

## Rapid Detection of *Shigella* Species in Environmental Sewage by an Immunocapture PCR with Universal Primers

Xuanxian Peng,\* Wen Luo, Jianying Zhang, Sanying Wang, and Shengcai Lin

The Key Laboratory of Education Ministry for Cell Biology & Tumor Cell Engineering, Department of Biology, School of Life Sciences, Xiamen University, Xiamen 361005, People's Republic of China

Received 25 July 2001/Accepted 20 January 2002

**In the present study, we developed a quick, highly specific method for detection of *Shigella* species by combining immunocapturing of the bacteria and a universal primer PCR. The method drastically enhances test sensitivity, and it can be used not only for identification of *Shigella* species in the environment but also for rapid detection of other pathogens.**

Dysentery caused by *Shigella* species is one of the most common infectious diseases in developing countries and in travelers to tropical countries (18, 19). One statistic showed that about 50% of Spanish travelers who visited developing countries developed dysentery, and *Shigella* species were among the main etiological agents (3, 4). In the People's Republic of China, *Shigella* species are the second leading cause of intestinal infectious diseases. The dysentery bacilli include four *Shigella* species, *S. dysenteriae*, *S. flexneri*, *S. sonnei*, and *S. boydii*; *S. sonnei*, *S. dysenteriae*, *S. flexneri*, and *S. boydii* have 15, 10, 8, and 1 serotypes, respectively. The symptoms of shigellosis are mild or severe depending on the species causing the infection. Therefore, early rapid identification of *Shigella* species and their serotypes is very important for public health, epidemiological investigations, and clinical treatment.

Traditionally, identification of pathogenic bacteria has been based on phenotypic characteristics and mainly involves analyses of differential metabolic properties and reactions of specific antibodies. Recently, molecular analysis of phylogenetic markers has been recognized as a very useful tool for identification of bacterial genera, species, or subspecies (2, 4, 14, 17). Among these markers, 16S rRNAs are particularly useful because these molecules are present in every living cell and their function is highly conserved. However, an approach based on utilization of universal primer PCR (UPPCR) for conserved regions, such as 16S rRNA genes, can be used to study almost all bacteria (5, 8). The bacteria have to be characterized further by subsequent steps, including restriction fragment length polymorphism analysis, single-strand conformation polymorphism analysis, or sequencing analysis (4, 10, 11, 12). These extra steps make the detection procedure more complex and tedious.

In this paper, we report development of a new technique for rapid and efficient detection and differentiation of dysentery bacilli in environmental sewage. The new method, termed immunocapture UPPCR (iUPPCR), employs UPPCR amplification to detect bacteria captured by specific antibodies coupled to polystyrene 96-well plates. The specificity of coating anti-

bodies distinguishes specific cell types, while the conserved 16S rRNA contributes to the universality of bacterial detection. We believe that this method will have broad application for detection and differentiation of pathogenic organisms in the environment.

The bacteria used in this study included *S. dysenteriae* serotype 1, *S. flexneri* serotypes 1a, 2a, 3a, 4, 5, and Y variant, *S. sonnei*, and *S. boydii* serotype 1; these organisms were purchased from the Public Health Station of Fujian Province, People's Republic of China. Cultures were grown at 37°C in L-broth medium. Then immune capture and conventional treatment of bacteria were carried out. For immunocapturing procedures, monoclonal antibodies (purchased from Institute of Lanzhou Biological Products, Lanzhou, People's Republic of China) against *S. dysenteriae* 1, *S. flexneri* 1a, 2a, 3a, 4, 5, and Y variant, *S. sonnei*, and *S. boydii* 1 were separately coupled to polystyrene 96-well plates using 0.05 M carbonic acid buffer (pH 9.6) at 4°C for 18 h. The plates were then washed with phosphate-buffered saline containing 0.05% Tween 20 and incubated with 20- $\mu$ l portions of bacterial cultures at 37°C for 1 h. Following washing, the wells were incubated with 20  $\mu$ l of sterile double-distilled H<sub>2</sub>O and heated at 100°C for 5 min to denature the bacterial DNA templates. For conventional procedures, 20- $\mu$ l portions of bacterial cultures were separately centrifuged at 3,000  $\times$  g for 20 min following one wash with sterile 0.85% NaCl. The pellets were each dissolved in 20  $\mu$ l of sterile double-distilled H<sub>2</sub>O and were heated at 100°C for 5 min to denature the bacterial DNA templates. Eighteen microliters of a suspension resulting from the immunocapturing or conventional treatment was used as the template for UPPCR amplification.

