嘉南學報第三十一期
第19～27頁，民國九十四年
CHIA-NAN ANNUAL BULLETIN
VOL. 31, PP. 19－27, 2005

Specific Primers for Rapid and Sensitive Identification of *Vibrio vulnificus*

Yu-Chung Chen*, Jer-Horng Su*, Wen-Teish Chang** and Jiunn-Kae Chang*

*Department of Biotechnology,
Chia-Nan University of Pharmacy and Science,
Tainan, Taiwan 71710, R.O.C.
**Department of Food Science and Technology,
Tung Fang Institute of Technology,
Kaoshiung, Taiwan, R.O.C.

ABSTRACT

A primer pair designed from *vvbfp* (*Vibrio vulnificus* blue fluorescent protein) of *V. vulnificus* amplified a 251 bp PCR fragment from all clinical isolates of *V. vulnificus* and failed to amplify any fragment from other clinical pathogens. The sensitivity of the primer pair is as little as 1 fg of chromosomal DNA and 60 cells of *V. vulnificus*. In a scientific evaluation of specimens, a total of 22 samples including serum specimens and wound aspirates from septicemic mice were screened by PCR. Thirteen (92.9%) of the 14 *V. vulnificus* culture-positive specimens gave positive results. Three (37.5%) of the 8 culture-negative specimens yielded positive results. The primer set gives high sensitivity and specificity in a one-step PCR protocol, an important criterion for unambiguous bacterial identification.

Key words: Bacterial identification, Blue fluorescent protein, PCR, *Vibrio vulnificus*

INTRODUCTION

Vibrio vulnificus, a highly virulent marine bacterium, is the causative agent of seafood-related diseases in many areas of the world[1,2,3,4]. There has recently been a dramatic increase in the number of cases due to this pathogen in Taiwan[5,6,7]. Three major clinical syndromes have been described in infection associated with *V. vulnificus*: primary septicemia, wound infection, and gastroenteritis[5,7,8,9]. Although gastroenteritis is often self-limited, primary septicemia and wound infections are highly lethal syndromes that occur most often among patients with liver disease or other immunocompromising conditions[5,7,8,9]. Primary septicemia is acquired through consumption of contaminated raw or undercooked seafood, especially raw oysters, with the mortality exceeding 50%[4,7,8,10]. Wound infection is caused by exposure of wounds to seawater or seafood products, or by trauma during preparation of marine animals, and may result in secondary sepsis with a mortality of about 25%[4,7,8,10]. *V. vulnificus* causes a rapid and severe disease often complicated by extensive soft tissue infections such as necrotizing fasciitis and myonecrosis coupled with

hemorrhagic bullous formation[7,10]. Most of the mortality cases die within 2 days after hospitalization [7,9,10].

Because *V. vulnificus* causes a rapidly progressing and fulminating course during infection, it is crucial to start early definitive antimicrobial therapy based on the identification of the causative bacteria. Klontz et al. [10] emphasized the importance of antibiotic treatment at the earliest time of infection course, because of the high mortality in patients where therapy is delayed. For clinical diagnosis, even with rapid presumptive detection that use differential media such as thiosulfate-citrate-bile salts-sucrose (TCBS) agar and MacConkey agar, more than 2 days are needed for identification of *V. vulnificus* from clinical specimens. Therefore, a reliable and rapid diagnostic assay to detect *V. vulnificus* specifically is clinically and epidemiologically important. Here we report the development of a highly sensitive and specific PCR based method for the identification of *V. vulnificus* in clinical specimens.

MATERIAL AND METHODS

1. Clinical strains and media

A total of 49 isolates, including 30 *V. vulnificus* and 19 non-*V. vulnificus* isolates, comprising different species were used in this study (Table 1). All isolates were obtained from the Department of Clinical Pathology, Chi Mei Foundation Medical Center; Department of Microbiology, National Cheng Kung University Hospital, Tainan, Taiwan. These bacteria were cultured in Luria-Bertani (LB) broth at 37℃ for 12 h in this study.

Table 1 Bacterial strains used to test the specificity and sensitivity of PCR in identifying *V. vulnificus*

Bacteria	Source and strains* (No. of isolates)
Vibrio vulnificus	CKM-1 (1)
	CKUH (18)
	CMMC (10)
	ATCC 27562 (1)
Vibrio aliginolyticus	CKUH (1)
Vibrio cholerae	CMMC (3)
Vibrio parahaemolyticus	CMMC (3)
Aeromonase hydrophila	CKUH (1)
Escherichia coli	ATCC 25922 (1)
	CMMC (2)
Enterobacter cloacae	CMMC (1)
Klebsiella pneumoniae	CKUH (2)
	ATCC 33495 (1)
Pseudomonas aeruginosa	CMMC (1)
	ATCC 27853 (1)
Salmonella enteritidis	CKUH (2)

*ATCC, American Type Culture Collect; CKUH, Chung Kung University Hospital, Tainan, Taiwan; CMMC, Chi Mei Foundation Medical Center, Tainan, Taiwan.

