Rapid identification of three waterborne pathogens in water samples by multiplex touchdown PCR

Hou Yanjiao [1, 2, a], Li Guanlin[3, b], Shao Chen[1, c], Shao Shihe[1, d*]

[1]School of Medicine, Jiangsu University, No. 301 Xuefu Road, Zhenjiang 212013, Jiangsu Province, China

[2] Dezhou People's Hospital, No.1751 New Lake Street, Dezhou 253014, Shandong Province, China

[3]Department of Environmental Science and Ecological Engineering, Korea University, Seoul 136-713, Korea

[a]email: houyanjiao@126.com, [b]email:Guanlin.li1207@gmail.com,

[c]email: 54236100@qq.com, [d*]email: shaoshihe2006@163.com

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Abstract The purpose of this study was to develop a multiplex touchdown PCR to identify three waterborne pathogens in water samples. Specific primers based on VP1332, *inv* and *ipaH* gene sequences were designed for *V. parahaemolyticus*, *Y. enterocolitca* and *Shigella*, respectively. By optimizing reaction conditions, assays obtained high specifity, sensitivity and repeatability. The detection limit can be as low as 0.005ng/μl *V. parahaemolyticus* and 0.5ng/μl *Y. enterocolitca* and 0.005ng/μl of *Shigella* respectively. The detection process was about 3~4 hours, saving a lot of time compared with microbiological culture method that required 2~3 days. The system could be useful for the detection of multiple pathogens in water samples to protect public health.

Introduction

Waterborne pathogens have posed a considerable threat to public health worldwide. From May to June 2012, a waterborne illness outbreaking in drinking water in Seoul induced watery diarrhea and vomiting of the local residents[1]. In March 2012, more than 3600 people developed gastroenteritis symptoms in a district with 37 264 inhabitants in central Greece[2]. The United States is one of the safest countries in the drinking water, they take a series of measures to clean it, but the disease outbreaks caused by drinking water still occurred.

Among the waterborne pathogens currently indwelled in water samples, *V. parahaemolyticus, Y. enterocolitca* and *Shigella* are frequently reported as the causative agents. *V. parahaemolyticus* was a significant cause of human gastrointestinal disorders worldwide, *Y. enterocolitca* and *Shigella* could cause inflammatory diarrhea and sepsis[3,4].

A real time PCR assay for the detection of *V. parahaemolyticus* in seafood samples was

developed. Quantitative PCR (qPCR) methods were used to detect *Y. enterocolitica* in surface water, and drinking untreated water[5]. Duplex PCR assay for the detection of pathogenic *Y. enterocolitca* strains has been developed. *Shigella* DNA was detected by real-time PCR in Nha Trang. Real-time quantitative PCR have successfully been performed to identify pathogens. However, they are very expensive for routine use in common testing laboratories.

In this study, a multiplex touchdown PCR with high specificity, sensitivity and repeatability was developed to identify *V. parahaemolyticus*, *Y. enterocolitca* and *Shigella* simultaneously in water samples. The expected sizes of PCR amplifications were 870bp, 393bp and 193bp, respectively. The detection limit can be as low as 0.005ng/μl *V. parahaemolyticus* and 0.5ng/μl *Y. enterocolitca* and 0.005ng/μl of *Shigella* respectively. the approach could provide a powerful supplement to conventional methods for more quick detection and monitoring of waterborne pathogens infection.

Materials and Methods

Strains and growth media Strains used in this study were listed in Table 1. *Y. enterocolitca* was kindly provided by Dr. Shi Lining; other reference strains were collected and preserved in our laboratory[6] . *V. parahaemolyticus* was cultured on Thiosulfate citrate bile salts sucrose agar culture medium(TCBS) (Hopebio, China) in 37℃ for18~24h, *Y. enterocolitca* was prepared in Luria–Bertani medium (LB) (Oxoid, UK) in 28℃ for 18~24h, Shigella and *Salmonella paratyphi A* were propagated and maintained on Salmonella-Shigella medium (SS) (Hang Wei, China) in 37℃ for 18~24h , other strains were cultured on Chocolate agar and Blood agar Blood agar in 37℃ for 18~24h.

