

Short communication

A Multiplex PCR Assay for Differentiation of *Streptococcus parauberis* Serotypes

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ABSTRACT—A multiplex PCR assay for differentiation of *Streptococcus parauberis* serotypes was developed. The three primer pairs for subserotypes Ia and Ib/Ic and serotype II were designed from the serotype-specific sequences of the *wzy* gene in the loci for capsular polysaccharide biosynthesis. All of 188 *S. parauberis* isolates from Japanese flounder showed positive reaction with the expected size of PCR product for each serotype, which was consistent with the results of agglutination test using rabbit antisera. The nontypeable isolates, which are not differentiated by agglutination test, could be identified as subserotype Ib/Ic or serotype II. Other streptococci including *S. parauberis* derived from cow and major bacterial fish pathogens showed negative reaction.

Key words: *Streptococcus parauberis*, multiplex PCR, serotyping, streptococcosis

Streptococcus parauberis, an etiologic agent of streptococcosis in fish, was isolated for the first time from turbot *Scophthalmus maximus* in Spain (Domeénech *et al*., 1996). Thereafter, *S. parauberis* has caused high economic losses in cultured turbot in Spain and the Japanese flounder *Paralichthys olivaceus* in Korea (Toranzo *et al*., 2005; Baeck *et al*., 2006). In Japan, *S. parauberis* has been recognized as a pathogen of the Japanese flounder since 2002 (Kanai *et al*., 2009).

The Japanese *S. parauberis* isolates can be classified into five serological phenotypes (subserotypes Ia, Ib and Ic, serotype II and nontypeable) according to the difference and presence/absence of capsular polysaccharide antigens (Kanai *et al*., 2015). To date, slide agglutination test using rabbit anti-*S. parauberis* sera has been applied to serotype differentiation as well as identification of the organism isolated from fish. The serotype differentiation will be of benefit to the chemotherapy of *S. parauberis* infection, because all of the serotype II *S. parauberis* isolates are tetracycline-resistant due to harboring Tn*916* (Meng *et al*., 2009). However, the differentiation of *S. parauberis* serotypes by slide agglutination test is sometimes difficult, because most institutes and laboratories do not have the antisera for serotyping. Thus, an alternative diagnostic method is required.

Recently it was revealed that there were three distinct capsular polysaccharide biosynthesis loci (*cps* loci) in *S. parauberis* isolated from the Japanese flounder, which corresponded to subserotypes Ia and Ib/Ic and serotype II, respectively (Tu *et al*., 2015). Moreover, the nontypeable (NT) strains of clusters I and III in *sma*I-PFGE analysis (Kanai *et al*., 2015) had similar *cps* loci to subserotype Ib/Ic and serotype II, respectively. Based on these findings, a multiplex PCR assay for the differentiation of *S. parauberis* serotypes, Ia, Ib/Ic and II, was developed.

Materials and Methods

Bacteria

A total of 188 *S. parauberis* isolates from diseased and apparently healthy Japanese flounder used in the previous study (Kanai *et al*., 2015) were employed. To verify the specificity of the multiplex PCR, the *S. parauberis* strain ATCC13386 derived from cow, the other Gram-positive cocci, *S. agalactiae* JCM5671, *S. thermophilus* JCM17834, *S. uberis* ATCC19436, *S. iniae* NUF631 from the Japanese flounder, *S. dysgalactiae* NUF1015 from greater amberjack *Seriola dumerili, Enterococcus faecalis* JCM5803, *Lactococcus lactis* subsp. *lactis* JCM5805 and *L. garvieae* NUF699 from the Japanese flounder, and the Gram-negative pathogens, *Edwardsiella tarda* NUF251 from the Japanese flounder, *Vibrio anguillarum* NUF652 from greater amberjack and *Photobacterium damselae* subsp. *piscicida* NUF89 from yellowtail *Seriola quinqueradiata*, were used as reference strains. Strains of JCM were provided by Japan Collection of Microorganisms, RIKEN BRC which is participating in the National BioResource Project of the MEXT, Japan. For the cultivation of Gram-positive cocci and Gram-negative pathogens, Todd Hewitt agar and heart infusion agar (Difco) were used, respectively. The bacteria were cultured at 27°C for 24 h.

