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Journal of Microbiological Methods 65 (2006) 612–618
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Note

Multiplex PCR for simultaneous detection of five virulence hemolysin genes in *Vibrio anguillarum*

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Received 4 April 2005; received in revised form 18 August 2005; accepted 13 September 2005
Available online 20 October 2005

Abstract

A multiplex PCR was developed for detection of hemolysin-producing *Vibrio anguillarum* using primers targeting five hemolysin genes (*vah*1, *vah*2, *vah*3, *vah*4 and *vah*5). This method was successful in amplifying reactions containing as little as 100 fg of genomic template DNA. The direct detection of *V. anguillarum* in clinical specimens by this multiplex PCR was also successful in reactions containing as few as 10 bacterial cells. This multiplex PCR method can be a rapid and sensitive method for detecting pathogenic *V. anguillarum*.

Keywords: *Vibrio anguillarum*; Multiplex PCR; Hemolysins; Virulence gene

Vibrio anguillarum is a pathogenic bacterium that causes vibriosis or hemorrhagic septicemia in fishes. Vibriosis has caused economic losses to the worldwide fish-farming industry, particularly salmonid farming (Actis et al., 1999).

In order to prevent and control outbreak of vibriosis, development of effective diagnostic method is an important step. Multiplex PCR method is one of the most effective methods used for detection of bacterial pathogens (Edwards and Gibbs, 1994). Multiplex PCR methods have been developed for rapid detection of fish pathogens associated with warm-water Streptococcosis (Mata et al., 2004) and other three major fish pathogens (*Aeromonas salmonicida*, *Flavobacterium psychrophilum* and *Yersinia ruckeri*) (Del Cerro et al., 2002).

Recently, complete nucleotide sequences of four types of hemolysin genes in *V. anguillarum* strain H775-3 have been identified and characterized. All genes were confirmed to be contributed to the virulence of *V. anguillarum* (Rodkhum et al., 2005). The described hemolysin genes not only provided a better understanding in the virulence of *V. anguillarum*, but they also made it possible to detect hemolysin-producing pathogenic strains by multiplex PCR.

Conventional PCR amplification based on a single hemolysin gene (*vah*1) of *V. anguillarum* was first reported by Hirono et al. (1996). However, the previous method could not detect some strains of *V. anguillarum* that lack of the *vah*1 gene. In order to correct this problem, we aimed to improve the efficacy of the PCR method by using multiplex PCR amplification

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doi:10.1016/j.mimet.2005.09.009

Table 1
Bacterial strains used in this study and their multiplex PCR results

Bacterial strain	Source of isolation	Multiplex PCR results[a]					Reference
		*vah*1	*vah*2	*vah*3	*vah*4	*vah*5	
V. anguillarum							
775 (ATCC 68554)	*Oncorhynchus kisutch,* Washington, USA	+	+	+	+	+	b
H775-3	Plasmidless derivative strain of strain 775	+	+	+	+	+	b
ATCC 19264	Ulcerous lesion in Cod, Denmark	+	+	+	–	–	MacDonell and Colwell, 1985
509	New Hampshire, USA	+	+	+	+	+	b
528	Maine, USA	+	+	+	+	+	b
R61	*Scophthalmus maximus*, Spain	+	+	+	+	+	b
PT 10	Diseased fish, Tokushima, Japan	–	+	+	+	–	c
PT 24	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 223	Diseased fish, Tokushima, Japan	+	+	+	+	+	c
PT 493	Diseased fish, Tokushima, Japan	–	+	+	+	–	c
PT 573	Diseased fish, Tokushima, Japan	+	+	+	+	+	c
PT641	Diseased fish, Tokushima, Japan	+	+	+	+	+	c
PT818	Diseased fish, Tokushima, Japan	–	+	+	+	–	c
PT 01027	Diseased fish, Tokushima, Japan	–	+	+	+	–	c
PT 01030	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 02025	Diseased fish, Tokushima, Japan	–	+	+	–	–	c
PT 02026	Diseased fish, Tokushima, Japan	–	+	+	–	–	c
PT 02039	Diseased fish, Tokushima, Japan	–	+	+	+	–	c
PT 7906	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 77052	Diseased fish, Tokushima, Japan	+	+	+	+	+	c
PT 77097	Diseased fish, Tokushima, Japan	+	+	+	+	+	c
PT 77022	Diseased fish, Tokushima, Japan	+	+	+	+	+	c
PT 80106	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 80146	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 80155	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 80162	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 80173	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 81070	Diseased fish, Tokushima, Japan	+	+	+	+	+	c
PT 81188	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 81208	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 85045	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 85050	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 86060	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 87016	Diseased fish, Tokushima, Japan	–	+	+	+	–	c
PT 87034	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 87041	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 88007	Diseased fish, Tokushima, Japan	–	+	+	+	–	c
PT 90015	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 90025	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 91029	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 92021	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 92047	Diseased fish, Tokushima, Japan	+	+	+	–	–	c
PT 98013	Diseased fish, Tokushima, Japan	+	+	+	+	–	c
WA 8114	Diseased fish, Wakayama, Japan	+	+	+	–	–	c
WA 8701	Diseased fish, Wakayama, Japan	+	+	+	+	+	c
WA 8703	Diseased fish, Wakayama, Japan	+	+	+	+	+	c
WA 8705	Diseased fish, Wakayama, Japan	+	+	+	–	–	c
WA 8709	Diseased fish, Wakayama, Japan	+	+	+	–	–	c
KS 7	Diseased fish, Japan	+	+	+	+	+	c
KS 19	Diseased fish, Japan	+	+	+	+	+	c
KG 8126	Diseased fish, Japan	+	+	+	+	+	c
MZM 8130	Diseased fish, Miyazaki, Japan	+	+	+	+	+	c
MZK 8103	Diseased fish, Miyazaki, Japan	+	+	+	+	+	c

