhou is also demonstrated.

2 Materials and Methods

2.1 Bacterial isolates, culture conditions and DNA extraction

The thirty-nine bacterials isolates examined in this study were listed in Table 1. Sources included clinical (blood, wound and faeces) and environmental samples obtained either from the American Type Culture Collection (ATCC), National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, China), Guangdong Entry-Exit Inspection and Quarantine Bureau (GDCIQ, China) or from Guangdong Institute of Microbiology (GIM, China). Campylobacter species were grown either on Brucella agar or Campylobacter blood free agar under microaerobic conditions. All other bacterial strains were cultured in Luria-Bertani broth at 37 ºC. After overnight, the optical density (at 600 nm) of bacterial cultures was adjusted to 1.0 and template DNA prepared. Total DNA was extracted from bacterial cultures either by boiling in sterile distilled water for 10 minutes or purified by phenol/chloroform extraction followed by ethanol precipitation[7].

2.2 PCR primers

A total of five sets of primers for the highly conserved regions, or toxic virulence genes of target pathogenic bacterials genome were used in this study. Three sets of primers were virulence genes: Inversion plasmid antigen H gene (IpaH), Inversion plasmid antigen B gene (IpaB) and Haemoly-ticuremic A gene (HlyA), were used for amplifying *Shigella*, *Salmonella* and *EHEC*, generating 611 bp, 315 bp and 366 bp PCR products, respectively. Two sets of primers were based on conserved nucleotide sequence, 16S – 23S rDNA IGS of *Vibrio prahaemolyticus* (Vpara) and outer membrane *Lipoprotein* (OprL), were used for amplying *Vibrio prahaemolyticus* and *Pseudomonas aeruginosa*, generating 165 bp and 504 bp PCR products, respectively. The sequence and relative locations of the primers were shown in Table 2. All oligonucleotide primers used in this study were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. Sequences of the five PCR primer pairs for multiplex PCR, their corresponding gene targets and size of expected amplification products were shown in Table 2.

2.3 Multiplex PCR assay

In the multiplex PCR assay, 2.5 μl of DNA was added to 2.5 μl of 10 × Hotstar Taq DNA polymerase Buffer (Qiagen, Netherlands). 2 μl of five sets of specific primers (10 μM each) in monoplex PCR (and in multiplex PCR: 1.0 μl each of IpaH and OprL, and 2.0 μl each of IpaB and HlyA, and 2.5 μl each of Vpara), 2.5 U of Hot Star Taq polymerase (Qiagen), 1.0 μl (10 mM) of dNPTs (Promega, USA), 5 μl of $MgCl_2$ (25 mM), and made up to 50 μl with MilliQ water. PCR amplification was performed in a Gene Cycle (ABI, USA) under the following conditions: first heat denaturation at 94 ºC for 3 minutes, followed by 35 cycles of touchdown PCR (first four cycles: denaturation at 94 ºC for 50 seconds, primer annealing at 63 ºC for 50 seconds and extension at 72 ºC for 100 seconds, then primer annealing at 1 ºC by 4 cycles, until the annealing temperature 58 ºC, then another 15 cycles). This was followed by incubation at 72 ºC for 10 minutes and cooling at 4 ºC. Negative control reaction mixtures contained sterile distilled water in place of template DNA.