Genomic DNA preparation

The genomic DNA of the 188 *S. parauberis* isolates and reference strains were prepared using Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions.

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Primer design

Based on the multiple sequence alignment of polysaccharide polymerase gene (*wzy*) in the serotype-specific region of the four *S. parauberis cps* loci (DDBJ/EMBL/GenBank accession no., LC060252 to LC060255), three pairs of serotype-specific primers were designed using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) in order to allow simultaneous amplification in the same conditions with specificity (Fig. 1). The designed primer pairs were named For-Ia (5´-ATTGTTAGTCATTCAGTTGT-3´) and Rev-Ia (5´-AATTATAGTCAACAGTCCAG-3´), For-Ib/Ic (5´-ATTTCTACCAGGTTACTTTG-3´) and Rev-Ib/Ic (5´-ACATCTCGAAACTTCATATT-3´) and For-II (5´-GAACTACTTAGGTTTAGCAT-3´) and Rev-II (5´-AACTTGTAAATAGGATTGCT-3´). They will give the amplification products of 213, 303 and 413 bp for subserotypes Ia and Ib/Ic and serotype II, respectively.

PCR amplification

The PCR was performed on C1000 Thermal Cycler (Bio-Rad) with *TaKaRa Ex Taq* Hot Start Version (Takara). The PCR reaction mixture (a total volume of 50 μL) contained 5 μL of ×10 Ex Taq buffer (Mg^{2+} plus), 4 μL of dNTP mixture (0.2 mM each), six primers (0.5 μM each), 1 μL of template DNA and 1.25 U of *Ex Taq* DNA polymerase. The condition of PCR amplification was denaturation at 95°C for 3 min, 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s and extension at 72°C for 30 s and a final extension at 72°C for 7 min. The PCR products were electrophoresed in 2.0% agarose gel followed by staining with ethidium bromide.

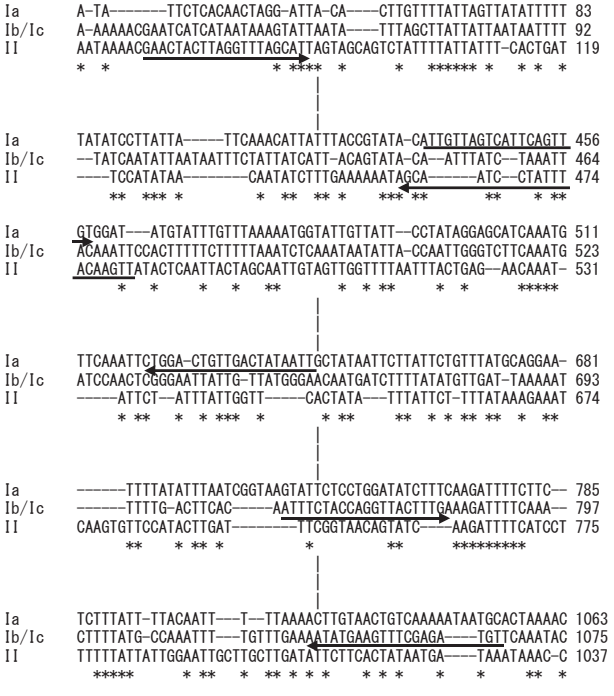

Fig. 1. Multiple sequence alignment of *wzy* genes in the serotype-specific region of *S. parauberis cps* loci. The primers used for the multiplex PCR and their directions were indicated under the sequences with arrows. Only the sequences where the primer sequences are included are shown. Numbers on the right represent the number of nucleotide from the start codon.

