

Assessment of pig saliva as a *Streptococcus suis* reservoir and potential source of infection on farms by use of a novel quantitative polymerase chain reaction assay

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Streptococcus suis, a gram-positive coccus, is a major pathogen of pigs that causes various diseases such as septicemia, meningitis, and endocarditis,¹ which result in marked economic losses for the swine industry. In addition, *S suis* is an emerging pathogen for humans because life-threatening or fatal *S suis* infections have been reported in people engaged in slaughtering pigs and working in the pork industry.^{2,3} However, *S suis* inhabits several sites (eg, nasal cavities, tonsils, and genital and digestive tracts) of pigs without causing disease.¹ It sometimes exerts

ABBREVIATIONS

qPCR Quantitative PCR
recN Recombination-repair protein-coding gene

OBJECTIVE

To evaluate colonization of *Streptococcus suis* and *Streptococcus parasuis* on pig farms in Japan and to identify sources of infections.

SAMPLE

Saliva, feces, and vaginal swab samples from 84 healthy pigs of several growth stages on 4 farms and swab samples of feed troughs and water dispensers at those farms.

PROCEDURES

Samples were collected from August 2015 to June 2016. Two quantitative PCR (qPCR) assays (one for *S suis* and the other for *S parasuis*) were designed for use in the study. The novel qPCR assays were used in combination with previously described qPCR assays for *S suis* serotype 2 or 1/2 and total bacteria. Relative abundance of bacteria in each sample was evaluated.

RESULTS

Streptococcus suis was detected in all saliva samples and some of the other samples, whereas *S parasuis* was not detected in any of the samples, including saliva samples, which indicated a difference in colonization preference. The ratio of *S suis* to total bacteria in saliva appeared to increase with age of pigs. *Streptococcus suis* serotype 2 or 1/2 was detected in a few saliva samples and feed trough swab samples at 2 farms where *S suis* infections were prevalent.

CONCLUSIONS AND CLINICAL RELEVANCE

Saliva, especially that of sows, appeared to be a reservoir and source of *S suis* infection for pigs. The qPCR assay described here may provide an effective way to monitor for *S suis* in live pigs, which could lead to effective disease control on pig farms. (*Am J Vet Res* 2018;79:941–948)

its pathogenic effects on the host. Certain serotypes of *S suis* (eg, serotypes 2, 1/2, 3, 7, 9, and 14) are frequently detected in diseased pigs in North and South America, Europe, and Asia. In particular, *S suis* serotype 2 is the most prevalent and highly virulent among all serotypes.¹

Streptococcus suis may colonize pigs through vertical transmission during parturition⁴ or through horizontal transmission by aerosolization.¹ Experimental exposure to airborne *S suis* causes infection.⁵ It is also possible that *S suis* can infect susceptible hosts through feces excreted by infected pigs, similar to the manner by which infection with the hepatitis E virus is spread among pigs through the fecal-oral route.⁶ Previous studies^{7–9} were conducted primar-

ily to isolate *S suis* from the organs of healthy or diseased pigs. However, the main source of infection has yet to be elucidated.

Specially designed primers for PCR assays and loop-mediated isothermal amplification procedures have been used to detect *S suis*.^{10,11} These methods do not detect bacterial taxa that should be removed from the *S suis* classification. Such taxa include *Streptococcus parasuis*, *Streptococcus orisratti*, and *Streptococcus ruminantium*. Recently, *S parasuis* (formerly recognized as *S suis* serotypes 20, 22, and 26) was reclassified as a new species and isolated from healthy and diseased pigs.¹² *Streptococcus orisratti* includes serotypes formerly classified as *S suis* serotypes 32 and 34.¹³ In addition, *S suis* serotype 33 was reclassified recently as *S ruminantium*.¹⁴ Among these closely related bacteria, the etiologic importance and colonization preference of *S parasuis* remain unknown, compared with the information known for *S suis*.^{12,15} Several qPCR assays have been used to detect specific pig-related bacteria such as enterobacteria, lactobacilli, *Leptospira interrogans*, and *Bifidobacterium* spp.^{16–18} These qPCR assays have been useful for monitoring microbial shifts and have been applied for the high-throughput detection of multiple bacterial species.¹⁹ Studies^{20–22} have provided descriptions of the design of qPCR assay primers for *S suis*, although the taxa that should be removed from the *S suis* classification are inevitably also detected by use of these qPCR assays.