2.4 Specificity testing of the five primer pairs

Specificity testing was carried out for the five positive

Table 1. Bacterial strains for the evaluation of specificity of PCR primers

Strains	Source	IpaH	OprL	HlyA	IpaB	Vpara
A. radiobacter	GIM-Ar	–	–	–	–	–
Aeromonas hydrophila	ATCC 23211	–	–	–	–	–
Aeromonas ltydrophila	ATCC 23213	–	–	–	–	–
Proteus	GIM-Pv	–	–	–	–	–
Bacillus foecalis alkaligenes	GIM-Af	–	–	–	–	–
Bacillus polymyxa	ATCC 21551	–	–	–	–	–
Campylobacter jejuni	ATCC 29428	–	–	–	–	–
	ATCC 43429	–	–	–	–	–
Pseudomonas aeruginosa	ATCC 9027	–	+	–	–	–
	ATCC 15442	–	+	–	–	–
	NICPBP 10104	–	+	–	–	–
	GIM-Ps	–	+	–	–	–
P. effluvium aeruginosa	GIM-Pp	–	–	–	–	–
Shigella sp.	ATCC 49071	+	–	–	–	–
	ATCC 29029	+	–	–	–	–
	NICPBP 51592	+	–	–	–	–
	GIM-Shi1	+	–	–	–	–
	GIM-Shi2	+	–	–	–	–
Salmonella sp.	ATCC 10749	–	–	–	+	–
	ATCC 19430	–	–	–	+	–
	NICPBP 50115	–	–	–	+	–
	NICPBP 47001	–	–	–	+	–
Vibrio rahaemolyticus	GIM-Vp	–	–	–	–	+
	ATCC 17802	–	–	–	–	+
Vibrio cholerae	ATCC 14035	–	–	–	–	–
	ATCC 25837	–	–	–	–	–
E. coli	ATCC 43889	–	–	+	–	–
	GDCIQ-O157-1	–	–	+	–	–
	GDCIQ-O157-2	–	–	+	–	–
	O26	–	–	–	–	–
	ATCC 10536	–	–	–	–	–
	ATCC 35401	–	–	–	–	–
	ATCC 8739	–	–	–	–	–
	ATCC 25922	–	–	–	–	–
Sarcina lutea	MIG1.18	–	–	–	–	–
Staphylococcus aureus	ATCC 6538	–	–	–	–	–
	ATCC 929737	–	–	–	–	–
Yersinia enterocolitica	ATCC 27739	–	–	–	–	–
Klebsiella pneumoniae	57	–	–	–	–	–

Table 2. The comparative results of the monologue among five primer pairs

V. prahaemo-lyticus	96 Vpara-F 120	270 Vpara-R 251	
	5'-***GCTGACAAAACAACAATTTATTGTT***-3'	5'-**GGAGTTTCGAGTTGATGAAC**-3'	
AF429304	GGAAA ***GCTGACAAAACAACAATTTATTGTT*** GTT……CTT ***CCTCAAAGCTCAACTACTTG*** TTCAGT		165 bp
NC004603	GGAAA ***GCTGACAAAACAACAATTTATTGTT*** GTT……CTT ***CCTCAAAGCTCAACTACTTG*** TTCAGT		
BA000032	GGAAA ***GCTGACAAAACAACAATTTATTGTT*** GTT……CTT ***CCTCAAAGCTCAACTACTTG*** TTCAGT		
Salmonella sp.	723 IpaB-F 742	1037 IpaB-R 1017	
	5'-***GGACTTTTTAAAAGCGGCGG***-3'	5'-***GCCTCTCCCAGAGCCGTCTGG***-3'	
U66877	AACAC ***GGACTTTTTAAAAGCGGCGG*** ATAAA……GGACT ***CGGAGAGGGTCTCGGCAGACC*** TTAAA		315 bp
CP000026	AACAC ***GGACTTTTTAAAAGCGGCGG*** ATAAA……GGACT ***CGGAGAGGGTCTCGGCAGACC*** TTAAA		
NC003197	AACAC ***GGACTTTTTAAAAGCGGCGG*** ATAAA……GGACT ***CGGAGAGGGTCTCGGCAGACC*** TTAAA		
EHEC	529 HlyA-F 548	894 HlyA-R 872	
	5'-***CAGTAGGGAAGCGAACAGAG***-3'	5'-***AAGCTCCGTGTGCCTGAAGC***-3'	
AY495950	GGTGT ***CAGTAGGGAAGCGAACAGAG*** AAAAT……TAATA ***TTCGAGGCACACGGACTTCG*** AGTAG		366 bp
AF074613	GGTGT ***CAGTAGGGAAGCGAACAGAG*** AAAAT……TAATA ***TTCGAGGCACACGGACTTCG*** AGTAG		
AB011549	GGTGT ***CAGTAGGGAAGCGAACAGAG*** AAAAT……TAATA ***TTCGAGGCACACGGACTTCG*** AGTAG		
NC002128	GGTGT ***CAGTAGGGAAGCGAACAGAG*** AAAAT……TAATA ***TTCGAGGCACACGGACTTCG*** AGTAG		
P. aeruginosa	212 OprL-F 232	715 OprL-R 695	
	5'-***GATGGAAATGCTGARATTCGGC***-3'	5'-***CTTCTTCAGYTCGACGCCACG***-3'	
Z50191	TACAT ***GATGGAAATGCTGAAATTCGGC*** AAATT.....AGAAT ***GAAGAAGTCGAGCTGCGCTGC*** CAAGA		504 bp
NC002947	TACAT ***GATGGAAATGCTGAAGTTCGGC*** AAATT.....AGAAT ***GAAGAAGTCAAGCTGCGCTGC*** CAAGA		
AE016778	TACAT ***GATGGAAATGCTGAAGTTCGGC*** AAATT…..AGAAT ***GAAGAAGTCAAGCTGCGCTGC*** CAAGA		
Shigella sp.	376 IpaH-F 396	986 IpaH-R 965	
	5'-***CCTTGACCGCCTTTCCGATAC***-3'	5'-***CAGCCACCCTCTGAGAGTACTC***-3'	
M76444	GCGTT ***CCTTGACCGCCTTTCCGATAC*** CGTCT……GGCCA ***GTCGGTGGGAGACTCTCATGAG*** TAAGA		611 bp
NC004761	GCGTT ***CCTTGACCGCCTTTCCGATAC*** CGTCT……GGCCA ***GTCGGTGGGAGACTCTCATGAG*** TAAGA		
NC004337	GCGTT ***CCTTGACCGCCTTTCCGATAC*** CGTCT……GGCCA ***GTCGGTGGGAGACTCCCATGAG*** TAAGA		
NC004851	GCGTT ***CCTTGACCGCCTTTCCGATAC*** CGTCT……GGCCA ***GTCGGTGGGAGACTCTCATGAG*** TAAGA		