Table1. Related strains for evaluation of specificity of PCR primers

Organism	VP1332	*inv*	*ipaH*
Acinetobacter baumannii ATCC®19606TM	—	—	—
Moraxella (Branhamella) catarrhalis ATCC® 25238TM	—	—	—
H. influenza ATCC® 10211TM	—	—	—
S. pneumoniae ATCC® 49619TM	—	—	—
Staphylococcus aureus ATCC® 25923TM	—	—	—
Escherichia coli ATCC® 25922TM	—	—	—
Pseudomonas aeruginosa ATCC® 27853TM	—	—	—
Klebsiella pneumoniae ATCC® 700603TM	—	—	—
Enterococcus faecalis ATCC® 29212TM	—	—	—
M. bovis	—	—	—
M. tuberculosis	—	—	—
M. smegmatis	—	—	—
M. bovis BCG	—	—	—
Salmonella paratyphi A	—	—	—
V. parahaemolyticus	+	—	—
Y. enterocolitca	—	+	—
Shigella	—	—	+

Oligonucleotide primers Basing on these sequences designed specific primers using Primer Premier 6.0,the primers sequences are listed in Table 2. The specificity of the designed primers was

tested using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 2. Primer sequences and product sizes used in this study

Bacteria	Target gene	DNA sequences	Primers	Sequence(5'-3')	Product sizes (bp)
V. parahaemolyticus	VP1332	NC_004603	VP1332-F VP1332-R	TACGCTGAAGATAAACTAACGG TGCGGAAGAAGACTTACGAG	870
Y . enterocolitica	*inv*	X53368	*Inv*-F *Inv*-R	ACCACGGCAATAGTTCTAATC CGGCAACAATATCAGATGGAGA	393
Shigella	*ipaH*	NC_004741	*ipaH*-F *ipaH*-R	CGCCATATCAGAGTCATCACT TGCGTGCAGAGACGGTAT	193

Genomic DNA extraction Genomic DNA from pure cultures was extracted using minibest bacterial genomic DNA extraction (TRANS, CHINA); for water samples, the bacteria of 100 mL water samples were firstly concentrated by membrane filtration, then extracting genomic DNA from the membrane with boiling methods. As follows, we put the membrane filtration into a 1.5ml eppendorf tube, and added 500ul sterile distilled water into eppendorf tube, making the membrane fully immersed in water. Then the eppendorf tube was boiled for 15 minutes to release the bacterial DNA, through centrifugation at 12000 rpm for 10 minutes collected the bacterial DNA and, stored at -20 ℃ until required for further study.

Results

Optimization of multiplex touchdown PCR system After systematic optimization of amplification parameters, the best results were obtained with 25μl volume of PCR mixture containing 1X PCR buffer, 2.5mM MgCl2, 0.3mM dNTP, 1.5 U Taq DNA polymerase, 0.2μM of VP1332 primer set, 0.2μM of ipaH primer set, 1.2μM of inv primer set, 0.1μg/μl BSA, 2μl DNA template and sterile distilled water. The system was performed using the touchdown PCR conditions: an initial denaturation at 94 ℃ for 5 min, followed by 20 cycles of 94 ℃ for 30 s, 61 ℃ (0.5 ℃ decrease per cycle) for 30s, 72 ℃ for 1 min, then another 20 cycles of 94 ℃ for 30s, 51 ℃ for 30s, 72 ℃ for 1 min, and a final extension step at 72 ℃ for 7 min.

Specificity of the multiplex touchdown PCR assay To evaluate the specificity of primers used in the study, DNA template prepared from other 14 strains in addition to objective strains (shown in Table 1) were tested using the established multiplex touchdown PCR system. As shown in Fig. 1, the multiplex touchdown PCR produced specific bands of *V. parahaemolyticus, Y. enterocolitica, Shigella*. No cross amplification with other species was observed.