The purpose of the study reported here was to create a novel qPCR assay to detect and quantify *S suis* in pigs and their living environment. A novel qPCR assay was designed to detect *S suis* but not bacterial taxa that formerly belonged to the *S suis* classification. Subsequently, the qPCR assay was used to compare the abundance of *S suis* between piglets and sows, among piglets of various growth stages, and among farms and to estimate the ratio of the number of *S suis* to the number of total bacteria. In addition, quantification of *S suis* serotype 2 or 1/2 and *S parasuis* was used to compare colonization preferences.

Materials and Methods

Sample

From August 2015 to June 2016, samples of saliva and feces and vaginal swab specimens (ie, body samples) were collected from sows and piglets on 4 farms in Japan (**Figure 1**). Swab specimens of the feed troughs and water dispensers (ie, environmental samples) at those 4 farms were also collected. Owner consent was provided for use of the animals and collection of samples. All experiments conformed to the Guidelines for Animal Experiments of the University of Tokyo and were approved by the Animal Research Committee of the University of Tokyo (No. P16-289).

Piglets were categorized into 3 growth stages for the period from birth (day 0) to day 70: suckling piglets, postweaning piglets, and growing piglets (**Figure 1**). Ages of piglets in the various stages differed

among the 4 farms. For example, 2 farms included a category of growing piglets, whereas the other 2 farms only had categories for suckling and postweaning piglets. Use of antimicrobials also differed among the 4 farms. Antimicrobials were not used for treating diseases but were added in the feed at all farms enrolled in the study, except for 1 farm (pigs at that farm did not receive any feed containing antimicrobials). All pigs appeared to be healthy; none of the animals had signs of disease.

Sample collection

Saliva samples were collected with handmade applicators. A piece of cotton was affixed with twine onto disposable wooden chopsticks, and the handmade applicators were sterilized in a pouch. Each handmade applicator was removed from the sterilization pouch immediately before sample collection. Investigators held onto the distal 5 cm of the end of the applicator opposite the cotton. The applicator was wiped on the inner surface of the oral cavity of a pig for 2 to 3 minutes to collect saliva. Three applicators were used for each pig.

A commercially available swab^a was used for collecting swab samples from the vagina of pigs as well as feed troughs and water dispensers. Approximately 1 g of feces was collected from the rectum of each pig by use of a spoon.^b All samples were immersed in a storage reagent^c in conical tubes to prevent bacterial growth and degradation of DNA.^{23,24} Tubes were stored at -20°C until use.

Bacterial strains and culture conditions

Bacteria used in the study included 45 strains of *S suis*, 5 strains of *S parasuis*, 3 strains of *S orisratti*, 2 strains of *S ruminantium*, 2 strains of *Escherichia coli*, and 1 strain each of *Streptococcus acidominimus*, *Streptococcus dysgalactiae* subsp *equisimilis*, *Streptococcus entericus*, *Streptococcus gallinae*, *Streptococcus minor*, *Streptococcus oralis*, *Streptococcus ovis*, *Streptococcus pluranimalium*, *Streptococcus plurextorum*, *Streptococcus porci*, *Streptococcus porcicus*, *Streptococcus pyogenes*, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Brachyspira hyodysenteriae*, *Erysipelothrix rhusiopathiae*, *Erysipelothrix tonsillarum*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Mycoplasma hyosynoviae*, *Salmonella enterica* subsp *enterica* serovar Choleraesuis, and *Staphylococcus hyicus* (**Supplementary Table S1**, available at avmajournals.avma.org/doi/suppl/10.2460/ajvr.79.9.941). All strains were part of our laboratory stock, purchased from culture collections, or provided by other researchers. Bacteria were cultured as described previously.¹¹ *Streptococcus suis* strains were genotyped by use of a PCR assay for 16S rRNA genes of 35 serotypes²⁵ and *recN*.¹⁰ This procedure was followed by serotyping with a coagglutination test and a direct agglutination test with commercial antisera.^{26,d}

