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Yamada, Ryoko ; Le Hong Thuy Tien ; Arai, Sakura ; Tohya, Mari ; Ishida-Kuroki, Kasumi ; Nomoto, Ryohei ; Kim, Hyunjung ; Suzuki, Eriko…

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FULL PAPER

Bacteriology

Development of PCR for identifying *Streptococcus parasuis*, a close relative of *Streptococcus suis*

Ryoko YAMADA[1,5], Le Hong Thuy TIEN[2], Sakura ARAI[1,6], Mari TOHYA[1,7], Kasumi ISHIDA-KUROKI[1], Ryohei NOMOTO[3], Hyunjung KIM[1], Eriko SUZUKI[1], Ro OSAWA[4], Takayasu WATANABE[1] and Tsutomu SEKIZAKI[1]*

[1]Research Center for Food Safety, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
[2]Department of Biotechnology, Nong Lam University, Quarter 6, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam
[3]Department of Infectious Diseases, Kobe Institute of Health, Minatojima-Nakamachi 4-6-5, Chuo-ku, Kobe, Hyogo 650-0045, Japan
[4]Department of Bioresource Sciences, Graduate School of Agricultural Sciences, Kobe University, Rokko-dai 1-1, Nada-ku, Kobe, Hyogo 657-8501, Japan
[5]Present address: Laboratory of Veterinary Ethology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
[6]Present address: Division of Microbiology, National Institute of Health Sciences, Tonomachi 3-25-26, Kawasaki-ku, Kawasaki, Kanagawa 210-9501, Japan
[7]Present address: Pathogenic Microbe Laboratory, Research Institute, National Center for Global Health and Medicine, Toyama 1-21-1, Shinjuku-ku, Tokyo 162-8655, Japan

ABSTRACT. *Streptococcus parasuis* has recently been removed taxonomically from *Streptococcus suis*, a zoonotic pathogen. *S. parasuis* has been detected in healthy pigs and in diseased pigs, which suggests that *S. parasuis* is involved in the normal microbiota of pigs and has potential pathogenicity. However, the pathogenicity of *S. parasuis* in pigs is unclear because of the lack of appropriate detection methods that discriminate *S. parasuis* from *S. suis*. In this study, we developed a PCR method that is specific for *S. parasuis*. The detection limit of the PCR was 350 CFU per reaction. Bacteria isolated from the saliva of eight pigs were collected and examined by PCR. Sixty-four isolates positive for PCR were obtained from the samples of all pigs. Thirteen of the 64 isolates were genetically confirmed as *S. parasuis*, and biologically and biochemically had nearly the same features of known *S. parasuis* strains, which suggested that strains positive for PCR were *S. parasuis*. Among the 64 isolates, 28 isolates were serotypes 20, 22, or 26 in the *S. suis* serotyping scheme. The remaining 36 isolates were untypeable, which suggested the presence of novel serotypes or a capsule-negative form. Therefore, the PCR method described in this study is a useful tool for identifying *S. parasuis*, and can be used in etiological studies on this bacterium.

KEY WORDS: PCR, pig saliva, *recN*, *Streptococcus parasuis*, *Streptococcus suis*

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Streptococcus suis is a zoonotic pathogen that causes inflammatory and invasive diseases in pigs and humans [8]. *Streptococcus suis* has diverse serotypes and genotypes, which has raised questions regarding its taxonomy and has led to a reclassification of several serotypes as novel species [17]. First, the *S. suis* reference strains of serotypes 32 and 34 are *Streptococcus orisratti* [10]. Second, *S. suis* serotypes 20, 22, and 26 were reclassified as *S. parasuis* [15, 17]. Third, it has recently been proposed that *S. suis* serotype 33 should be reclassified as *Streptococcus ruminantium* [18].