2. PCR template preparation

Genomic DNA used as PCR templates were extracted and purified from various isolates as described previously[11]. Briefly, bacteria were harvested at the logarithmic growth phase and washed twice with phosphate-buffered saline. They were then serially diluted from 6.0×10^7 to 6 cells/100μl. Each aliquoted dilution was pelleted and DNA extracted by boiling in 100 μl of 1 mM EDTA solution. Five microlitre of this was used as template for PCR. For primer specificity assay, chromosomal DNA (0.02 ng/μl) was used as PCR template.

3. Specimen collection and preparation

BALB/c mice (8 to 10 weeks old) purchased from the animal center of the College of Medicine at National Cheng Kung University were infected by intraperitoneal (i.p.) or subcutaneous (s.c.) injection of bacterial suspension. Mice were given 0.2 ml of bacterial suspension (1.0×10^7 cells/ml) per mouse, and specimens (blood and wound aspirates) were collected at 24 postinfection. These specimens were divided into two parts for bacteriological culture and PCR analysis. The volumes of specimens used for the PCR were about 0.5 to 3 ml. The wound aspirates were diluted with 3X volume of saline (0.85% NaCl). For the chromosomal DNAs extraction, these specimens were spun down at $1,000 \times g$ for 15 s. Supernatant was centrifuged at $15,000 \times g$ for 5 min. The pellect was washed twice with saline and resuspended in 100 μl of 1 mM EDTA solution. Bacterial DNAs were prepared via single-step extraction method and PCR amplification was performed as described next.

4. Oligonucleotide primers and PCR amplification parameters

The primers were designed based on the nucleotide sequence of the *vvbfp* (*Vibrio vulnificus* blue fluorescent protein) from *V. vulnificus* CKM-1 (GenBank accession no. AF080431). The sequences of the primers were as follows: sense P1, 5'-GGA TCA CAA AAT GAA AAA ATT AGT CG-3'; antisense P2, 5'-CGT TGT TGA CTA ATA CGT CC- 3'. The target of the primers is a 251-bp DNA fragment specific for the *vvbfp* gene (720 nucleotides) from positions 2656 to 2906. Primers were synthesized with a DNA synthesizer (Nucleic acid analysis and synthesis Core Laboratory, National Cheng Kung University Hospital, Tainan, Taiwan, Republic of China).

For PCR amplification, a reaction mixture containing 5 μl of template, PCR 10X reaction buffer (100 mM Tris-HCl, pH9.0 at 25℃, 500 mM KCl, 0.1% (w/v) gelatin, 15 mM $MgCl_2$ and 1% Triton X-100), 0.5 μM each primer, 250 μM each deoxyribonucleoside triphosphate (TaKaLa, Shiga, Japan) and 5U of ProTaq DNA polymerase (PROtech Technology, Inc., Taipei, Taiwan) in a total volume of 50 μl was prepared. The amplification reaction included an initial denaturation step 96℃ for 5 min, followed by 50 cycles at 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s, followed by a final incubation step at 72℃ for 2 min. PCR was performed with a model PE 2400 automated thermocycler (Perkin-Elmer Cetus, Norwalk, USA). A 10-μl portion of the PCR product was electrophoresed on a 1.5% agarose gel with 1X Tris-borate-EDTA electrophoresis buffer (TBE; 90 mM Tris-borate, 1mM EDTA) and a 100-bp DNA ladder (PROtech Technology, Inc., Taipei, Taiwan) as molecular size markers. Negative controls with all components of the reaction mixture except the template DNA were included in each experiment. All experiments were performed at least twice. DNA sequences of these amplified products were confirmed by DNA sequencing.

RESULTS AND DISCUSSION

A gene (designated *vvbfp*) encoding the blue fluorescent protein of *V. vulnificus* CKM-1 has recently been cloned and characterized[12]. Using the Blast program analysis[13], we could not detect any significant similarity in a region of 251 bp of the *vvbfp* gene compared to other *Vibrio* species and non-*Vibrio* species. This unique characteristic makes it very desirable as a species-specific diagnostic tool. In this study we evaluated the use of this partial sequence from *vvbfp* as a target for identification. We designed a pair of primers based on this unique region. The effectiveness and specificity of the primer pair was first assessed in PCR assays with 30 *V. vulnificus* isolates using directly isolated or purified DNA. All the *V. vulnificus* strains produced 251-bp fragment (Fig.1), while other bacteria including various vibrio species did not give any PCR products (data not shown).