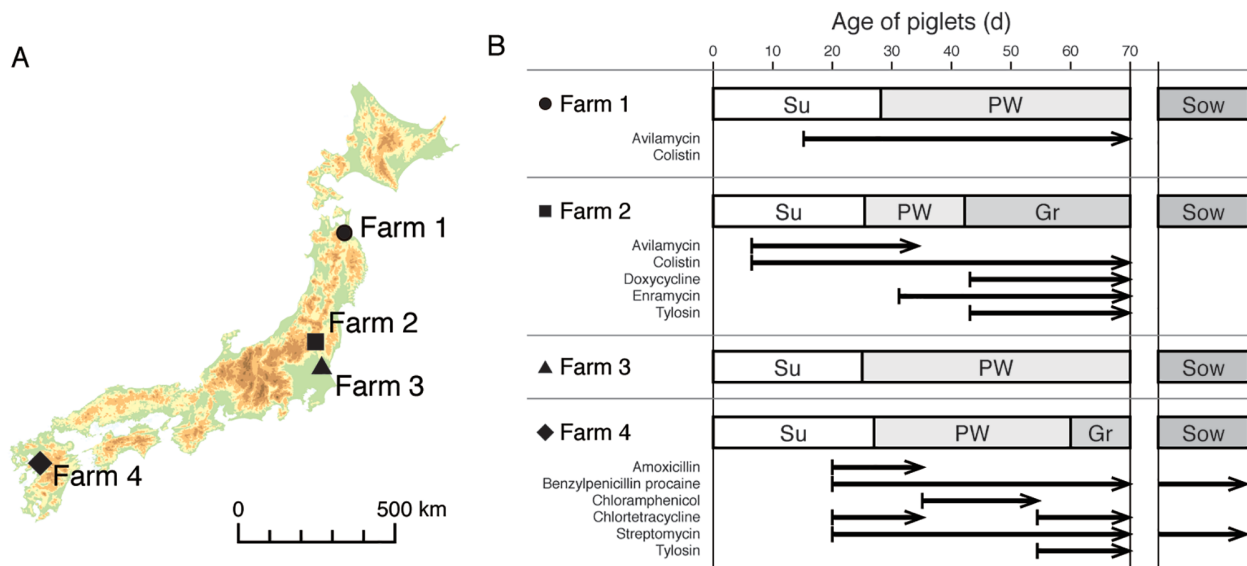


Figure 1—Location of the 4 pig farms in Japan included in the study (A), and the description of the pigs and antimicrobials used at each farm (B). The map in panel A was publicly available from the Geospatial Information Authority of Japan. In panel B, the various growth stages of the piglets are indicated. Notice the time course for administration of antimicrobials (arrows). Gr = Growing piglets. PW = Postweaning piglets. Su = Suckling piglets.

Extraction of DNA

Frozen samples were thawed and centrifuged at 20,630 X g for 10 minutes at 4°C. The pellet in each tube was washed twice with sterile saline (0.85% NaCl) solution and then resuspended in 1 mL of the saline solution. An aliquot (800 µL) was centrifuged again; that pellet was resuspended in 350 µL of solution from a commercially available kit^c and used for the extraction of total DNA in accordance with the manufacturer's instructions, with a few modifications. Beads provided in the kit were not used. Instead, 400 µL of 0.5-mm-diameter zirconia beads^f and two 5-mm-diameter zirconia beads^f was used to crush bacterial cells. Bacteria were crushed with a homogenizer^g at 3,200 rpm for 10 minutes. Total DNA was eluted in 100 µL of the elution buffer provided in the kit^c and stored at -20°C until use. The concentration of DNA was determined by use of a fluorometer^h with reagents.ⁱ Quality of DNA was verified by measuring the ratio of the absorbances at 260 and 280 nm with a spectrophotometer.^j Samples with a ratio of 1.8 to 2.0 were subsequently used for qPCR assays.