To date, *S. parasuis* has been isolated from healthy pigs and diseased pigs, and has characteristics similar to those of *S. suis*; however, it differs in enzymatic activity and acid production [7, 15, 19]. The presence of *S. parasuis* in diseased pigs with pneumonia or systemic infection (meningitis, arthritis, endocarditis or septicemia) [19] suggests it may have pathogenicity in pigs. However, *S. parasuis* is often isolated from healthy pigs, which has led to the notion that *S. parasuis* may be included in the normal microbiota of pigs. Since being proposed as a novel species [15], the biological and pathological features of *S. parasuis* have remained unclear because of the lack of a detection method specific for *S. parasuis*. In this study, we developed a novel

*Correspondence to: Sekizaki, T.: asekizak@mail.ecc.u-tokyo.ac.jp

polymerase chain reaction (PCR) method to specifically target *S. parasuis* and applied it to identify field isolates from healthy pigs.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains and isolates used in this study are listed in Table 1. *Streptococcus parasuis* strains and isolates were cultured in Todd–Hewitt (TH) agar (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) at 37°C under 5% carbon dioxide, when necessary, supplemented with *Streptococcus* Selective Supplement (Oxoid, Basingstoke, U.K.). The remaining bacterial strains were cultured, as described previously [11]. *S. parasuis* isolates were serotyped by using the coagglutination test, as described previously with commercial antisera (Statens Serum Institut, Copenhagen, Denmark) [2].

Sample collection

Saliva samples were collected from eight pigs: Six sows (age unknown) and 2 boars (104-month-old and 80-month-old) on two farms in Japan for the isolation of *S. parasuis*. Handmade applicators were formed by fixing one piece of cotton (5 × 10 cm) onto disposable wooden chopsticks. For collection of the saliva, the inner surface of the pigs' oral cavity was wiped with the handmade applicators for 2–3 min. Three applicators per pig were used. The applicators were placed in conical tubes, which were centrifuged at 10,000 *g* for 10 min at room temperature to collect the saliva.

DNA extraction

Genomic DNA from *S. parasuis* strains and isolates was extracted, using the procedure described in a previous report [14]. In brief, colonies of pure cultured *S. parasuis* on TH agar were suspended in saline–ethylenediaminetetra-acetic acid (EDTA) buffer (pH 8.0) consisting of 0.15 M sodium chloride and 0.1 M sodium EDTA. A 20-*µl* mixture consisting of 50 mg/m*l* lysozyme and 200 units/m*l* mutanolysin from *Streptomyces globisporus* ATCC 21553 (Sigma-Aldrich, St. Louis, MO, U.S.A.) was added and incubated for 1 hr at 37°C. Twenty percent (w/v) sodium dodecyl sulfate was added to the mixture and incubated for 10 min at 60°C. Five hundred microliters of phenol/chloroform/isoamyl alcohol (25:24:1) was then added and mixed well with inversion. The mixture was centrifuged at 6,300 *g* for 10 min. Five hundred microliters of phenol/chloroform/isoamyl alcohol (25:24:1) was then added to the collected supernatant and mixed slowly. The mixture was centrifuged under the aforementioned condition, and 1 m*l* of cold 99.5% (v/v) ethanol was added and mixed with inversion. The resulting precipitate was washed with 70% (v/v) ethanol and 99.5% (v/v) ethanol. After removing the ethanol, the resulting DNA was dried in a dryer (Spin dryer mini VC-15s; TAITEC, Saitama, Japan), and suspended with 100 *µl* of distilled water. The DNA solution was stored at −20°C until used. Genomic DNA from the remaining strains was extracted, as described previously [11]. The concentration of the extracted genomic DNA was measured using the Quantus fluorometer (Promega, Madison, WI, U.S.A.) with the QuantiFluor dsDNA System (Promega), based on the manufacturer's instructions.