Nucleotide sequence alignment of the reference strains of *Salmonella sp.*, *Shigella sp.*, *P. aeruginosa*, *V. prahaemolyticus* and *EHEC* at consense and antisense primer regions. Conserved nucleotides among strains are given in bold italic letters. The position numbers of the five primer pairs indicated correspond to representative strains, GeneBank accession number of the reference strains are U66877, CP000026, NC003197; M76444, NC004761, NC004337, NC004851; Z50191, NC002947, AE016778; AF429304, NC004603, BA000032 and AY495950, AF074613, AB011549, NC002128, respectively. Within nucleotide sequences of primers, R: A or G, Y: C or T.

controls: first, each primer pair was tested by PCR on DNA templates prepared from a panel of 39 different bacterial isolates (including the control strains). The analysis indicated that all primer pairs showed specificities only for their corresponding target organisms (Table 1).

Secondly, for the five positive controls, various combinations were tested: the PCR mixture containing five primer pairs and each bacterial DNA (name as A mixture), each primer pair and five bacterial DNA which were synthesized from a mixture of *Shigella*, *Salmonella*, *Vibrio prahaemolyticus*, *Pseudomonas aeruginosa* and *EHEC* (B mixture), and the five bacterials DNA and five primer pairs (C mixture), by using the same thermal cycler program described above. The final concertrations of each kind of premixes were the same for the procedures of multiplex PCR mentioned above, and MillQ water was

Table 3. The components of PCR premix for the specificity testing

25 μl of PCR premix (μl)	A mixture (μl)	B mixture (μl)	C mixture (μl)
DDW	6.5	14	6.5
10 × Taq buffer	2.5	2.5	2.5
Mg^{2+}	2.5	2.5	2.5
dNTP (2.5 mM)	2	2	2
Primer mixture (10 μM)	8.5[a]	1.0[b]	8.5[a]
Taq (2.5 U/μl)	0.5	0.5	0.5
Template	2.5[c]	2.5[d]	2.5[d]

[a]The mixture of five sets of specific-primers (0.5 μl IpaH each, 0.5 μl OprL each, 1.0 μl IpaB each, 1.0 μl HlyA each, 1.25 μl Vpara each).
[b]The mixture of single specific-primer (0.5 μl of 10 μmol/L each).
[c]The single DNA, such that contain every kind of DNA of this five bacteria.
[d]Synthesized from the mixture of *Shigella sp*., *P. aeruginosa*, *EHEC*, *Salmonella sp*. and *V. prahaemolyticus*, such that contains the five kinds of DNA.

added to give a total volume of 25.0 μl (Table 3).

2.5 Determination of detection sensitivity

Log-phase cultures of five reference bacterial pathogens in LB were mixed in equal numbers (previously enumerated by plate counting on LB agar) and were serially 10-fold diluted in sterile saline. Each serial dilution (100 μl) was seeded into 100 ml of autoclaved water, and the bacterial mixtures from each dilution were harvested by centrifugation at 7000 rpm for 20 minutes and washed three times with sterile distilled water. Total DNA was extracted by phenol chloroform, ethanol precipitated and washed twice with 70% (v/v) ethanol.