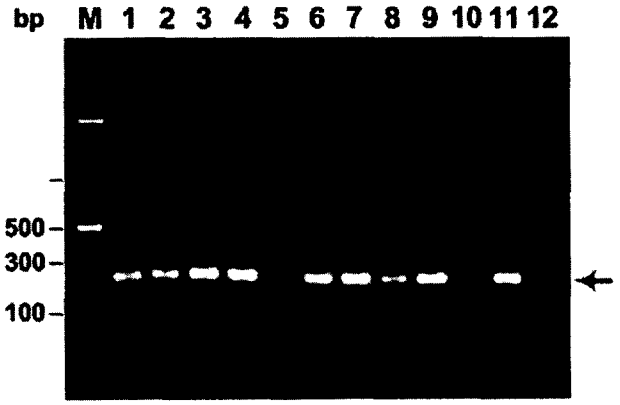

Fig. 1 PCR assay of DNAs from some *V. vulnificus* using P1-P2 primer set. Lane M, 100-bp ladder molecular size markers; sizes (in bp) indicated on left; lanes 1-10, template DNA from 10 clinical *V. vulnificus* strains; lane 1, positive control (template DNA from *V. vulnificus* CKM-1); lane 12, negative control.

To determinate the sensitivity of the primer pair, 10-fold dilutions of *V. vulnificus* CKM-1 were prepared and used as the templates for PCR amplification. Under PCR conditions, the PCR primer pair could detect as little as 1 fg of *V. vulnificus* DNA (Fig. 2A). To reduce the total processing time, we chose an effective single-step DNA extraction method to extract *V. vulnificus* DNA. The *V. vulnificus* CKM-1 were serially diluted, pelleted, and DNAs were extracted by boiling in 100 μl of 1 mM EDTA solution. These bacterial dilutions were also plated on LB plate to compare bacterial number with PCR sensitivity. Employing this direct DNA extraction method, the primer set could detect as few as 60 cells of *V. vulnificus* (Fig. 2B).

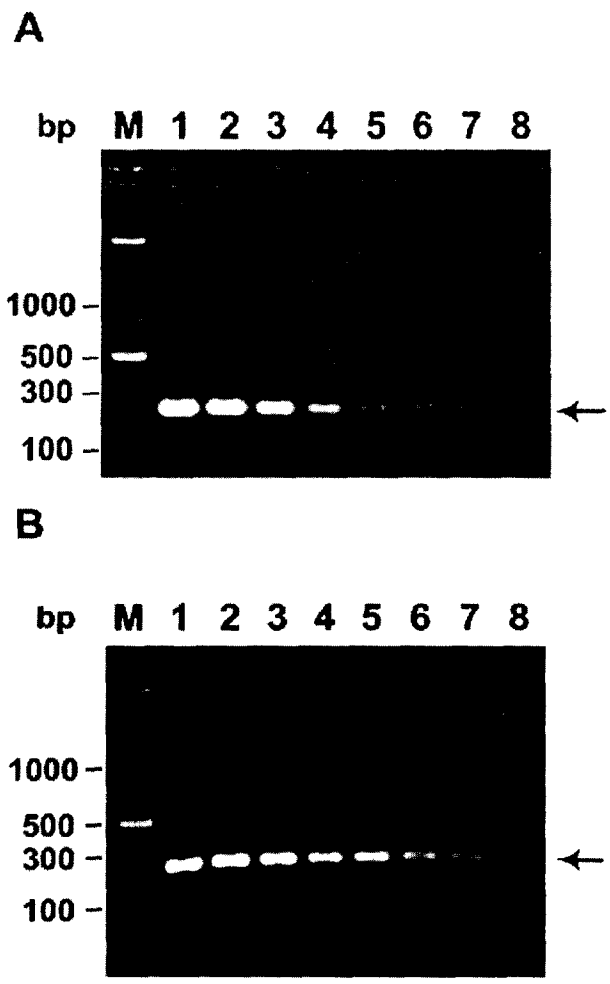

Fig. 2 Sensitivity of PCR with the P1-P2 primer set at different concentration of purified chromosomal DNA (A) or directly extracted chromosomal DNA (B) from *V. vulnificus* CKM-1. Lane M, 100-bp ladder molecular size markers; size (in bp) indicated on left. For panel A, lanes 1-7, purified chromosomal DNA serially diluted 10-fold from 1 ng to 1 fg; lane 8, negative control. For panel B, lanes 1-7, 10-fold serial dilutions of *V. vulnificus* CKM-1 from 6.0×10^7 to 60 cells; lane 8, negative control.