Nucleotide sequence data analysis, primer design, and DNA sequencing

Nucleotide sequences of the recombination/repair protein-coding gene (*recN*) were retrieved from the National Center for Biotechnology Information (NCBI) GenBank (Bethesda, MD, U.S.A.). These sequences were aligned via the ClustalW program using GENETYX v13.0.4 processing software (Genetyx, Tokyo, Japan) with the default parameters. The primers for the PCR were searched to have no mismatch or one mismatch to all strains of the target species, and more than two mismatches to the other species. Specificity of nucleotide sequences of the designed primers was checked *in silico* by a BLASTN search against the NCBI Nucleotide Collection. The primers were also checked for dimers by using the Multiple Primer Analyzer (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

The genetic regions of partial 16S rRNA gene were amplified from the genomic DNA of 13 *S. parasuis* isolates using primers F1 and R13, as described previously [3]. The PCR products were purified with NucleoSpin Gel and a PCR clean-up kit (both by MACHEREY-NAGEL, Düren, Germany). The amplified sequences were determined using the following primers: Fow1 (5′-TGGCGGCGTGCCTAATACATGCA-3′), Rev1 (5′-ACCTTCCGATACGGCTACCTTGT-3′), 522F (5′-AAGGGACGGCTAACTACGTGCCA-3′), 522R (5′-TGGCACGTAGTTAGCCGTCCCTT-3′), 1104F (5′-AGATGTTGGGTTAAGTCCCGCAA-3′), and 1104R (5′-TTGCGGGACTTAACCCAACATCT-3′). Sequencing was achieved using the Big Dye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific). The sequence products were purified with ethanol/EDTA/sodium acetate precipitation, based on the manufacturer's instructions. The nucleotide sequences were determined using the ABI PRISM 3130 genetic analyzer (Thermo Fisher Scientific). These nucleotide sequences were deposited in DDBJ, EMBL, and GenBank under accession number LC144931-LC144943. The sequences were assembled with Sequencher v4.8 software (Hitachi Software Engineering, Yokohama, Japan) and aligned with GENETYX (Genetyx) using the default parameters. The resulting sequences (1,276 bp) were used for later analysis. Molecular Evolutionary Genetics Analysis 7 software (Center for Evolutionary Medicine and Informatics, Tempe, AZ, U.S.A.) [12] was used for the estimation and visualization of a phylogenetic tree with the neighbor-joining method. The tree construction was estimated with 1,000 bootstrap replicates.

PCR conditions

PCR was performed using a total volume of 25 *µl* containing 1–5 *µl* of a DNA template, 0.4 *µ*M of each primer, 12.5 *µl* of Quick Taq HS DyeMix (Toyobo, Osaka, Japan), and 6.5–10.5 *µl* of distilled water. The T100 thermal cycler (Bio-Rad Laboratories,

Table 1. Bacterial strains and isolates used in this study

Species	Strain and isolate	*Streptococcus parasuis* serotype and its close relatives	Source or reference
Streptococcus parasuis	SUT-286^T	20	[15]
	86-5192	20	[6, 15]
	SUT-443	20	This study
	88-1861	22	[6, 15]
	SUT-380	22	[15]
	SUT-458, SUT-516, SUT-523	22	This study
	89-4109-1	26	[5, 15]
	SUT-503, SUT-529	26	This study
	SUT-7	22/26	[15]
	SUT-319	20/22	[15]
	SUT-328	20/22	[15]
	SUT-447, SUT-462, SUT-479, SUT-481, SUT-483, SUT-488, SUT-507	a	This study
Streptococcus suis	NCTC 10237	1	[16]
	NCTC 10234^T	2	[16]
	4961	3	[16]
	6407	4	[16]
	11538	5	[16]
	2524	6	[16]
	8074	7	[16]
	14636	8	[16]
	22083	9	[6]
	4417	10	[6]
	12814	11	[6]
	8830	12	[6]
	10581	13	[6]
	13730	14	[6]
	NCTC 10446	15	[6]
	2726	16	[6]
	93A	17	[6]
	NT77	18	[6]
	42A	19	[6]
	14A	21	[6]
	89-2479	23	[5]
	88-5299A	24	[5]
	89-3576-3	25	[5]
	89-5259	27	[5]
	89-590	28	[5]
	92-1191	29	[9]
	92-1400	30	[9]
	92-4172	31	[9]
	2651	1/2	[16]
Streptococcus orisratti	EA1172.91	32	[9, 10]
	92-2742	34	[9, 10]
Streptococcus ruminantium	EA1832.92	33	[9, 18]
Streptococcus acidominimus	ATCC 51725^T	b	c
Streptococcus dysgalactiae subsp. *equisimilis*	ATCC 35666	b	[1]
Streptococcus entericus	JCM 12180^T	b	[11]
Streptococcus gallinaceus	JCM 12181^T	b	d
Streptococcus minor	CCUG 47487^T	b	e
Streptococcus oralis	JCM 12997^T	b	[11]
Streptococcus ovis	CCUG 39485^T	b	e
Streptococcus pluranimalium	FKI 2012	b	[11]
Streptococcus plurextorum	CECT 7308^T	b	[11]
Streptococcus porci	CECT 7374^T	b	[11]
Streptococcus porcinus	ATCC 43138^T	b	[11]
Streptococcus pyogenes	ATCC 12344^T	b	[11]