(continued on next page)

Table 1 (*continued*)

Bacterial strain	Source of isolation	Multiplex PCR results[a]					Reference
		*vah*1	*vah*2	*vah*3	*vah*4	*vah*5	
MZK 8118	Diseased fish, Miyazaki, Japan	+	+	+	+	–	c
SH 86049	Diseased fish, Japan	+	+	–	–	–	c
SH 86073	Diseased fish, Japan	+	+	+	–	–	c
SG 43	Diseased fish, Shiga, Japan	+	+	+	–	–	c
SG 8122	Diseased fish, Shiga, Japan	+	+	+	+	+	c
SG 8125	Diseased fish, Shiga, Japan	+	+	+	+	+	c
SG 8126	Diseased fish, Shiga Japan	+	+	+	+	+	c
SG 8606	Diseased fish, Shiga, Japan	+	+	+	–	–	c
MV-3	Diseased fish, Japan	+	+	+	+	+	c
AV 21	Diseased fish, Japan	+	+	+	–	–	c
PB 15	Diseased fish, Japan	+	+	+	+	+	c
PB 28	Diseased fish, Japan	+	+	+	+	+	c
ET-1	Diseased fish, Japan	+	+	+	+	+	c
030491	Amberjack (*Seriola dumerili*), Japan	+	+	+	+	+	c
030571	yellowtail, (*Seriola quinqueradiata*), Japan	+	+	+	+	+	c
030841	Yellowtail, Japan	+	+	+	+	+	c
030842	Yellowtail, Japan	+	+	+	+	+	c
V. ordalii							
ATCC33509	Coho salmon kidney, WA, USA	–	+	+	–	–	Schiewe et al., 1981
V. vulnificus							
ATCC 27562	Human blood, Florida, USA	–	–	–	–	–	Okada et al., 1987
V. parahaemolyticus							
ATCC 17802	Shirasu food poisoning, Japan	–	–	–	–	–	Fujino et al., 1965
EP 25	Black Tiger shrimp (*Penaeus monodon*), Thailand	–	–	–	–	–	Saitanu et al., 1999
SMV 45	Sediment of shrimp (*Penaeus monodon*) pond, Thailand	–	–	–	–	–	Saitanu et al., 1999
V. costicolus							
ATCC 33508	Bacon curing brine	–	–	–	–	–	Mellado et al., 1996
V. cholerae non O1							
SMV 128	Sediment of shrimp (*Penaeus monodon*) pond, Thailand	–	–	–	–	–	Saitanu et al., 1999
V. fluvialis							
STV-25	Soft shell turtle (*Pelodiscus sinensis*)	–	–	–	–	–	Rodkhum et al., 2001
V. cambellii							
ATCC 25920	Seawater	–	–	–	–	–	Baumann et al., 1971
V. alginolyticus							
NCIMB 1903 (ATCC 17749)	Spoiled horse mackerel which caused food poisoning, Japan	–	–	–	–	–	Sakazaki, 1968
V. nereis							
ATCC 25917	Seawater enriched with propanol	–	–	–	–	–	Baumann et al., 1971
V. diazotrophicus							
ATCC 33466	Sea urchin gastrointestinal tract, Canada	–	–	–	–	–	Guerinot and Patriquin, 1981
Photobacterium leiognathi							
ATCC 25521	Light organ of teleostean fish	–	–	–	–	–	Boisvert et al., 1967

