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PCR and Restriction Fragment Pattern of 16S rRNA gene of *Vibrio vulnificus*

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Abstract

A Pair of designed primers (sequences from Gene Bank) amplified 16S rRNA gene of *V. vulnificus* within polymerase chain reaction (PCR) machine. This PCR product is about 1.3kb DNA fragment. Six enzymese (*BamH* I, *Alu* I, *Sau3A* I, *Hind* III, *Sal* I, *Sma* I) were used for restriction pattern analysis of amplified 16S rRNA gene of *V. vulnificus* ATCC 27562. Digested fragments are resolved by 3% agarose gel. *BamH* I did not show digested fragment so, there was no cutting site of *BamH* I in PCR product. *Alu* I produced three small fragments from 400 bp to 200 bp. *Sau3A* I produced three fragments larger than *Alu* I from 70 bp to 450 bp. *Hind* III showed two different molecular size fragments wnich are 800 bp and 500 bp. One of fragments of *Sal* I was same with 500 bp of *Hind* III fragment and the other was 750 bp. *Sma* I showed two fragments of 800 bp and 470 bp.

The profile of digested fragments of 16S rRNA of *V.vulnificus* ATCC 27562 will may be able to use standard profile for identification of *V. vulnificus.*

Key words : PCR, Restriction fragment pattern, V. vulnificus.

INTRODUCTION

The family of *Vibrionacea* is one of the most important bacterial group in marine environments. This group presented every place of marine as seawater, intestine of marine animal and so on. In addition, some members of the *Vibrionacae* are important pathogens for marine animals and human. In case of fish farming, *Vibrio* sp. caused large mortality.[2,3]

Identification of *Vibrio* sp. has been conducted on biochemical characteristics and serological things of purposed bacteria. Recently, Application of genetic methods effect to identification of *Vibrio* sp. For example, 16S rRNA gene sequence typing is used for taxonomy and shows phylogenetic relationship of *Vibrio sp.*[4]

In accordance with developing of PCR, genetic identification has been developed rapidly.[1] Designed useful primer effect to PCR identification for Vibrio sp.[3]

RFLPs (Restriction fragment length polymorphisms) has been afflicted in bacterial identification since it has been discovered for selection of mutated gene of human.[5,6] In this report we examined restriction fragment patterns of *Vibrio vulnificus* ATCC 27562. 16S rRNA gene was amplified with designed primers. PCR product was restricted by six enzymes. We tried to use restriction fragment map for identification of *Vibrio vulnificus* ATCC 27562 from other bacteria.

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MATERIALS AND METHODS

Strains and Culture

Vibrio vulnificus ATCC 27562 was used for PCR and restriction fragment analysis. It was cultured in 2216E medium (Table 1) at 26℃ for 18~24 hr.

Table 1. The composition of 2216E medium

COMPOSITION	AMOUNT
Yeast extract	1g
Peptone	5g
$FePO_4$	0.01g
NaCl	3%
Agar	2%
D.W.	1000ml

Extraction of genomic DNA from bacterial cell

Cultured bacteria were harvested and washed with saline in 5,000×g of centrifugation. Cells were resuspended in 500µl of TE (10 mM Tris-Cl, 100 mM EDTA, pH8) lysis buffer. 25µℓ of 10% sodium dodesyl sulfate and 1µℓ of RNase were added to lysate then incubated at 37℃ for 1hr. 4µℓ of Proteinase K(100 g/ml, Sigma) was added to lysate and icubated at 55℃ for another 1hr.

DNA was purified by phenol-chloroform extraction and 95% ice ethanol precipitation. After dried completely, the DNA was resuspended in sterile distilled water. The quality of extracted DNA was assessed by 0.8% agarose gel electrophoresis and UV meter at 260 nm.

PCR amplification

A pair PL1 and PL2 were used for PCR amplification of 16S rRNA gene of *V. vulnificus* (Table 2). Sequences of primers were based on 16S rRNA gene sequences of eubacteria from EMBL and GENE BANK.

PCR solution was performed total 50µℓ using 5µℓ of template (20 ng/ l), 5µℓ of 10× buffer, 4µℓ of dNTPs (2.5 mM), 2.5µℓ of each primer (10 pmol) and 1µℓ of *Taq* polymerase (1 unit).