Table 1. Continue

Species	Strain and isolate	*Streptococcus parasuis* serotype and its close relatives	Source or reference
Actinobacillus pleuropneumoniae	FBPM-460	b	[11]
Bordetella bronchiseptica	FBPM-462	b	[11]
Brachyspira hyodysenteriae	ATCC 27164^T	b	[11]
Erysipelothrix rhusiopathiae	Fujisawa	b	[11]
Erysipelothrix tonsillarum	ATCC 43339^T	b	[11]
Escherichia coli	MC-1	b	[11]
Haemophilus parasuis	FBPM-463	b	[11]
Mycoplasma hyopneumoniae	J^T	b	[11]
Mycoplasma hyorhinis	BTS-7^T	b	[11]
Mycoplasma hyosynoviae	S16^T	b	[11]
Salmonella enterica subsp. *enterica* serovar Choleraesuis	FBPM-477	b	[11]
Staphylococcus hyicus	FBPM-464	b	[11]

a) Untypeable. b) Not applicable. c) Purchased from American Type Culture Collection (Manassas, VA, U.S.A.). d) Purchased from RIKEN BioResource Center via the National Bio-Resource Project of MEST (Tokyo, Japan). e) Y. Kawamura, School of Pharmacy, Aichi Gakuin University (Aichi, Japan).

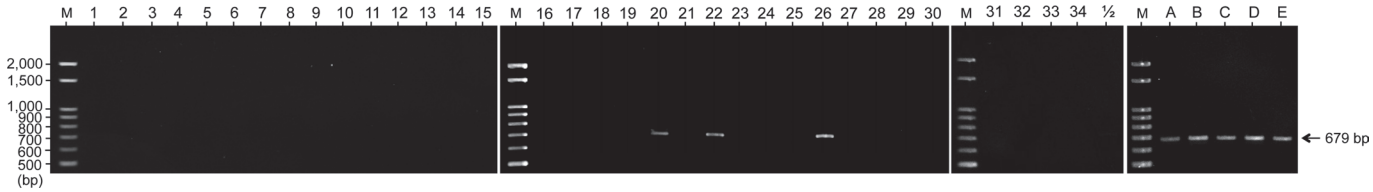

Fig. 1. The PCR products. Lanes 1–19, 21, 23–25, 27–31 and 1/2 contain *S. suis* reference strains with serotypes 1–19, 21, 23–25, 27–31 and 1/2, respectively. Lanes 20, 22 and 26 contain *S. parasuis*. Lanes 32 and 34 contain *S. orisratti*. Lane 33 contains *S. ruminantium*. Lanes A–E contain *S. parasuis* strains (A: SUT-286^T; B: SUT-7; C: SUT-319; D: SUT-328; E: SUT–380). Lane M is the molecular size marker (100-bp ladder; Wako, Osaka, Japan).