Results

Using the designed primer set, PCR products with the expected size specific for subserotypes Ia, Ib and Ic, and serotype II were obtained (Fig. 2). In all 188 *S. parauberis* isolates from the Japanese flounder, the

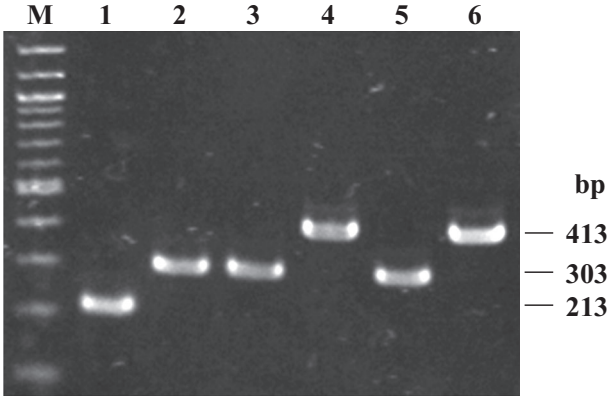

Fig. 2. Multiplex PCR products of *Streptococcus parauberis*. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed under UV light. M, 100-bp DNA ladder markers; 1, subserotype Ia; 2, subserotype Ib; 3, subserotype Ic; 4, serotype II; 5 and 6, nontypeable strains of clusters I and III in *sma*I-PFGE analysis, respectively.

Table 1. The results of the multiplex PCR assay for differentiation of the *Streptococcus parauberis* serotypes

Bacteria (no. of strain)	Primer pair for amplification		
	For/Rev-Ia	For/Rev-Ib/Ic	For/Rev-II
S. parauberis from flounder			
subserotype Ia (6)	+	–	–
subserotype Ib (91)	–	+	–
subserotype Ic (7)	–	+	–
serotype II (62)	–	–	+
NT/Cluster I[*1] (9)	–	+	–
NT/Cluster III[*1] (13)	–	–	+
S. parauberis from cow			
ATCC13386	–	–	–
Other species (11)[*2]	–	–	–

+, positive amplification; –, negative amplification; NT, nontypeable.

[*1] Clusters in *sma*I-PFGE analysis (Kanai *et al*., 2015).

[*2] *S. agalactiae* JCM5671, *S. thermophilus* JCM17834, *S. uberis* ATCC19436, *S. iniae* NUF631, *S. dysgalactiae* NUF1015, *Enterococcus faecalis* JCM5803, *Lactococcus lactis* subsp. *lactis* JCM5805, *L. garvieae* NUF699, *Edwardsiella tarda* NUF251, *Vibrio anguillarum* NUF652 and *Photobacterium damselae* subsp. *piscicida* NUF89.

results were consistent with those of agglutination test using rabbit antisera. The NT isolates yielded PCR products whose size was the same as subserotype Ib/Ic or serotype II. All the reference strains including the *S. parauberis* ATCC13386 derived from cow were negative in the PCR. The test results are summarized in Table 1.

Discussion

In streptococci, the PCR assays developed for serotype differentiation were focused on the *cps* locus, because in many cases the construction and nucleotide sequence of *cps* locus were different according to serotypes (Smith *et al*., 1999; Wang *et al*., 2011). In *S. parauberis,* three genes encoding initial glycosyltransferase, polysaccharide polymerase (Wzy) and flippase (Wzx) were present in the *cps* loci of all *S. parauberis* serotypes (Tu *et al*., 2015), indicating that these genes were essential for the construction of the capsular polysaccharides of *S. parauberis*. A multiple sequence alignment of these genes showed that the *wzy* gene was less similar among the serotypes than the other two genes. Accordingly, the primers for the multiplex PCR were designed from the *wzy* gene. However, the primer pair for differentiation between subserotypes Ib and Ic could not be designed, because the sequences of *cps* loci of these subserotypes were almost identical (Tu *et al*., 2015). The same case was found in *S. suis* (Liu *et al.,* 2013). Antisera may be required in such situation to aid distinction.

