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FULL PAPER

Bacteriology

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

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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*

*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
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/ml lysozyme and 200 units/ml mutanolysin from *Streptomyces globisporus* ATCC 21153 (Sigma-Aldrich, St. Louis, MO, U.S.A.) was added and incubated at 10°C for 1 hr. Twenty percent (v/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 10,000 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 ml 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 Quatus 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 recombinant/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: Fowl 1 (5′-TGGCGGGCTGACTAACTACATGCA-3′), Rev1 (5′-AAGGACGGCTAACTACGTGCCA-3′), 522F (5′-CCACGCATGTTAGTCGCTCC-3′), 1104F (5′-AGATGTTGGTGAATCCGCAA-3′), and 1104R (5′-TTGCGGGACCTTACACCCACATCT-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 µM 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,
| Species                          | Strain and isolate | Streptococcus parasuis serotype and its close relatives | Source or reference |
|---------------------------------|--------------------|--------------------------------------------------------|--------------------|
| *Streptococcus parasuis*        | SUT-286T           | 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 10234T        | 2                                                      | [16]               |
|                                 | 4961               | 3                                                      | [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 51725T        | b                                                      | e                  |
| *Streptococcus dysgalactiae subsp. equisimilis* | ATCC 35666 | b                                                      | [1]                |
| *Streptococcus entericus*       | JCM 12180T         | b                                                      | [11]               |
| *Streptococcus gallinaceus*     | JCM 12181T         | b                                                      | d                  |
| *Streptococcus minor*           | CCUG 47487T        | b                                                      | e                  |
| *Streptococcus oralis*          | JCM 12997T         | b                                                      | [11]               |
| *Streptococcus ovis*            | CCUG 39485T        | b                                                      | e                  |
| *Streptococcus pluranimalium*   | FKI 2012           | b                                                      | [11]               |
| *Streptococcus plurextorum*     | CECT 7308T         | b                                                      | [11]               |
| *Streptococcus porci*           | CECT 7374T         | b                                                      | [11]               |
| *Streptococcus porcinus*        | ATCC 45138T        | b                                                      | [11]               |
| *Streptococcus pyogenes*        | ATCC 12344T        | b                                                      | [11]               |
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 pg of the genomic DNA of bacteria listed in Table 1 as the template. 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.

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'-CAACTGCTGGATAGTTTTCGG-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
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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-286T 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-286T [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. 2. The PCR products from 10-fold serial dilutions of the S. parasuis SUT-286T genomic DNA containing the indicated number of CFU. M, molecular size marker (100-bp ladder; Wako, Osaka, Japan).
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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