Hercules, CA, U.S.A.) or the My Cycler thermal cycler 580BR 10803 (Bio-Rad) was used for amplification. The PCR program consisted of an incubation for 2 min at 94°C, 30 cycles of 30 sec at 94°C, 10 sec at 54°C, and 1 min at 68°C, and a final extension for 10 min at 68°C. The PCR products were analyzed by 2.0 or 2.5% (w/v) agarose gel electrophoresis in tris-phosphate EDTA buffer at 100 V for 25–50 min. The gel was stained with GelRed (Biotium, Fremont, CA, U.S.A.) for 15 min, and then photographed on an ultraviolet illuminator. The sizes of the PCR products were compared with the Gene Ladder 100 (Wako, Osaka, Japan) as the molecular size standard.

Specificity and sensitivity of PCR

The specificity of PCR was evaluated by using 10 *p*g of the genomic DNA of bacteria listed in Table 1 as the template. The sensitivity of PCR was evaluated by using 10-fold serial dilutions of a DNA extract from a culture of *S. parasuis* reference strain SUT-286^T at a titer of 7.5×10^8 CFU/m*l*. Genomic DNA from the culture (500 μ*l*) was prepared, as described previously [11]. Five microliters of the DNA extract was tested.

Isolation and characterization of S. parasuis

Ten microliters of pig saliva collected by a cotton applicator was centrifuged and the pellet was spread on TH agar supplemented with *Streptococcus* Selective Supplement (Oxoid). Colonies visually similar to those of *S. parasuis* were selected and purified by single colony isolation three times under the same culture condition without the selective supplement. Phenotypic and biochemical features were examined, as described previously [15].

RESULTS

Specificity and sensitivity of PCR

A forward primer (5′-CAACTGCTGGATAGTTTCGG-3′) and reverse primer (5′-GTCTGGCTGAGCTTAATTGG-3′) were designed for the PCR to amplify a 679-bp fragment of *recN*. The PCR was positive for all eight strains of *S. parasuis* and negative

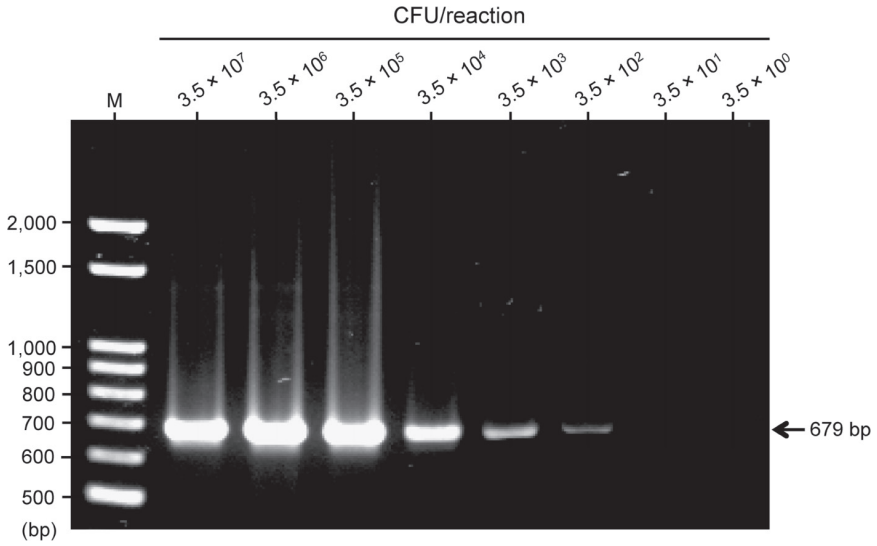

Fig. 2. The PCR products from 10-fold serial dilutions of the *S. parasuis* SUT-286^T genomic DNA containing the indicated number of CFU. M, molecular size marker (100-bp ladder; Wako, Osaka, Japan).

for *S. suis*, *S. orisratti*, *S. ruminantium* (Fig. 1) and other bacterial species (data not shown). Among the 10-fold serial dilutions of *S. parasuis* SUT-286^T genomic DNA, the detection limit of the PCR was 350 CFU per reaction (Fig. 2).