Genomic DNA from the bacterial strains used in the study was extracted by use of a previously described method²⁷ for *Streptococcus* spp or a boiling method¹¹ for other bacterial species. Concentration of the extracted genomic DNA was measured with a fluorometer.^h

Primer design and PCR assay conditions

The qPCR assay for total bacteria (designated as the qPCR_{TB} assay) and the qPCR assay for *S suis* serotype 2 or 1/2 (designated as the qPCR_{2j} assay) have been described elsewhere,^{28,29} whereas primers and probes of 2 qPCR assays (a qPCR assay for *S suis*

[designated as the qPCR_{SS} assay] and a qPCR assay for *S parasuis* [designated as the qPCR_{SP} assay]) were designed for the present study (**Appendix**). Nucleotide sequences of *recN* in the bacterial species used in the study were retrieved from the National Center for Biotechnology Information GenBank. These sequences were aligned by use of a commercially available software program^k with the default parameters. Nucleotide sequences spanning 80 to 150 bp were searched for primers corresponding to the appropriate loci. Specificity of the designed primers was confirmed in silico by conducting a search^l of the nucleotide collection of the National Center for Biotechnology Information.

Probes were labeled with a fluorescent reporter dye^m at the 5' end, an internal quencher dyeⁿ in the middle, and a dark quencher dye^o at the 3' end. Each qPCR assay was performed in a total volume of 20 µL, which contained 2 µL of DNA template, 0.4 µM each primer (0.2 µM each primer for the qPCR_{TB} assay), 0.2 µM probe, 1X reference dye,^p and 1X qPCR assay buffer-enzyme mixture.^q Melting temperatures were estimated with the calculator provided by open-source software.^r The qPCR assay conditions were 1 minute at 95°C, which was followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A thermal cycler^s was used for amplification. Distilled water^t was the template for the negative control sample. All reactions were performed in triplicate.

Evaluation of the specificity and sensitivity of qPCR assays

Specificity of the qPCR_{SS} and qPCR_{SP} assays was evaluated by use of 0.2 ng of the genomic DNA of the bacteria as the template. Sensitivity of the qPCR_{SS} and

Table 1—Results of a qPCR assay for *Streptococcus suis* (designated as the qPCR_{SS} assay) for samples collected from pigs and the environment on 4 pig farms in Japan.

Category	Sample	Growth stage	Farm 1	Farm 2	Farm 3	Farm 4	
Body samples	Saliva	Suckling piglets	9/9	9/9	5/5	15/15	
		Postweaning piglets	9/9	9/9	NC	NC	
		Growing piglets	NC	9/9	NC	NC	
	Feces	Sows	6/6	3/3	5/5	3/3	
		Vaginal swab specimens	Sows	3/6	0/3	0/1	NC
		Feed trough swab specimens	Sows	NC	NC	3/6	NC
			Postweaning piglets	3/3	3/3	NC	NC
Environmental samples	Feed trough swab specimens	Growing piglets	NC	3/3	NC	NC	
		Sows	3/6	2/3	1/1	NC	
		Water dispenser swab specimens	Postweaning piglets	2/3	3/3	NC	NC
	Water dispenser swab specimens	Growing piglets	NC	3/3	NC	NC	
		Sows	3/6	0/3	1/1	NC	

Values reported represent the number of samples with a positive result for the qPCR_{SS} assay/number of samples collected. NC = Not collected.

qPCR_{SP} assays was evaluated by use of 10-fold serial dilutions of genomic DNA of *S suis* P1/7 and *S parasuis* SUT-286^T, respectively, as the template.