Table 1 (*continued*)

Bacterial strain	Source of isolation	Multiplex PCR results[a]					Reference
		*vah*1	*vah*2	*vah*3	*vah*4	*vah*5	
Yersinia ruckeri							
332-053	Diseased fish	–	–	–	–	–	c
Aeromonas hydrophila							
ATCC 7966	Tin of milk with a fishy odor	–	–	–	–	–	Popoff and Veron, 1976
ATCC 14715	Juvenile silver salmon (*Oncorhynchus kisutch*) intestine	–	–	–	–	–	Popoff and Veron, 1976
ATCC 15467	Used oil emulsions	–	–	–	–	–	Popoff and Veron, 1976
ATCC 19570	Intestine of pike	–	–	–	–	–	Popoff and Veron, 1976
Photobacterium damselae subsp. piscicida							
ATCC 51736	Spleen of young yellowtail , Japan	–	–	–	–	–	Gauthier et al., 1995
P97-008	Yellowtail, Japan	–	–	–	–	–	c
P98-025	Yellowtail, Japan	–	–	–	–	–	c
P98-039	Yellowtail, Japan	–	–	–	–	–	c
P98-042	Yellowtail, Japan	–	–	–	–	–	c
P99-021	Yellowtail, Japan	–	–	–	–	–	c
P97-018	Yellowtail, Japan	–	–	–	–	–	c
P97-019	Yellowtail, , Japan	–	–	–	–	–	c
P88-006	Yellowtail, , Japan	–	–	–	–	–	c
P88-011	Yellowtail, Japan	–	–	–	–	–	c
Edwardsiella tarda							
ET-54	Diseased fish, Japan	–	–	–	–	–	c
Renibacterium salmoninarum							
ATCC 33209	Yearling Chinook salmon *(Oncorhynchus tshawytsch)*, OR, USA	–	–	–	–	–	Sanders and Fryer, 1980

[a] +, PCR product obtained; −, no PCR product obtained.
[b] Bacterial culture collection, Crosa's lab, Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR, USA.
[c] Bacterial Culture Collection, Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Tokyo, Japan.

based on a multi-hemolysin loci system. This was done by simultaneously targeting five different hemolysin toxin genes (*vah*1, *vah*2, *vah*3, *vah*4 and *vah*5) present in the *V. anguillarum* chromosome in a single PCR reaction.

A total of 70 *V. anguillarum* strains and 30 other bacterial strains mainly isolated from diseased fish and aquatic environments in Japan, the United states, Canada, Europe and Thailand were used in this study (Table 1). *V. anguillarum* strains were cultured at 25 °C in Tryptic soy broth (TSB) and/or in Tryptic soy agar (TSA) (Difco, USA) containing 2% NaCl. Medium and culture condition of another bacteria were made following the recommendation of American Type Culture Collection (ATCC).

Genomic DNA of bacteria was prepared according to the standard method of Ausubel et al. (1987). Specific oligonucleotide primers were designed from the full length of five hemolysin genes (*vah*1, *vah*2, *vah*3, *vah*4 and *vah*5) published in DDBJ (DNA Data Bank of Japan) (Table 2).