Streptococcus parasuis isolates from pig saliva

From eight pig saliva samples, 223 isolates (range, 16–56 isolates per pig) were collected and examined by PCR. The isolates positive for the PCR were obtained from all samples examined. Sixty-four isolates (3.6–79.0% in the isolates from each pig) were positive for PCR. Of these 64 isolates, two isolates were serotype 20; 23 isolates, serotype 22; and three isolates, serotype 26. However, the remaining 36 isolates were untypeable. Among them, 13 representative isolates listed in Table 1 were selected using the criteria that isolates from all pigs were included and the isolates contained all serotype variations in each pig. A phylogenetic tree was inferred from the nucleotide sequences of the *16S rRNA* gene for 13 isolates and other streptococcal species. In the phylogenetic tree, 13 isolates were within the same cluster of the *S. parasuis* strains (Fig. 3). The phenotypic features of the 13 isolates such as morphology in gram staining and enzymatic activities were very similar to those previously described [15]. However, the biochemical features of the 13 isolates, compared to those reported for *S. parasuis* SUT-286^T [15], differed in the following features: hydrolysis of arginine; acid production from cyclodextrin, melibiose, and trehalose; detection of esterase lipase (C8), and the activity of cystine arylamidase, valine arylamidase, α-glucosidase, and acid phosphatase.

DISCUSSION

Based on the nucleotide sequence comparison of *recN* with *S. parasuis* and its close relatives, we were able to design PCR primers specific for *S. parasuis*. The *recN* gene has a low degree of sequence similarity with *Streptococcus* species, compared to other housekeeping genes [4, 17]. In fact, the PCR was positive for only *S. parasuis*, including eight strains and 64 field isolates, whereas it was negative for 29 *S. suis* types and reference strains; two *S. orisratti* strains; one *S. ruminantium* strain; other streptococcal species, including taxonomically close relatives, based on the *recN* sequence [4, 18], and some pig pathogens. Furthermore, the field isolates obtained from the saliva of pigs had similar characteristics. The phylogenetic tree based on the *16S rRNA* gene showed that these isolates were involved in the same cluster as the *S. parasuis* strains. Biological and biochemical features of the isolates were nearly the same as those reported for *S. parasuis* [15], which indicated that the isolates were *S. parasuis*. These results suggested that the PCR method described in this study was highly specific for *S. parasuis*, and could be used to identify field isolates.

To date, the *S. suis* strains have been typed into 35 serotypes (i.e., serotypes 1–34 and serotype 1/2), based on their capsular polysaccharide antigenicity [5, 6, 9]. However, the classification of serotypes 20, 22, 26 and 32–34 were reevaluated. Among these, bacteria including the *S. suis* reference strains of serotypes 20, 22 and 26 have been reclassified as *S. parasuis*.

The *S. suis* reference strains of serotypes 20, 22 and 26 have been isolated from diseased calves and pigs [5, 6]; however, previous studies [13, 15] and our present study showed that clinically healthy pigs usually carry *S. parasuis* in their saliva. This evidence suggests that *S. parasuis* can be concomitantly isolated from diseased pigs and the bacterium itself has a low degree of virulence.

Precise bacterial identification using the PCR method described in this study, in combination with previously reported PCR detecting authentic *S. suis* [11], will be a clue in identifying and characterizing the pathogenicity of the *S. parasuis* field isolates.