Each UPPCR mixture (total volume, 25  $\mu$ l) consisted of 2.5  $\mu$ l of 10 $\times$  PCR buffer, 3 mM MgCl<sub>2</sub>, each deoxynucleoside triphosphate at a concentration of 200  $\mu$ M, each primer at a concentration of 250 nM, 1 U of *Taq* DNA polymerase (MBI Fermentas, Inc., Amherst, N.Y.), and 18  $\mu$ l of DNA template in a thin-wall 600- $\mu$ l tube. The UPPCR mixtures were subjected to 40 cycles of 94°C for 1 min (denaturation), 51°C for 1 min (annealing), and 70°C for 2.5 min (extension). The amplified DNA products were separated by electrophoresis on 2.5% agarose gels. DNA was visualized by ethidium bromide staining. Oligonucleotide primers were designed by using the

\* Corresponding author. Mailing address: School of Life Sciences, Xiamen University, Xiamen 361005, People's Republic of China. Phone: 86-592-2183805. Fax: 86-592-2181015. E-mail: wangpeng@xmu.edu.cn.

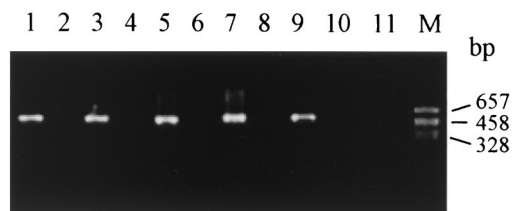


FIG. 1. Representative cross-reaction analysis showing the specificity of iUPPCR for identification of serotypes of *S. flexneri*. Lane M, PGEM-7zf(+)/*Hae*III marker; lane 1, *S. flexneri* 1a added to well coated with antibody against *S. flexneri* 1a; lane 2, *S. flexneri* 2a added to well coated with antibody against *S. flexneri* 1a; lane 3, *S. flexneri* 2a added to well coated with antibody against *S. flexneri* 2a; lane 4, *S. flexneri* 3a added to well coated with antibody against *S. flexneri* 2a; lane 5, *S. flexneri* 3a added to well coated with antibody against *S. flexneri* 3; lane 6, *S. flexneri* 4 added to well coated with antibody against *S. flexneri* 3; lane 7, *S. flexneri* 4 added to well coated with antibody against *S. flexneri* 4; lane 8, *S. flexneri* 5 added to well coated with antibody against *S. flexneri* 4; lane 9, *S. flexneri* 5 added to well coated with antibody against *S. flexneri* 5; lane 10, *S. flexneri* Y variant added to well coated with antibody against *S. flexneri* 5; lane 11, *S. flexneri* Y variant added to well coated with antibody against *S. flexneri* 6.

conserved regions of the 16S rRNA sequence from bp 909 to 1410. The primer sequences were 5'-AAACTCAAAGGAAT TGAC-3' for the forward primer and 5'-GACGGGCGGTGT GTACAA-3' for the reverse primer. The expected DNA fragments (about 500 bp) were amplified by this method. The fidelity of PCR was further checked by digestion of PCR products with *Hae*III. The lengths of the restriction fragments were identical to the expected lengths. No PCR product was obtained with the control samples.

Specificity and sensitivity tests were repeated three times. Differences between groups were tested for significance by using a chi-square test with Yates's continuity correction for small numbers, and two levels of significance ( $P < 0.05$  and  $P < 0.01$ ) were used.