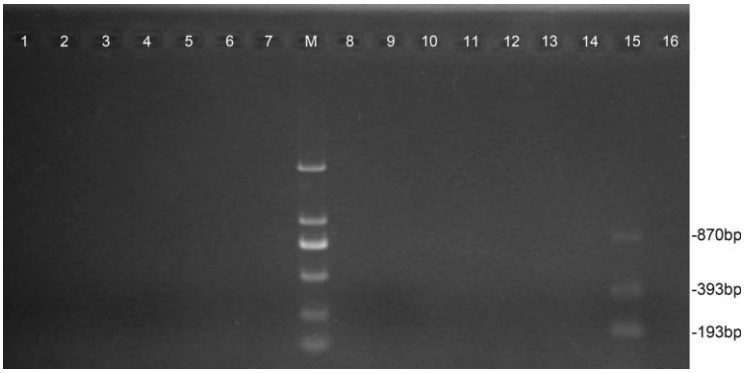

Fig.1. Specificity of the multiplex touchdown PCR assay performed on DNA from pure cultures.

Lane M: DL2000 marker; Lanes 1-14: *Staphylococcus aureus* ATCC® 25923TM; *Moraxella (Branhamella) catarrhalis* ATCC® 25238TM; *Streptococcus pneumoniae* ATCC® 49619 TM; *Klebsiella pneumoniae* ATCC® 700603TM; *Acinetobacter baumannii* ATCC®19606TM; *Enterococcus faecalis* ATCC® 29212TM; *H. influenza* ATCC® 10211TM; *M. smegmatis*; *M. bovis*;*Escherichia coli* ATCC® 25922TM; *Pseudomonas aeruginosa* ATCC® 27853TM; *M. tuberculosis*; *M. bovis BCG*; *Salmonellaparatyphi A*; Lane15: *V. parahaemolyticus*, *Y. enterocolitca*, *Shigella*; lane16: Sterile distilled water.

Sensitivity of the multiplex touchdown PCR assay Different concentrations of DNA templates were tested with multiplex touchdown PCR , and then the lowest DNA concentration that produced visible band was considered as the detection limit. The results displayed that the detection limit of DNA was as few as 0.005ng/μl *V. parahaemolyticus* and 0.5ng/μl *Y. enterocolitca* and 0.005ng/μl of *Shigella* respectively (Fig. 2).

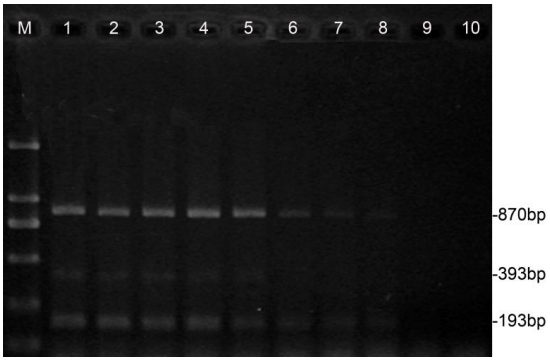

Fig. 2. Sensitivity of the multiplex touchdown PCR assay

Lane M:DL2000 marker; Lane 1-10 the serial dilutions of chromosomal DNA extracted from *V. parahaemolyticus*, *Y. enterocolitca*, *Shigella* strains, from left to right, 10, 5, 2.5, 1.5, 0.5, 0.15, 0.015, 0.005, 0.001, 0.0005ng/μl.

Repeatability of the multiplex touchdown PCR assay Using the established multiplex touchdown PCR detected many times to the same sample, and obtained consistent results. It indicated that the assay had good repeatability.

Multiplex touchdown PCR applied to single and multiple pathogens infection In fact, Infection can be caused by single pathogen or multiple pathogens together. In order to verify the practicality of the detection, different random combinations of pathogens were added in drinking water, then genomic DNA of these pathogens were detected by optimized multiplex touchdown PCR. Different combinations of pathogens wouldn't interfere with the stability and accuracy of the system (Fig. 3). This method has a good practicality, and promotional value.

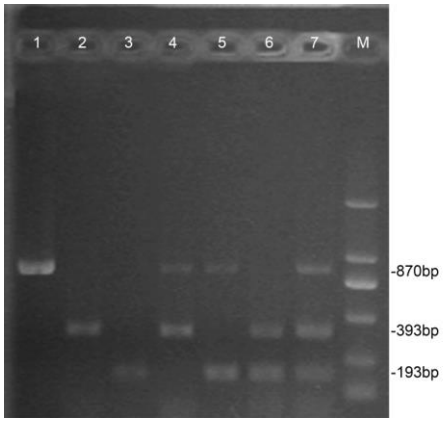

Fig. 3. Multiplex touchdown PCR applied to single and multiple pathogens infection.