2.6 Sensitivity testing of the multiplex PCR and the monoplex PCR

To compare the sensitivity level of the multiplex PCR and the monoplex PCR, 10-fold serial dilutions (10^6 – 10^0) in MilliQ water of the five different bacterial DNA. The two types of PCR mixture were: one was five primer pairs and each bacterial DNA for one dilution, and the other was one primer pairs and each bacterial DNA for this dilution bacterial cultures. The multiplex PCR and the monoplex PCR were performed simultaneously for the same dilution series, by using the same PCR machine and in the same program described above.

2.7 Electrophoresis

PCR products were electrophoresed in 1.5% agarose gel, followed by staining with ethidium bromide (EtBt, 0.5 μg/ml) for 20 minutes then visualized under ultraviolet light, and the results were recorded by photography.

3 Results

3.1 Specificity of the five primer pairs

The specificity of each set of primers used in this study was tested. For the five positive controls, various combinations, exactly, the PCR reaction mixture containing five primer pairs and each DNA (Figure 1, Lanes 1 – 5), the five DNA and each primer pair (data not shown), and the five DNA and five primer pairs (Figure 1, Lane 6), amplified each expected product independently and specifically. No cross-reaction was found between the individual primers and non-target pathogens in the monoplex PCR (data not shown). Each PCR product was obtained as a clear band at 175, 315, 366, 504 and 611 bp generated by Vpara, IpaB, IpaH, OprL and HlyA respectively (Figure 1). For the negative controls *Vibrio prahaemolyticus*, *Salmonella sp*., *EHEC*, *Pseudomonas aeruginosa* and *Shigella sp.*, none amplicon was generated.

3.2 Determination of detection sensitivity

The detection sensitivity of the multiplex PCR system for the five bacterial pathogens was subsequently examined in serial 10-fold dilutions of bacterial mixtures in autoclaved seawater. Cell suspensions containing 10^0 – 10^6 cfu each of the 5 reference strains plus 10^3 cfu of *E.coli* (to mimic mixed culture conditions) were harvested by centrifugation. Total DNA from bacterial mixtures at each serial dilution was extracted by phenol-chloroform and tested by multiplex PCR. As shown in Figure 2, a detection limit of 10^1 cfu was noted for *EHEC* and *Salmonella sp.*, 10^2 cfu for *Shigella sp*., *Vibrio prahaemolyticus* and *Pseudomonas aeruginosa*. Similar results were obtained in at least five independent experiments. Attempts at improving the detection sensitivity by increasing the number of amplification cycles or amount of Hot Star Taq polymerase by 2 – 5 fold (data not shown), or by means of the Pit-Stop PCR strategy[8] were unsuccessful. Nevertheless, our multiplex PCR system compared favorably with other monoplex PCR-based assays in which a detection sensitivity of 10 cfu of *Salmonella*, and 10 cfu of *Shigella*, 10 cfu of *V. parahaemolyticus*, 10^2 cfu of *Pseudomonas aeruginosa* have been reported. Although the infectious concentration varied between pathogen types, it is generally believed that most bacterial pathogens are able to cause infection when more than 10^3 infectious cells are ingested[9]. Thus, the detection sensitivity of the multiplex assay described in this study was within the infectious concentration of most enteric pathogens.

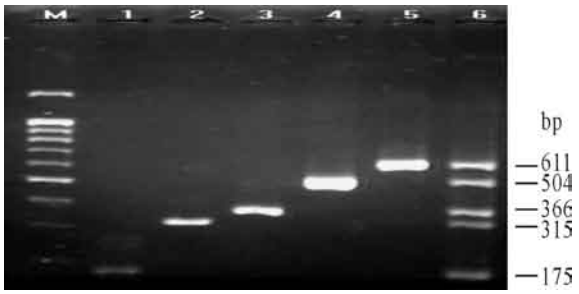

Figure 1. Lanes 1: *V. prahaemolyticus*; Lanes 2: *Salmonella sp.*, Lanes 3: *EHEC*; Lanes 4: *P. aeruginosa*; Lanes 5: *Shigella sp.*; Lane 6: The mixture of *V. prahaemolyticus*, *Salmonella sp.*, *EHEC*, *P. aeruginosa*, *Shigella sp.* Lane M: DNA molecular size markers.