The NT isolates of *S. parauberis* which agglutinate with both serotypes I and II rabbit antisera are thought to be noncapsulated (Kanai *et al*., 2015). About one-third of them were derived from apparently healthy fish. We suspected that their *cps* loci were different from those of capsulated strains, but the sequencing of the *cps* loci revealed that the NT strains had almost the same sequences as subserotype Ib/Ic or serotype II (Tu *et al*., 2015). Accordingly, it is suggested that the NT isolates are originally subserotype Ib/Ic or serotype II *S. parauberis* and that their capsule production is suppressed by mutation in the gene(s) for capsule production. As expected, all of NT isolates belonging to cluster III of *sma*I-PFGE analysis, which consisted of serotype II isolates, were tetracycline-resistant like serotype II isolates (data not shown).

Although the multiplex PCR developed in this study could not differentiate subserotypes Ib and Ic, the other serotypes including the nontypeable type could be differentiated accurately and specifically, and no positive amplification was detected in the reference strains used. So, it would be a useful tool for diagnosis and serotyping of fish-pathogenic *S. parauberis*. And it might be able to apply this method for diagnosis using bacterial DNA directly extracted from the internal organs or legions of infected fish.

References

Baeck, G. W., J. H. Kim, D. K. Gomez and S. C. Park (2006): Isolation and characterization of *Streptococcus* sp. from diseased flounder (*Paralichthys olivaceus*) in Jeju Island. *J. Vet. Sci.*, **7**, 53–58.

Domeénech, A., J. F. Dernández-Garayzábal, C. Pascual, J. A. Garcia, M. T. Cutuli, M. A. Moreno, M. D. Collins and L. Dominguez (1996): Streptococcosis in cultured turbot, *Scopthalmus maximus* (L.), associated with *Streptococcus parauberis*. *J. Fish Dis.*, **19**, 33–38.

Kanai, K., M. Yamada, F. Meng, I. Takahashi, T. Nagano, H. Kawakami, A. Yamashita, S. Matsuoka, Y. Fukuda, Y. Miyoshi, I. Takami, H. Nakano, T. Hirae, K. Shutou and T. Honma (2009): Serological differentiation of *Streptococcus parauberis* strains isolated from cultured Japanese flounder in Japan. *Fish Pathol.*, **44**, 33–39. (In Japanese with English abstract)

Kanai, K., C. Tu, N. Katayama and K. Suga (2015): Existence of subserotypes in *Streptococcus parauberis* serotype I. *Fish Pathol.*, **50**, 75–80.

Liu, Z., H. Zheng, M. Gottschalk, X. Bai, R. Lan, S. Ji, H. Liu and J. Xu (2013): Development of multiplex PCR assays for the identification of the 33 serotypes of *Streptococcus suis*. *PLoS One.*, **8**, e72070.

Meng, F., K. Kanai and K. Yoshikoshi (2009): Structural characterization of Tn*916*-like element in *Streptococcus parauberis* serotype II strains isolated from diseased Japanese flounder. *Lett. Appl. Microbiol*., **48**, 770–776.

Smith, H. E., V. Veenbergen, J. van der Velde, M. Damman, H. J. Wisselink and M. A. Smits (1999): The *cps* genes of *Streptococcus suis* serotypes 1, 2, and 9: Development of rapid serotype-specific PCR assays. *J. Clin. Microbiol.*, **37**, 3146–3152.

Toranzo, A. E., B. Magariños and J. L. Romalde (2005): A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, **246**, 37–61.

Wang, K., W. Fan, H. Wisselink and C. Lu (2011): The *cps* locus of *Streptococcus suis* serotype 16: Development of a serotype-specific PCR assay. *Vet. Microbiol.*, **153**, 403–406.

Tu, C., K. Suga and K. Kanai (2015): Structure of genetic loci for capsular polysaccharide biosynthesis in *Streptococcus parauberis* isolated from Japanese flounder. *Fish Pathol.*, **50**, 192–199.