Lane M: DL2000 marker; Lane 1: *V. parahaemolyticus*; Lane 2: *Y. enterocolitica*; Lane3: *Shigella* ; Lane 4: *V. parahaemolyticus* and *Y. enterocolitica* ; Lane 5: *V. parahaemolyticus* and *Shigella* ; Lane 6: *Y. enterocolitica* and *Shigella* ; Lane 7: *V. parahaemolyticus*, *Y. enterocolitica* and *Shigella*.

Multiplex touchdown PCR detection applied to different types of water samples Genomic DNA from four types of water samples was extracted as the template of multiplex touchdown PCR assay; specific bands can be successfully amplified. The test results were consistent with traditional separation, but traditional identification needed 2~3 days, multiplex touchdown time PCR detection required only 3~4 h, so the detection time was greatly reduced. The results were as follows (Fig. 4).

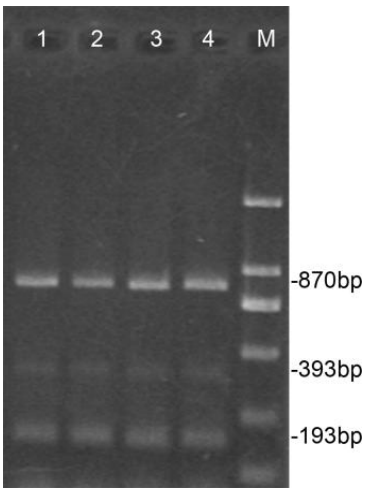

Fig. 4. Multiplex touchdown PCR detection applied to different types of water samples.
Lane M: DL2000 marker; Lane 1-4: lake water, tap water, distilled water, bottled purified water.

Discussion

The waterborne pathogens often caused many lethal human diseases and could be an important water safety issue in the worldwide. A rapid and accurate detection method is very important to identify and trace outbreaks of bacterial pathogens within the water supply.

Multiplex PCR could be applied to detect multiple target organisms in a single tube reaction to save time and labor[7], Touchdown PCR approach exploits the exponential nature of PCR, the appearance of additional mismatched products reduced or effectively abrogate. Multiplex touchdown PCR can save a great deal of time, and had a lower risk of cross-contamination.

In this work, we used VP1332 as a target gene of *V.parahaemolyticus*. the virulence gene did not exist in other *Vibrio* species. The positive rate of *inv* tested in 160 isolates *Y. enterocolitca* was 100% , hence, we Chose *inv* gene as the target. It has been considered that the *ipaH* gene was the main target for detection of *Shigella*. We Chose *ipaH* gene as the target.

Hossain *et al.* have reported that the detection limit of mixed primers for detection of *V. parahaemolyticus* strains was 200 pg[8], the detection limit of *V. parahaemolyticus* in our system was found to be lower, which was 0.005ng, namely 5pg. Although Chen et al. have stated the detection limit of *Shigella* to be 73.35fg[9], we were unable to obtain as low as their values (0.005ng). We have ever used the ail of *Y. enterocolitca* as the target, and its detection limit (5fg) was much lower than that of *inv*(0.5ng) in this study, but positive rate of virulence genes of *ail* gene was 94%, not 100%(*inv*), some *Y. enterocolitca* strains lacked the *ail* gene. The possibility of false-negative results may occur, so we still chose the *inv* as the target. Although the infectious dose

varies from pathogen types, the detection sensitivity of the multiplex assay in this study is within the infectious dose of most pathogens.

The test results were consistent with traditional separation. But the detection time is greatly reduced. The established system was proved to have specificity, sensitivity, good repeatability and high reliability. This method had a good practicality, and promotional value.

Although this method is rapid and time-saving, it is a fact that present PCR approaches cannot completely substitute all the advantages of conventional microbiological diagnostics. In addition, a broad spectrum of possible other bacterial pathogens could be missed when only multiplex touchdown PCR is conducted.

Conclusion

This method suggests a simple, fast, inexpensive, and reliable system for the practical detection of three pathogens in water samples. Further studies on the detection of other important pathogens and other samples will be developed.

Acknowledgements

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