Recently several research groups developed primers targeting various *V. vulnificus*-specific genes to detect the bacteria in various environmental sources[11,14,15,16,17]. The sensitivity of some primer pairs was so low that incubation of samples was required for proper detection. In one of the above studies, the specificity of some primer pairs still needed to be improved[11]. Lee et al. reported a nested PCR for rapid detection of the bacterium in clinical specimens, even so there were smears of DNA fragments at sufficient but lower template DNA concentrations, around the detection limit[11]. In addition, some nonspecific bands often appeared in this method. A serious disadvantage with nested PCR is that multiple primers are often needed in more than one PCR process. To evaluate the potential for our PCR-based assay as a diagnostic

test, we used specimens from mice infected by *V. vulnificus*. Although Lee et al. have shown that specific primer targeting the *V. vulnificus* hemolysin/cytolysin gene can identify *V. vulnificus* in clinical specimens (serum and bulla aspirate) [11]. However, most cases of diseases caused by *V. vulnificus* present with cellulitis, local wound, necrotizing fasciitis or myonecrosis, it is desirable to detect *V. vulnificus* from the wound sites of clinical specimens. A total of 22 specimens were collected and analyzed in this study (Fig. 3). The PCR results were compared with those of bacteriological culture (Table 2). The PCR results showed that 9 (90%) serum specimens were positive for the 10 serum specimens from blood culture-positive *V. vulnificus* septicemia mice, whereas three of the eight (37.5%) culture-negative serum specimens became positive. In addition, all wound aspirates (100%) showed positive in PCR and bacteriological culture. In our study, three (37.5%) of the 8 culture-negative serum specimens yielded positive results. The culture negativity could be result from *V. vulnificus* transformation during infection. Because PCR detects genomic DNA rather than the metabolism of culturable bacteria, the nonculturable *V. vulnificus* inhibited by host factors can be identify by PCR detection. In the other thing, the PCR was negative for blood culture-positive specimen, and this result likely was due to very low bacterial density in the blood specimen.

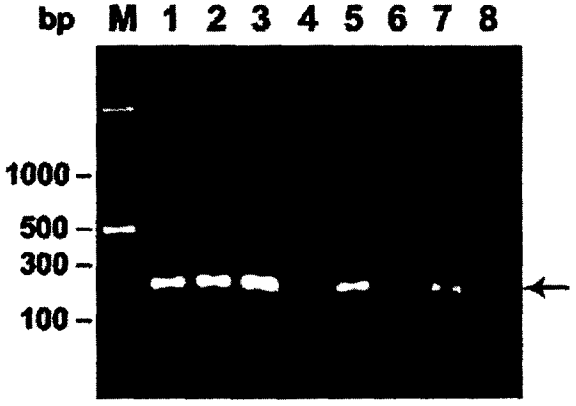

Fig. 3 Identification of *V. vulnificus* DNA from different specimens source. Lane M, 100-bp ladder molecular size markers; size (in bp) indicated on left; lane 1, positive control (pellet of *V. vulnificus* CKM-1); lanes 2, 3 and 4, serum samples; lane 5, 6 and 7, wound aspirates; lane 8, negative control.

Table 2 Detection of *V. vulnificus* DNAs by one-step PCR method and bacteriologic culture

Specimen type	Culture result	No. of specimens with the following result by PCR		
		Positive	Negative	Total
Serum	Positive	9	1	10
	Negative	3	5	8
Wound aspirate	Positive	4	0	4

This study represents the first attempt to detect *V. vulnificus* in wound aspirate by PCR using primers that are superior to other previously reported primers targeting different *V. vulnificus* genes. Apart from the high sensitivity and specificity of the primer pair, this method is a very rapid one-step PCR protocol thereby reducing the waiting time considerably when compared to culture which is the gold standard. The utilization of the *vvbfp* as a probe will provide a valuable diagnostic target for *V. vulnificus*.

ACKNOWLEDGEMENT

We acknowledgement M. C. Chang and Y. C. Chuang for providing bacterial strains and BALB/c mice.