PCR amplification was performed in a Takara PCR thermal cycler MP (Takara, Japan) with 200-µl microcentrifuge tubes. Purified genomic DNA from 70 strains of *V. anguillarum* and 16 genera of taxonomically related bacterial species ($n=30$ strains) were used as DNA template for multiplex PCR. The PCR was carried out in a 50-µl reaction mix containing 100 ng of bacterial genomic DNA, 250 µM of each deoxynucleoside triphosphate (dNTP), 10 pmol of each primer (Table 2), 5 µl of 10× *rTaq* buffer with $MgCl_2$, 0.75 U of *Taq* DNA polymerase (*rTaq*) (Bioneer, Korea) and Milli-Q water up to 50 µl. The PCR annealing temperatures was varied from 48 to 62 °C. The appropriate annealing temperature that did not interfere with annealing of any of the primers was 58 °C. Therefore, 58 °C was used as the annealing temperature for all primer pairs. The PCR thermocycling was one cycle of

Table 2
Specific primers for multiplex PCR used in this study

Gene	Primer	Melting temperature (T_m)	Amplicon size (bp)	Accession number	Reference
*vah*1	Sense 5′-ACCGATGCCATCGCTCAAGA-3′	48.7	490	S83534	Hirono et al., 1996
	Antisense 5′-GGATATTGACCGAAGAGTCA-3′	44.6			
*vah*2	Sense 5′-ATGAACGAAGATAACCCCCAGA-3′	47.9	876	AB189395	This study
	Antisense 5′-TCACTCTTCTGCTATCACTGG-3′	47.3			
*vah*3	Sense 5′-ATGACTTCTTCTAAATTTTCGTTATGTGCG-3′	51.1	1128	AB189396	This study
	Antisense 5′-GATAGAGCGGACTTTGCTTG-3′	51.9			
*vah*4	Sense 5′-ATGAAAACCATACGCTCAGCATCT-3′	48.9	603	AB189397	This study
	Antisense 5′-TCACGCTTGTTTTTGGTTTAAATGAAATCG-3′	51.1			
*vah*5	Sense 5′-ATGCTCACGATAAGCCCTTTTAGAT-3′	49.3	1758	AB189398	This study
	Antisense 5′-TCAAGGGTTAGGCGCGTGAT-3′	48.7			

initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, and subsequently, extension by one cycle of 72 °C for 7 min. After amplification, 3 μl of each reaction mixture was separated by electrophoresis on a 1.5% agarose gel containing 0.2 μg of ethidium bromide per milliliter. The gel was run in 1× Tris–Borate–EDTA (TBE) buffer at 100 V with standard DNA size markers for 40 min and visualized with a densitograph (Bioinstrument, ATTO, Japan).

Each of the five amplicons was also amplified separately with the same PCR program to ensure that all target loci can be amplified efficiently by this PCR condition. The PCR products showed distinct DNA bands of *vah*1, *vah*2, *vah*3, *vah*4 and *vah*5 with predicted sizes of 490, 876, 1,128, 603, 1,758 bp, respectively.

A total of 70 strains of *V. anguillarum* were tested with multiplex PCR using five hemolysin genes targets. All strains of *V. anguillarum* showed target DNA bands of *vah*2 (70/70), while other bacteria did not show any targeted band except for one strain of *Vibrio ordalii* ATCC 33509, the closest species which produced the same target amplicons of *vah*2 and *vah*3. Nine of 70 strains (9:70) did not produce the *vah*1 amplicon, one (1:70) did not produce the *vah*3 amplicon, thirty-one (31:70) did not produce the *vah*4 amplicon and forty (40:70) did not produce the *vah*5 amplicon. Therefore, the prevalences of the hemolysin genes, *vah*1, *vah*2, *vah*3, *vah*4 and *vah*5, were 87.14%, 100%, 98.57%, 55.71% and 42.86%, respectively. The details of the presented hemolysin genes in *V. anguillarum* were shown in Table 1.

The DNA template was added to each PCR reaction mixture by varying the DNA template concentration from 1 μg/μl to 1 fg/μl with 10-fold dilution method. Only the reactions that amplified all five of the hemolysin genes were considered as positive. Multiplex PCR was successful with PCR reactions containing as little as 100 fg of genomic DNA template per reaction (Fig. 1).