Neutralization and coating antibody replacement tests were used to check the specificity of iUPPCR. In the neutralization test, cell suspensions (20  $\mu$ l) containing 20 CFU were separately mixed with equal volumes of monoclonal antibodies against dysentery species (50, 100, and 200  $\mu$ g/ml) prior to incubation at 37°C for 1 h and were then added to wells. The results of the neutralization test showed that all otherwise positive samples were negative. In the second test, monoclonal antibodies against dysentery species were replaced with monoclonal antibody against hepatitis B surface antigen as a coating antibody. No PCR products were detected in the positive samples. Moreover, two types of cross-reactive tests to determine the specificity of iUPPCR

TABLE 1. Cross-reactivities of *S. dysenteriae* 1, *S. sonnei*, *S. flexneri* 4, and *S. boydii* 1, as determined by iUPPCR

Antibody	Cross-reactivity			
	<i>S. dysenteriae</i> 1	<i>S. sonnei</i>	<i>S. flexneri</i> 4	<i>S. boydii</i> 1
Anti- <i>S. dysenteriae</i> 1	+	-	-	-
Anti- <i>S. sonnei</i>	-	+	-	-
Anti- <i>S. flexneri</i> 4	-	-	+	-
Anti- <i>S. boydii</i> 1	-	-	-	+

TABLE 2. Cross-reactivity used for identification of *S. flexneri* 1a, 2a, 3a, 4, 5, and Y variant by iUPPCR

Antibody	Cross-reactivities with <i>S. flexneri</i> serotypes					
	1a	2a	3a	4	5	Y variant
Anti- <i>S. flexneri</i> 1a	+	-	-	-	-	-
Anti- <i>S. flexneri</i> 2a	-	+	-	-	-	-
Anti- <i>S. flexneri</i> 3a	-	-	+	-	-	-
Anti- <i>S. flexneri</i> 4	-	-	-	+	-	-
Anti- <i>S. flexneri</i> 5	-	-	-	-	+	-
Anti- <i>S. flexneri</i> 6	-	-	-	-	-	-

were used in this study. One test was designed for cross-reactive detection of *S. dysenteriae* 1, *S. flexneri* 1a, *S. sonnei*, and *S. boydii* 1. Monoclonal antibodies against *S. dysenteriae* 1, *S. flexneri* 1a, *S. sonnei*, and *S. boydii* 1 were separately coated in polystyrene 96-well plates. Then *S. dysenteriae* 1, *S. flexneri* 1a, *S. sonnei*, and *S. boydii* 1 were separately added to the wells coated with the four different antibodies. The other test was used for cross-reactive detection of *S. flexneri* serotypes 1a, 2a, 3a, 4, 5, and Y variant. Monoclonal antibodies against *S. flexneri* 1a, 2a, 3a, 4, and 5 were separately coated and reacted with *S. flexneri* 1a, 2a, 3a, 4, 5, and Y variant. The results of the two types of cross-reaction tests showed that PCR products were detected only in the wells coated with antibodies to which cells containing the corresponding antigens were added (Fig. 1 and Tables 1 and 2).

Two methods were used to compare the sensitivities of iUPPCR and conventional UPPCR (cUPPCR). All tests were repeated three times. First, a bacterial pellet was diluted to obtain concentrations of 20, 50, 500, and 5,000 CFU per 20  $\mu$ l. All reaction tubes were positive as determined by iUPPCR or cUPPCR. Of the 120 tubes containing 10 or 5 CFU/20  $\mu$ l, which were divided into two groups, however, 60 tubes were found to be positive by using iUPPCR; cUPPCR was used to examine the other tubes, and in three tests 6, 6, and 7 of the 10 tubes containing 10 CFU/20  $\mu$ l and 2, 3, and 3 tubes of the 10 tubes containing 5 CFU/20  $\mu$ l were positive. Second, bacteria were diluted to obtain a concentration of 20 CFU per 360  $\mu$ l, evenly distributed into 20 wells for immunocapture, and then amplified by the two methods. The results indicated that when iUPPCR was used, 5, 4, and 4 of 10 tubes were positive in three tests, whereas all 10 tubes were negative in each test when cUPPCR was used. Samples without bacteria were used as controls, and all of them were negative. Table 3 shows the data obtained for the three tests. There were significant differences