REFERENCES

1. Bisharat N, Agmon V, Finkelstein R, Raz R, Ben-Dror G, Lerner L, Soboh S, Colodner R, "Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel", Lancet, 354:1421-1424, 1999.
2. Hlady WG, Mullen RC, Hopkin, RS, "*Vibrio vulnificus* infections associated with raw oyster consumption-Florida, 1981-1992", Arch Dermatol, 129: 957-958, 1993.
3. Morris JG, Jr, Black RE, "Cholera and other vibrios in the United States", N Engl J Med, 312:343-350, 1985.
4. Shapiro RL, Altekruse S, Hutwagner L, Bishop R, Hammond R, Wilson S, Ray B, Thompson S, Tauxe RV, Griffin PM, *Vibrio* Working Group, "The role of gulf coast oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988-1996", J Infect Dis, 178:752-759, 1998.
5. Chang JJ, Sheen IS, Peng SM, Chen PC, Wu CS, Leu, HS, "*Vibrio vulnificus* infection: report of 8 cases and review of cases in Taiwan", Chang Gung Med J, 17:339-346, 1994.
6. Chuang YC, Young C, Chen, CW, "*Vibrio vulnificus* infection", Scand J Infect Dis, 21:721-726, 1989.
7. Chuang YC, Yuan CY, Liu CY, Lan CK, Huang AH, "*Vibrio vulnificus* infection in Taiwan: report of 28 cases and review of clinical manifestations and treatment", Clin Infect Dis, 15:271-276, 1992.
8. Blake PA, Merson MH, Weaver RE, Hollis DG, Heublein PC, "Disease caused by a marine *Vibrio*: clinical characteristics and epidemiology", N Engl J Med, 300:1-5, 1979.
9. Bonner JR, Coker AS, Berryman CR, Pollock HM, "Spectrum of vibrio infections in a Gulf Coast community", Ann Intern Med, 99:464-469, 1983.
10. Klontz KC, Lieb S, Schreiber M, Janowski HT, Baldy LM, Gunn RA, "Syndromes of *Vibrio vulnificus* infections: Clinical and epidemiologic features in Florida cases, 1981-1987", Ann Intern Med, 109:318-323, 1988.
11. Lee SE, Kim SY, Kim SJ, Kim HS, Shin JH, Choi SH, Chung SS, Rhee JH, "Direct identification of *Vibrio vulnificus* in clinical specimens by nested PCR", J Clin Microbiol, 36:2887-2892, 1998.
12. Su JH, Chuang YC, Tsai YC, Chang MC, "Cloning and characterization of a blue fluorescent protein from *Vibrio vulnificus*", Biochem Biophys Res Commun, 287:359-365, 2001.
13. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic acids Res, 25:3389-3402, 1997.

14. Arias CR, Garay E, Aznar R, "Nested PCR method for rapid and sensitive detection of *Vibrio vulnificus* in fish, sediments, and water", Appl Environ Microbiol, 61:3476-3478, 1995.
15. Brauns LA, Hudson MC, Oliver JD, "Use of polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells", Appl Environ Microbiol, 57:2651-2655, 1991.
16. Hill WE, Keasler SP, Trucksess MW, Feng P, Kaysner CA, Lampel KA, "Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters", Appl Environ Microbiol, 57:707-711, 1991.
17. Lee JY, Eun JB, Choi SH, "Improving detection of *Vibrio vulnificus* in *Octopus variabilis* by PCR", J Food Sci, 62:179-182, 1997.

CHIA-NAN ANNUAL BULLETIN
VOL. 31, PP. 19－27, 2005

以專一性引子進行創傷弧菌的鑑別

陳昱仲* 蘇哲弘* 張文智** 張竣凱*

*嘉南藥理科技大學生物科技系
**東方技術學院食品科技系

摘 要

由創傷弧菌的藍色螢光蛋白基因序列設計一組引子，以聚合酵素鏈反應方法，這組引子可從所有來自臨床的創傷弧菌擴增獲得預期的核酸片段251鹼基對，而不會由其他的臨床致病菌中產生任何核酸片段。這組引子可偵測創傷弧菌染色體含量少至1豪微微克或少至60個細菌數。在生物體樣品的菌體檢測評估中，以血清和傷口抽取液共22件進行聚合酵素鏈反應檢測。在14件細菌培養有細菌存在的樣品中，有13件在聚合酵素鏈反應檢測中呈現正反應，比率高達92.9%。然而，在8件細菌培養無細菌存在的樣品中，有3件在聚合酵素鏈反應檢測可呈現正反應，比率有37.5%之高。在進行細菌鑑別時，這組引子除了具有高專一性的性質外，並且在一階段式的聚合酵素鏈反應仍具有高靈敏性之特質；因此，這組引子是一對可用來進行鑑別創傷弧菌的重要引子。

關鍵字：細菌鑑別、藍色螢光蛋白、聚合酵素鏈反應、創傷弧菌