Hemolysin-producing *V. anguillarum* from infected fish was also directly detected by multiplex PCR. Briefly, five yellowtail fishes (*Seriola quinqueradiata*; mean body weight 100 g) were injected intraperitoneally with *V. anguillarum* H775-3 dose approximately 10^7–10^8 cells of *V. anguillarum* strain H775-3/fish. Kidney and spleen were collected from infected fishes after 48 h of injection and divided into two parts for bacteriological culture and DNA extraction for PCR. The DNA of bacteria from tissues was extracted without a bacterial culturing step by using Instagene matrix (Bio-Rad, USA). The tissues were homogenized in sterile phosphate-buffered saline (PBS), pH 7.4. The number of bacteria/g of tissue were quantified by serial dilution

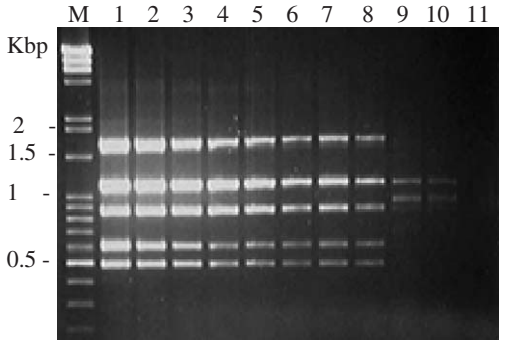

Fig. 1. Detection limit of multiplex PCR with five primer sets of *V. anguillarum* hemolysin genes as shown by agarose gel electrophoresis. The bands are assumed to correspond to *vah*1, *vah*2, *vah*3, *vah*4 and *vah*5, with predicted sizes of 490, 876, 1128, 603, 1758 bp, respectively. Lanes M, DNA markers; Lanes 1 through 10, multiplex PCR products of purified chromosomal DNA of *V. anguillarum* H7775-3 serially diluted 10-fold from 1 μg/μl to 1 fg/μl; Lane 11, no template (negative control).

and plating onto TSA containing 2% NaCl. The bacteria grown on the plates were confirmed to be *V. anguillarum* by conventional PCR based on *vah*2 gene. In order to calculate the detection limit of PCR for direct detection of *V. anguillarum* DNA in tissue samples, serial 10-fold dilutions of DNA extracted from tissue were prepared from the original tube (1 g of homogenized tissue). The DNA in each tube was used as a template for multiplex PCR. The tissues from non-infected fish were also homogenized in PBS and used as a negative control for multiplex PCR. The detection limit was evaluated from the PCR reaction containing the lowest amount of *V. anguillarum* DNA that amplified all five of the hemolysin genes. The direct detection of *V. anguillarum* in clinical specimens by this multiplex PCR was also successful in reactions containing as few as 10 bacterial cells, which is equivalent to 10^4 bacterial cells/g of tissue. This is an advantage of the multiplex PCR assay that can be performed with template DNA extracted directly from clinical specimens, without the time-consuming bacterial cultivation step. The low detection limit suggests that the method can detect *V. anguillarum* during early stages of infection.

A pathogenic *V. anguillarum* which cannot be detected by conventional PCR targeting a single hemolysin gene was shown to be detected by this multiplex PCR method. This suggests that multiplex PCR is more accurate method than conventional PCR for detecting hemolysin-producing *V. anguillarum*. However, multiplex PCR method also showed amplicons of *vah*2 and *vah*3 when tested with *V. ordalii*. Gonzalez et al. (2003) developed a conventional PCR method for identification of *V. anguillarum* based on the *rpo*N gene and they found that the amplification band was produced with *V. ordalii* NCIMB 2167 using an annealing temperature of 58 °C. They solved the problem by increasing the annealing temperature to 62 °C. However, in this study, non-specific bands were observed when the annealing temperature was changed to 62 °C or to temperatures other than 58 °C. Therefore, we could not use this multiplex PCR for differentiate some strains of *V. anguillarum* from *V. ordalii* due to similar target PCR products of *vah*2 and *vah*3 found in both genera. This suggests that some hemolysin genes similar to *vah*2 and *vah*3 of *V. anguillarum* may also exist on the *V. ordalii* chromosome. The information about genetic similarity between hemolysin genes of both genera should be further evaluated.

This multiplex PCR is a breakthrough for the detection of hemolysin-producing *V. anguillarum* because it is not only rapid and sensitive but it can be also provide comprehensive information about the prevalence of hemolysin genes present in several strains of pathogenic *V. anguillarum* isolated from several sources.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research (S) from the ministry of Education, Culture, Sports, Science, and Technology of Japan.

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