TABLE 3. Comparison of sensitivities of iUPPCR and cUPPCR based on detection of 10, 5, and 1 CFU per 20  $\mu$ l<sup>a</sup>

Method	No. tested	No. positive (%) with:		
		10 CFU/20 $\mu$ l	5 CFU/20 $\mu$ l	1 CFU/20 $\mu$ l
iUPPCR	30	30 (100.0)	30 (100.0)	13 (43.3)
cUPPCR	30	19 (63.3)	7 (23.3)	0
Control	15	0	0	0

<sup>a</sup> Comparison of rates of positive results between iUPPCR and cUPPCR at 10 CFU ( $\chi^2 = 13.47$ ;  $P < 0.01$ ), at 5 CFU ( $\chi^2 = 37.29$ ;  $P < 0.01$ ), and at 1 CFU ( $\chi^2 = 13.47$ ;  $P < 0.01$ ) per 20  $\mu$ l is shown.

TABLE 4. Identification of *Shigella* species in 35 sewage samples by iUPPCR and bacterial culture

Taxon	No. positive (%) as determined by:	
	iUPPCR	Bacterial culture
<i>S. dysenteriae</i> 1	2 (5.7)	0
<i>S. dysenteriae</i> 2	0	0
<i>S. sonnei</i>	3 (8.6)	0
<i>S. flexneri</i> 1	0	0
<i>S. flexneri</i> 2	15 (42.9)	5 (14.3) <sup>a</sup>
<i>S. flexneri</i> 3	3 (8.6)	0
<i>S. flexneri</i> 4	0	0
<i>S. flexneri</i> 5	0	0
<i>S. flexneri</i> 6	0	0
<i>S. boydii</i> 1-6	0	0
<i>S. boydii</i> 7-11	0	0
<i>S. boydii</i> 12-15	0	0
<i>S. boydii</i> 16-18	0	0
Total	23 (65.8)	5 (14.3) <sup>a</sup>

<sup>a</sup> *S. boydii* serotypes 1 to 6, 7 to 11, 12 to 15, and 16 to 18 were identified by antisera against *S. boydii* serotypes 1 to 6, 7 to 11, 12 to 15, and 16 to 18, respectively. Values are significantly different between iUPPCR and bacterial culture of *S. flexneri* 2 ( $\chi^2 = 8.10$ ;  $P < 0.01$ ) and from total *S. flexneri* 2 ( $\chi^2 = 16.06$ ;  $P < 0.01$ ).

between the two methods for samples containing 10, 5, or 1 CFU/20  $\mu$ l ( $P < 0.01$ ).

We further validated our method by performing a stool environment simulation test. Stool (1 g) from a healthy individual was diluted with 1 ml of saline. The suspension was centrifuged at  $2,500 \times g$  for 10 min. The supernatant was used for dilution of *Shigella* species. A dilution containing 200 CFU in 400  $\mu$ l was added to 20 wells for iUPPCR or cUPPCR analysis. Of 20 tubes divided into two groups, 8 were positive and 2 were negative as determined by iUPPCR, whereas 10 were negative when cUPPCR was used. The difference between the results was significant ( $P < 0.01$ ).