Table 2. The construction of used primers for PCR amplification

DESIGNATION	Position on *E. coli*	SEQUENCES
PL1	155	5 TTCGAAACGATCTGCTAATACCGC 3
PL2	1377	5 GAATTCACCGTGGCATTCTGATCC 3

PCR protocol was : 10 min of predenaturation of template with 50 of mineral oil then followed by 30 cycles of 70s at 94℃, 90s at 50℃, 120s at 72℃ and final extension at 72℃ for 5 min. 5µℓ of PCR products were electrophoresised in a 1.5% agarose gel.

Restriction fragment analysis of PCR product

Mineral oil of PCR product was removed by chloroform extraction. DNA was purified by phenol-chloroform extraction. DNA was treated by 95% of ice ethanol and incubated at -20℃ for 1h. Precipitated DNA was dried completely and resuspended in sterile distilled water. DNA was digested with 6 restriction enzymes of *Alu* I, *Sau3A* I, *BamH* I, *Hind* III, *Sal* I, Sma I (Promega, Table 3).

Table 3. The characteristics of restriction enzymes

ENZYME	BUFFER	TEMP(℃)	CUTTING SITE
Alu I	B	37	AGCT
Sau3A I	B	37	GATC
BamH I	E	37	GGATCC
Hind III	B	37	AAGCTT
Sal I	D	37	GTCGAC
Sma I	J	25	CCCGGG

Restriction enzyme solution was performed final 30µℓ using 20µℓ of PCR product, 3µℓ of ×10 buffer, 1.5µℓ (10 unit) of enzyme and 5.5µℓ of sterile distilled water. These tubes were incubated at optimum temperature of each enzyme for overnight. After incubation, 5µℓ of digested products were electrophoresised by 3% agarose gel with DNA marker (X74 DNA/*Hae* III, Promegma)

and photographed under UV.

RESULTS

PCR amplification of 16S rRNA gene from *V. vulnificus*

The location and sequences of primers for amplification of 16S rRNA of *V. vulnificus* are got from GENE BANK. A Pair of designed primers make 1.3Kb fragment of clear PCR product (Fig.1). GENE BANK send same size amplified 16S rRNA part with our PCR product.

Restriction pattern analysis

Six enzymes (*BamH* I, *Alu* I, *Sau3A* I, *Hind* III, *Sal* I, *Sma* I) were used for restriction pattern analysis of 16S rRNA gene of *V. vulnificus*. Digested fragments are resolved by 3% agarose gel (Fig.2).

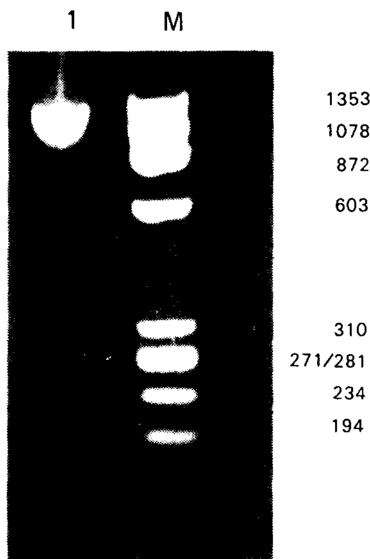

Fig. 1. Restriction map of PCR product of *V. vulnificus* ATCC 27562.
M : X74 DNA/Hae III DNA molecular size marker.
1 : Amplified 16S rRNA gene (First PCR product).

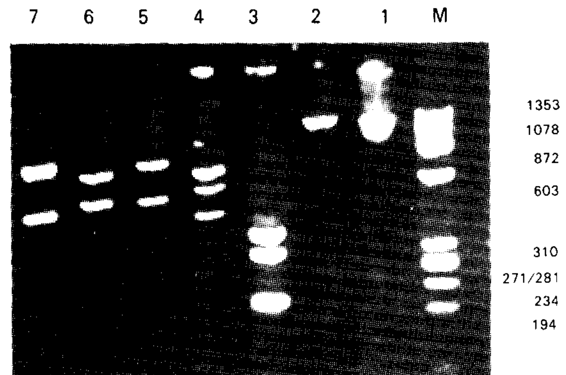

Fig. 2. Restriction map of PCR product of *V. vulnificus* ATCC 27562.
M : X74 DNA/Hae III DNA molecular size marker, 1 : Control (undigested PCR product), 2 : Fragment of *Bam H* I, 3 : Fragments of *Alu I*, 4 : Fragments of *Sau3A* I, 5 : Fragments of *Hind* III, 6 : Fragments of *Sal* I, 7 : Fragments of *Sma* I.