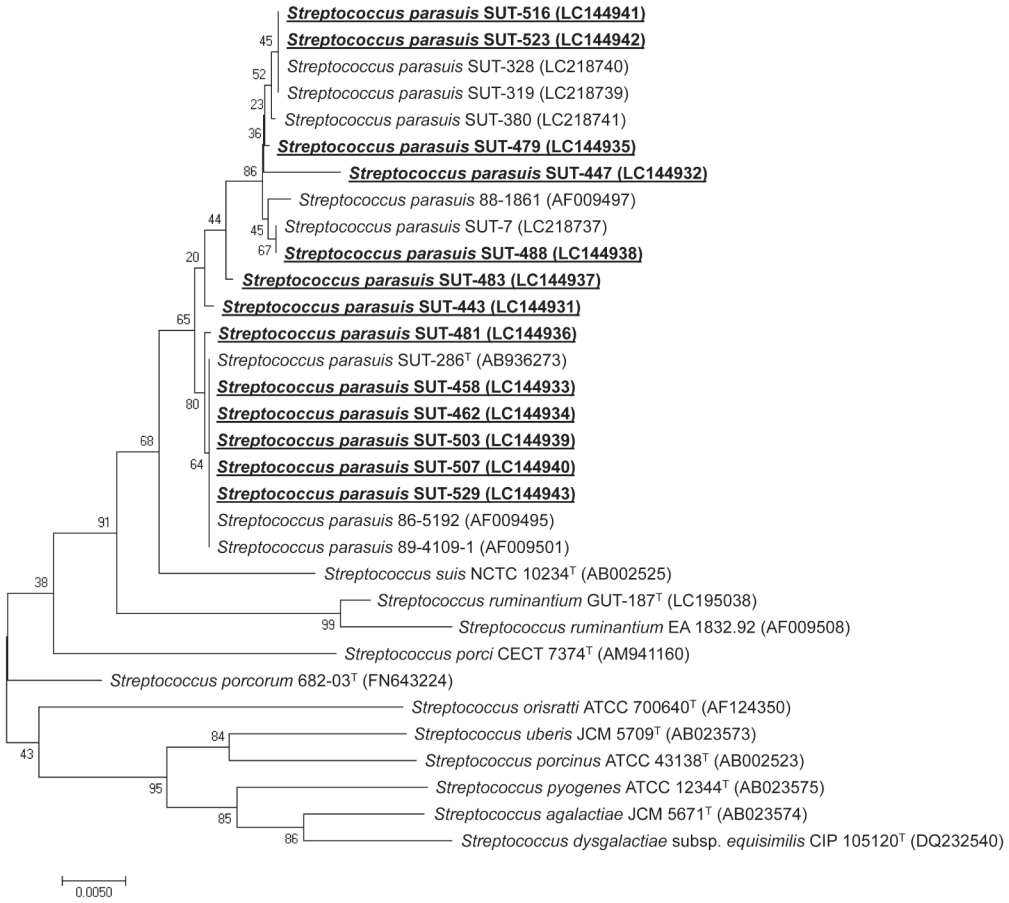

Fig. 3. Phylogenetic tree inferred from the *16S rRNA* gene sequence comparisons using the neighbor-joining method. It shows the relationships between the isolates positive for PCR (underlined and bold) and other streptococcal species. Accession numbers in the DDBJ/EMBL/GenBank are indicated in parentheses.

However, the specificity of PCR has been assessed using a limited number of known bacterial strains and species. Therefore, we recommend a presumptive identification of the test materials before performing PCR, which would include establishing a pure culture and performing gram staining, catalase testing, and oxidase testing.

In this study, more than one-half of the *S. parasuis* isolates from pig saliva were untypeable under the *S. suis* serotyping schema. Untypeable *S. suis* isolates from diseased pigs are either novel serotypes or capsule-negative forms because serotypes are determined by their capsular polysaccharides [20]. Since the PCR method described in this study can specifically detect untypeable *S. parasuis*, it enables collecting *S. parasuis* field isolates, which will confirm the presence of novel serotypes or a capsule-negative form of *S. parasuis*, and also be useful to examine the unknown bacterial features, such as pathogenicity.

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