Sewage samples were collected from sewage ditches near hospitals and in inhabited areas. Of 35 samples, 15 were collected from places around hospitals, and the other 20 were collected from residential areas. Five milliliters of each sewage sample was removed and kept in a sterile tube for 2 h. Then 20  $\mu$ l of the supernatant was used for iUPPCR, and 40  $\mu$ l was plated for conventional bacterial culture. Bacteria on agar plates were identified by using monoclonal antisera, colony lifting, and antibody reactions, followed by visualization with a microscope. Table 4 shows the prevalence of *Shigella* species in sewage as detected by the two different methods. Of the 35 samples, 23 were found to be positive by iUPPCR, whereas 5 samples were positive as determined by bacterial culture. The difference between the results was significant ( $P < 0.01$ ). In the iUPPCR procedure, monoclonal antibodies against different serotypes of *Shigella* species were used. *S. dysenteriae* 1, *S. sonnei*, *S. flexneri* 2, and *S. flexneri* 3 were shown to be positive by iUPPCR, and the positive rates were 5.7% (2 of 35 samples), 8.6% (3 of 35 samples), 42.9% (15 of 35 samples), and 8.6% (3 of 35 samples), respectively. Only *S. flexneri* 2 was found to be positive by bacterial culture, and its positive rate was 14.3% (5 of 35 samples). There was a significant difference in the positive rates for *S. flexneri* 2 between the two methods ( $P < 0.01$ ).

The iUPPCR detection method for *Shigella* species is at least four times more sensitive than the cUPPCR method, which is currently the conventional method used internationally. More importantly, the specificity provided by coated monoclonal antibodies makes the use of restriction fragment length polymorphism analysis, single-strand conformation polymorphism analysis, or sequencing analysis unnecessary and shortens the diagnosis time.

Similar procedures coupling immunocapturing to PCR have been reported for detecting virions and both DNA and RNA viruses; however, universal primers are not used in these procedures (1, 6, 7, 9, 15). We previously reported a new method termed immunocapture reverse transcription-PCR (iRT-PCR) for detection of the virions present in the circulating immune complexes of hepatitis patients and for analysis of specific immunoglobulin-bound hepatitis C virions (13). In this study, the iUPPCR method was used to detect serotypes of diarrheal pathogens or *Shigella*. Because the primers are universal, the iUPPCR used for detection of bacteria has advantages over the similar immunocapture PCR used for detection of viruses and the iRT-PCR used for detection of virions coupled to antibodies. Moreover, the iRT-PCR method was designed for detection of viruses bound with isotopes of immunoglobulin, while the iUPPCR technique for bacteria was developed for identification of free bacteria. Samples from sewage inevitably contained dead bacteria, and bacteria from different sample sources were unevenly distributed so that samples with fewer bacteria may have resulted in negative culture detection but positive detection by iUPPCR. These reasons may contribute to the fact that the iUPPCR is more sensitive for detection of bacteria in sewage samples than bacterial culture. It was reported previously that specific antibodies could determine whether target bacteria were alive or dead (16). By replacing a coating antibody with a monoclonal antibody that was specific for live cells, we could distinguish live bacteria from dead bacteria by this new method.

The new method described here provided increased sensitivity, specificity, and convenience for clinical diagnosis and epidemiological investigation of bacillary dysentery. More importantly, this method could be used directly to detect bacteria in environmental samples with no pretreatment, and the test results were obtained within 5 h.

This work was supported by grants from the Foundation of Chinese Education Ministry for Excellent Young Teachers, the State Foundation of China for Natural Science (grant 39770585), and IFS (grant A/2338-2).

#### REFERENCES

1. Bariana, H. S., A. L. Shannon, P. W. G. Chu, and P. M. Waterhouse. 1994. Detection of five seedborne legume viruses in one sensitive multiplex polymerase chain reaction test. *Phytopathology* **84**:1201-1205.
2. Carroll, N. M., E. E. M. Jaeger, S. Choudhury, A. A. S. Dunlop, M. M. Matheson, P. Adamson, N. Okhravi, and S. Lightman. 2000. Detection of and discrimination between gram-positive and gram-negative bacteria in intraocular samples by using nested PCR. *J. Clin. Microbiol.* **38**:1753-1757.
3. Gascon, J., J. Vila, M. E. Valls, L. Ruiz, J. Vidal, M. Corachan, G. Prats, and M. T. Jimenez de Anta. 1993. Etiology of traveller's diarrhea in Spanish travellers to developing countries. *Eur. J. Epidemiol.* **9**:217-223.
4. Gascon, J., M. Vargas, L. Quinto, M. Corachon, M. T. Jimenez de Anta, and J. Vila. 1998. Enterococcal *Escherichia coli* strains as a cause of traveller's diarrhea: a case-control study. *J. Infect. Dis.* **177**:1409-1412.
5. Greisen, K., M. Loeffelholz, A. Purohit, and D. Leong. 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *J. Clin. Microbiol.* **32**:335-351.