BamH I did not show digested fragment so, there was no cutting site of *BamH* I in PCR product. *Alu* I produced three small fragments from 400 bp to 200 bp. *Sau 3A* I produced three fragments larger than *Alu* I from 70 bp to 450 bp.

Hind III showed two different molecular size fragments 800 bp and 500 bp. One of fragments of *Sal* I was same with 500 bp of *Hind* III fragment and the other was about 750 bp. *Sma* I showed two fragments 800 bp and 470 bp. This result almost same with computer analysis (data not shown).

Sequences of 16S rRNA gene

Sequences of 16S rRNA of *V. vulnificus* were received from GENE BANK for analysis of restriction site. Between computer analysis and our experiment are not different in size (Fig. 3).

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   1 agagtttgat nntggctcag attgaacgct ggcggcaggc ctaacacatg caagtcgagc
  61 ggcagcacag agaaacttgt ttctcgggtg gcgagcggcg gacgggtgag taatgcctgg
 121 gaaattgccc tgatgtgggg gataaccatt ggaaacgatg gctaataccg catgatagct
 181 tcggctcaaa gagggggacc ttcgggcctc tcgcgtcagg atatgcccag gtgggattag
 241 ctagttggtg aggtaagggc tcaccaaggc gacgatccct agctggtctg agaggatgat
 301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
 361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcgggtt
 421 gtaaagcact ttcagtcgtg aggaaggtgg tagtgttaat agcactatca tttgacgtta
 481 gcgacagaag aagcaccggc taactccgtg ccagcagccg cggtaatacg gagggtgcga
 541 gcgttaatcg gaattactgg gcgtaaagcg catgcaggtg gtttgttaag tcagatgtga
 601 aagcccgggg ctcaacctcg gaactgcatt tgaaactggc agactagagt actgtagagg
 661 ggggtagaat ttcaggtgta gcggtgaaat gcgtagagat ctgaaggaat accggtggcg
 721 aaggcggccc cctggacaga tactgacact cagatgcgaa agcgtgggga gcaaacagga
 781 ttagataccc tggtagtcca cgctgtaaac gatgtctact tggaggttgt ggccttgagc
 841 cgtggctttc ggagctaacg cgttaagtag accgcctggg gagtacggtc gcaagattaa
 901 aactcaaatg aattgacggg ggcccgcaca agcggtggag catgtggttt aattcgatgc
 961 aacgcgaaga accttaccta ctcttgacat ccagagaatc tagcggagac gctggagtgc
1021 cttcgggaac tctgagacag gtgctgcatg gctgtcgtca gctcgtgttg tgaaatgttg
1081 ggttaagtcc cgcaacgagc gcaaccctta tccttgtttg ccagcgagta atgtcgggaa
1141 ctccagggag actgccggtg ataaaccgga ggaaggtggg gacgacgtca agtcatcatg
1201 gcccttacga gtagggctac acacgtgcta caatggcgca tacagagggc ggccaacttg
1261 cgaaagtgag cgaatcccaa aaagtgcgtc gtagtccgga ttggagtctg caactcgact
1321 ccatgaagtc ggaatcgcta gtaatcgtgg atcagaatgc cacggtgaat acgttcccgg
1381 gccttgtaca caccgcccgt cacaccatgg gagtgggctg caaaagaagt gggtagttta
1441 accttcggga ggacgctcac cactttgtgg ttcatgactg gggtgaagtc gtaacaaggt
1501 agcgctaggg gaacctggcg ctggatcacc tcctt
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Fig. 3. Sequeces of 16S rRNA gene of *Vibrio vulnificus* ATCC 27562 from GENE BANK.