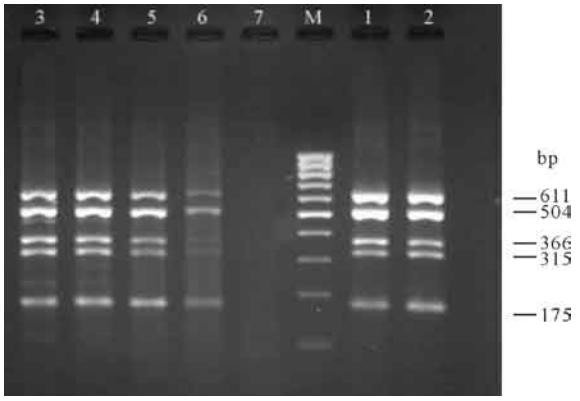

Figure 2. The sensitivity of multiplex PCR detection for five different bacterial pathogens. Lane 1: 10^6 cfu; Lane 2: 10^5 cfu; Lane 3: 10^4 cfu; Lane 4: 10^3 cfu; Lane 5: 10^2 cfu; Lane 6: 10^1 cfu; Lane 7: 10^0 cfu; Lane M, DNA molecular size markers.

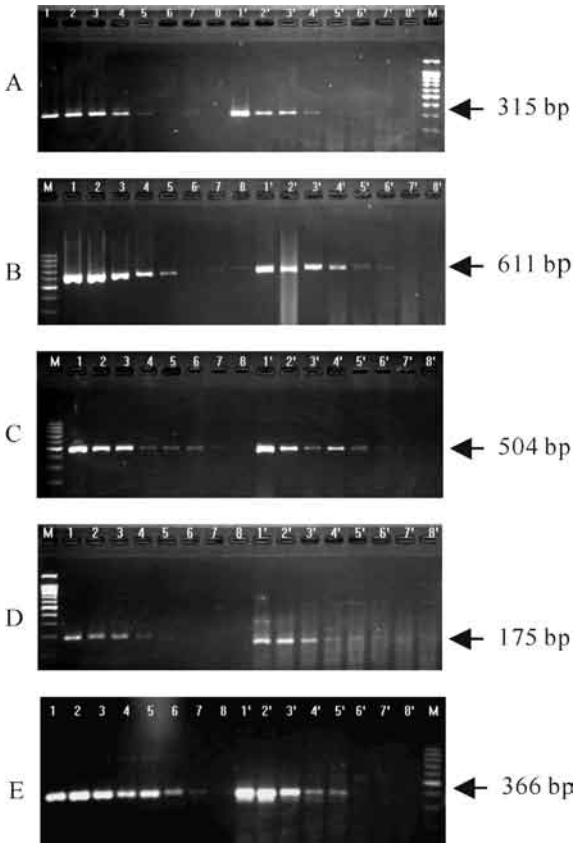

Figure 3. The comparision of the sensitivity between multiplex PCR and monoplex-PCR detection for five different bacterial pathogens. A: *Salmonella sp.*; B: *Shigella sp.*; C: *P. aeruginosa*; D: *V. prahaemolyticus;* E: *EHEC*. Lane 1 & 1': 10^6 cfu; Lane 2 & 2': 10^5 cfu; Lane 3 & 3': 10^4 cfu; Lane 4 & 4': 10^3 cfu; Lane 5 & 5': 10^2 cfu; Lane 6 & 6': 10^1 cfu; Lane 7 & 7': 10^0 cfu; Lane 8 & 8': negative control; Lane M: DNA markers.

3.3 Sensitivity of the multiplex PCR and the monoplex PCR

For the 10-fold dilution series, the highest dilution at which the mutiplex PCR exhibited a positive result was $10^1 – 10^2$ cfu for the former five kinds of pathogens, and the positive results of the highest dilution in the monoplex PCR were $10^0 – 10^1$ cfu for the pathogens. However, products of *Shigella sp.*, *Vibrio prahaemolyticus*, *Salmonella*, *EHEC* and *Pseudomonas aeruginosa* DNA in the multiplex PCR were poorer by visualization on agarose gels than that in the monoplex PCR (Figure 3). Overall, the sensitivity level of the multiplex PCR dropped about 10-fold compared with the monoplex PCR.

3.4 Detection of target bacteria in water samples by multiplex PCR

3.4.1 Detection of manual polluted water samples by multiplex PCR. Put 100 μl mixture with 10^6 cfu *Shigella sp.*, *Vibrio prahaemolyticus*, *Salmonella*, *EHEC* and *Pseudomonas aeruginosa* into 100 ml sea water, ponk water, tap water and distilled water, respectively. Total DNA was iso- lated from water samples in concentrating for filtrate membrane, then isolated from manual polluted water by phenol-chloroform extraction followed by ethanol precipitation. Using the same multiplex PCR machine and in the same cycler program described above, we got the expected species from every manual polluted water samples (Figure 4).