6. Harper, G., G. Dahal, G. Thottappilly, and R. Hull. 1999. Detection of episomal banana streak badnavirus by IC-PCR. *J. Virol. Methods* **79**:1–8.
7. Henrard, D. R., W. F. Mehaffey, and J. P. Allain. 1992. A sensitive viral capture assay for detection of plasma viremia in HIV-infected individuals. *AIDS Res. Hum. Retrovir.* **8**:47–52.
8. Marchesi, J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, and W. G. Wade. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.* **64**:795–799.
9. Mumford, R. A., and S. E. Seal. 1997. Rapid single-tube immuno-capture RT-PCR for the detection of two yam potyviruses. *J. Virol. Methods* **69**:73–79.
10. Osorio, C. R., M. D. Collins, A. E. Toranzo, J. L. Barja, and J. L. Romalde. 1999. 16S rRNA gene sequence analysis of *Photobacterium damsela* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. *Appl. Environ. Microbiol.* **65**:2942–2946.
11. Peng, X. X., H. Gao, S. Y. Wang, and W. Z. Zheng. 2000. Universal primer PCR with SSCP and RFLP for identification of fish disease pathogens. *J. Fish. China* **24**:345–348.
12. Peng, X. X., Y. Zhou, S. Y. Wang, and Y. L. Zhang. 2000. Quick identification for pathogenic vibrio of aquacultivated creatures by UPPCR-SSCP. *J. Oceanogr. Taiwan Strait* **19**:460–463.
13. Peng, X. X., H. Zhu, J. Y. Zhang, and S. Y. Wang. 2001. Analysis of HCV-immunoglobulin isotype complexes by a novel immuno-capture RT-PCR method. *Scand. J. Immunol.* **54**:409–413.
14. Rossi, M. C., A. Gori, G. Zehender, G. Marchetti, G. Ferrario, C. De Maddalena, L. Catozzi, A. Bandera, A. D. Esposti, and F. Franzetti. 2000. A PCR-colorimetric microwell plate hybridization assay for detection of *Mycobacterium tuberculosis* and *M. avium* from culture samples and Ziehl-Neelsen-positive smears. *J. Clin. Microbiol.* **38**:1772–1776.
15. Sharman, M., J. E. Thomas, and R. G. Dietzgen. 2000. Development of a multiplex immunocapture PCR with colourimetric detection for viruses of banana. *J. Virol. Methods* **89**:75–88.
16. Sølve, M., J. Boel, and B. Nørrung. 2000. Evaluation of a monoclonal antibody able to detect live *Listeria monocytogenes* and *Listeria innocua*. *Int. J. Food Microbiol.* **57**:219–224.
17. Song, Y. L., N. Kato, C. X. Liu, Y. Matsumiya, H. Kato, and K. Watanabe. 2000. Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group and species specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. *FEMS Microbiol. Lett.* **187**:167–173.
18. Tauxe, R. V., N. D. Puh, J. G. Wells, N. Hargrett-Bean, and P. A. Blake. 1990. Antimicrobial resistance of *Shigella* isolates in the USA: the importance of international travelers. *J. Infect. Dis.* **162**:1107–1111.
19. Vila, J., J. Gascon, S. Abdalla, J. Gomez, F. Marco, A. Moreno, M. Corachan, and T. Jimenez de Anta. 1994. Antimicrobial resistance of *Shigella* isolates causing traveler's diarrhea. *Antimicrob. Agents Chemother.* **38**:2668–2670.