DISCUSSION

Marine bacteria, *Vibrio* sp. is important to environment and disease. Some kinds of *Vibro* species is typical pathogen to human and marine animal like. Especially *V. vulnificus* cause serious septisemia.[2,4)]

So, identification method for *Vibrio* species have been developed in diversity ways. Recently many investigator use molecular biological method as DNA hybridization and direct sequencing and so on. In order to identify rapidly more than classical ways need bacterial culture.[5,6)]

PCR is suitable method for rapid identification of bacteria. Specific primer provide typical product in each bacterial stain. 16S rRNA part is conserved in same genus so it have been used at taxonomic study. KIMUKO [4)] *et al.* have ever reported phylogenetic relationship of family *Vibrionacea* on basis of 16S rRNA sequences.

Used primer was successful at amplification of 16S rRNA gene of *Vibrio* species. Here, about 1.3kb single fragment was showed as PCR product. In same species, results of PCR are same. So it is hard to separate or identify one strain by PCR. However Same PCR product produce different fragment by enzyme digestion because of its polymorphism on agarose gel electrophoresis.

In this report, we examined restriction pattern with six kinds of enzymes. Sequences of PCR product are compared with computer analysis. *BamH* I did not show any digested fragment as expect. *Alu* I and *Sau3A* I showed three fragment. *Hind* III, *Sal* I and *Sma* I showed two fragment. We can also expect these fragment on sequences of 16S rRNA of *V. vulnificus* from GENE BANK. But there was undetectable low molecule fragment on agarose gel. These bands should be resolved in high resolving gel like arylamide. The profile of enzyme digested fragment of 16S rRNA of *V.vulnificus* will be able to use standard profile for identification of *V. vulnificus.* But just six enzymes were used to only one strain. So, more enzymes and strain should be examined.

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REFERENCE

1. Armelle, D., Sylvie, P., Alain, K. and YVES, B. : PCR and restrictio fragment length polymorphism of *pel* gene as a tool to identify *Erwinia carorovora* in relation to potato disease, *Appl. Environ. Microbiol.* **60**, 1437~1443(1994).
2. BOCK, J. A. and Lightner, D. V. : Disease of Crustacea, Disease caused by microorganisms in animals, Willey & Sons, New York pp 245~349(1990).
3. JIE, K. : Identification of bacterial pathology by PCR-RFLPs.(1996)
4. KUMIKO KITA-TSUKAMOTO, HIROSHIOYAIZU, KENJI NANBA and USIO SIMIDU : Phylogenetic relationship of marine bacteria mainly members of the

family *Vibrionacea* determined on the basis of 16s RNA sequences, *Int. J. Sys. Bac.* 43, 819(1993).
5. RACHEL Peillon, Emmanual B. Drouet, Sylvie Bruneau, Gilles Panteix, Gerard Antoine, Denoyel and Henri P. De Montclos : Discrimination of *Mycobacterium aviumm-Mycobacterium* intracellular strain by genomic DNA fingerprinting with 16s RNA gene probe, *FEMS Microbiol. lett.* 124, 7580(1994).
6. RYTAS VILGALYS and MARK HESTER : Rapid genetic identification and mapping of enzymically amplified ribosomal DNA from several *Cryptococcus* species, *J. Bac.* 172, 4238~4246(1990).

초록 : *Vibrio vulnificus* ATCC 27562의 16S rRNA 유전자의 PCR과 제한효소절단 방식

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Vibrio vulnificus ATCC 27562의 16S rRNA 유전자를 PCR법과 제한효소절단법으로 분석 하여 얻은 결과는 다음과 같다.

1. 고안된 한쌍의 primer로 PCR을 시행하여 얻은 산물은 약 1.3kb 였다.
2. PCR산물을 여섯가지의 제한 효소로 절단하여 얻은 단편들은 아래와 같다. *BamH* I : 어떤 restriction fragment도 만들지 않았다.

Alu I : 약 400bp와 200bp의 두가지 fragment를 생산하였다.

Sau3A I : 약 70bp에서 450bp 사이에 세가지 fragment를 생산하였다.

Hind III : 약 800bp와 500bp의 약간 큰 두가지 fragment를 생산하였다.

Sal I : 약 500bp와 750bp의 두가지 fragment를 생산하였다.

Sma I : 약 800bp와 470bp의 두가지 fragment를 생산하였다.