3.4.2 Detection of water samples by multiplex PCR. Using the above protocol, multiplex PCR analysis of water samples from 16 sites from Guangzhou during 2005 April and 2005 December was carried out. For PCR analysis, bacterial cells from the same water samples were harvested by phenol-chloroform extractions. In total, 14

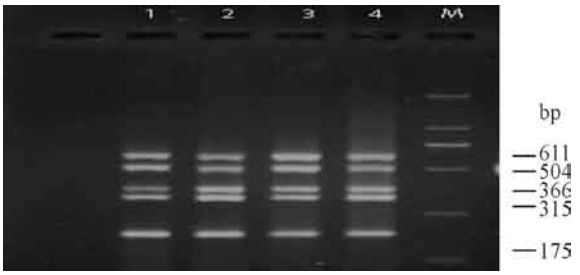

Figure 4. The electrophoretic analysis of multiplex PCR for different water samples. Lane M: Marker DL2000; Lanes 1 – 4: Five different bacterial pathogens from sea, pool, tap water and sterile water respectively.

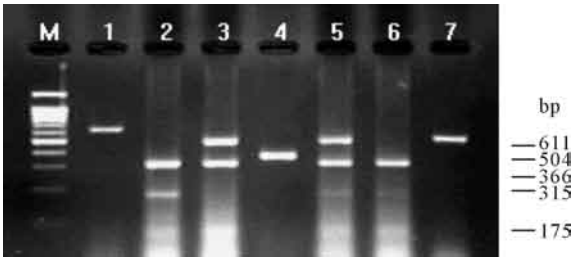

Figure 5. The electrophoretic analysis of multiplex PCR for positive samples. Lane 1: *Shigella*; Lane 2: *Salmonella* and *V. prahaemolyticus*; Lane 3: *P.aeruginosa* and *Salmonella*; Lane 4: *EHEC*; Lane 5: *P.aeruginosa* and *Salmonella*; Lane 6: *Salmonella*; Lane 7: *P.aeruginosa*; Lane M: 100 bp ladder.

out of 100 water samples were positive for the target bacterials (Figure 5), showing an example of *Salmonella*, *Paeruginosa*, *V. prahaemolyticus*, *EHEC* and *Shigella* samples, which out of the water samples collected using multiplex PCR. Infections occurred mainly in three months period, September, October and November. Co-infection of five or more different bacterials found in none of these samples.

4 Discussion

In this investigation, five pairs of oligonucleotide primers were designed to simultaneously detect five different types of water-borne pathogens by multiplex PCR in a single tube. The primer pairs showed significant affinities only for their target genes. To facilitate PCR product detection, the primers were designed so that the expected size of the products from each target gene would be different to permit size discrimined by gel electrophoresis. And the five monoplex PCR products were sequenced by ABI PRISM 377, the homoeology arrived 99% – 100% (data not shown).

This study has developed a rapid assay to detect simultaneously *Salmonella*, *Paeruginosa*, *V. prahaemolyticus*, *EHEC* and *Shigella*, the specificity of the mixture of five primer sets was verified by testing for the positive controls and also for the negative controls *E. coli* (ATCC8739, ATCC25922), *Listeria monocytogenes* and *L.murrayi* (data not shown) using the multiplex PCR. We have not found any products from the negative controls, and obtained specific product from positive control. To date, PCR assays have been shown to be useful diagnostic procedures for bacterial pathogens detection. It was reported that the sensitivity of the conventional PCR with specific primers was higher than that of conventional culture. However, the sensitivity of multiplex PCR assay could not be determined in absolute terms. In most cases, sensitivity of the multiplex PCR dropped 10 – 100 folds when compared with those of the monoplex PCR[11,12]. In our study, the multiplex PCR showed slightly less sensitive than the monoplex PCR, at which the sensitivity dropped about 10 folds (Figure 4). The detection limits for the single target of different pathogens were similar to other multiplex PCR assays previously reported.

5 Conclusion

The high throughput and cost-effective multiplex PCR system developed in this study could provide a powerful supplement to conventional methods for more accurate risk assessment and monitoring of pathogenic becteria in the marine environment. The ability to rapidly monitor various types of microbial pathogens would be extremely useful not only for routine assessment of water quality to protect public health, but also for assessments of water quality during the water treatment processes. Such tests may be further developed to include other important pathogens (e.g. *Vibrio pvulnificus*, Hepatitis A virus, etc) and extended to examination of shellfish and food samples.

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