Estimation of bacterial cell numbers

Ten-fold serial dilutions of genomic DNA of the following bacterial strains were used for each qPCR assay: *E coli* ATCC 25922 for the qPCR_{TB} assay, *S suis* P1/7 for the qPCR_{SS} and qPCR_{2j} assays, and *S parasuis* SUT-286^T for the qPCR_{SP} assay. For the standard curve, data obtained for the qPCR assays by use of the serial dilution templates were plotted against cell numbers, which were calculated from the DNA concentrations and the length (number of base pairs) of each genome, as described previously.³⁰ Cell numbers of the target species were estimated from the standard curve. For the qPCR_{TB} assay, cell numbers were estimated by use of *E coli*; therefore, we converted the number of *E coli* into the number of total bacteria as follows: number of total bacteria = number of *E coli* × 7/4.1858. The values 7 and 4.1858 represent the mean copy numbers of the 16S rRNA gene of *E coli* and total bacteria, respectively. The mean copy number for total bacteria was estimated from 1,690 publicly available complete bacterial genomes.³¹ Scatterplots were created by use of open-source software⁴⁴ and evaluated to determine the estimated cell number of *S suis*, *S suis* serotype 2 or 1/2, and *S parasuis* and their ratios to the number of total bacteria.

Results

Sample

Body samples were collected from 84 suckling piglets, postweaning piglets, growing piglets, and sows (Table 1). Saliva samples were collected from piglets and sows at all farms. Fecal samples were collected only from sows and only at 3 farms. Vaginal swab specimens were collected only from sows at 1 farm. Swab specimens were collected from feed troughs and water dispensers of growing piglets at 1 farm, postweaning piglets at 2 farms, and sows at 3 farms.

Specificity and sensitivity of the designed qPCR assays

Primers and probe of the qPCR_{SS} assay had no or only 1 mismatch with *S suis* and had ≥ 4 mismatches with the other species (Supplementary Table S2, available at avmajournals.avma.org/doi/suppl/10.2460/ajvr.79.9.941; Supplementary Figure S1, available at avmajournals.avma.org/doi/suppl/10.2460/ajvr.79.9.941). However, primers and probe of qPCR_{SP} had no mismatches with *S parasuis* and ≥ 8 mismatches with the other species (Supplementary Table S3; available at avmajournals.avma.org/doi/suppl/10.2460/ajvr.79.9.941). The qPCR_{SS} and qPCR_{SP} assays with the designed primers and probes amplified the desired regions of only the appropriate species (Supplementary Table S1). The detection limits of the qPCR_{SS} and qPCR_{SP} assays were 45 and 6.8 cells, respectively, in the 20-μL reaction.

Estimated bacterial cell numbers in samples

Body and environmental samples were evaluated by use of the qPCR assays. Bacterial cell numbers estimated by use of the qPCR_{TB} assay were > 1 × 10⁶ cells/reaction tube for all samples collected (Supplementary Figure S2, available at avmajournals.avma.org/doi/suppl/10.2460/ajvr.79.9.941). The qPCR_{SS} assay yielded positive results for all saliva samples but negative results for some of the other samples (Table 1). The qPCR_{SP} assay yielded negative results for some body and environmental samples. The qPCR_{2j} assay yielded positive results for 3 saliva samples and 2 swab specimens of feed troughs from 2 farms where *S suis* infections were prevalent.

Cell numbers estimated by use of the qPCR_{TB} assay were in the range of the log₁₀ value of 3 (Supplementary Figure S2). There was no apparent difference in the range of cell numbers among farms as estimated by use of each qPCR assay. Cell numbers in saliva samples estimated by use of the qPCR_{SS} assay apparently did not change with increasing age, whereas cell numbers in saliva samples estimated by

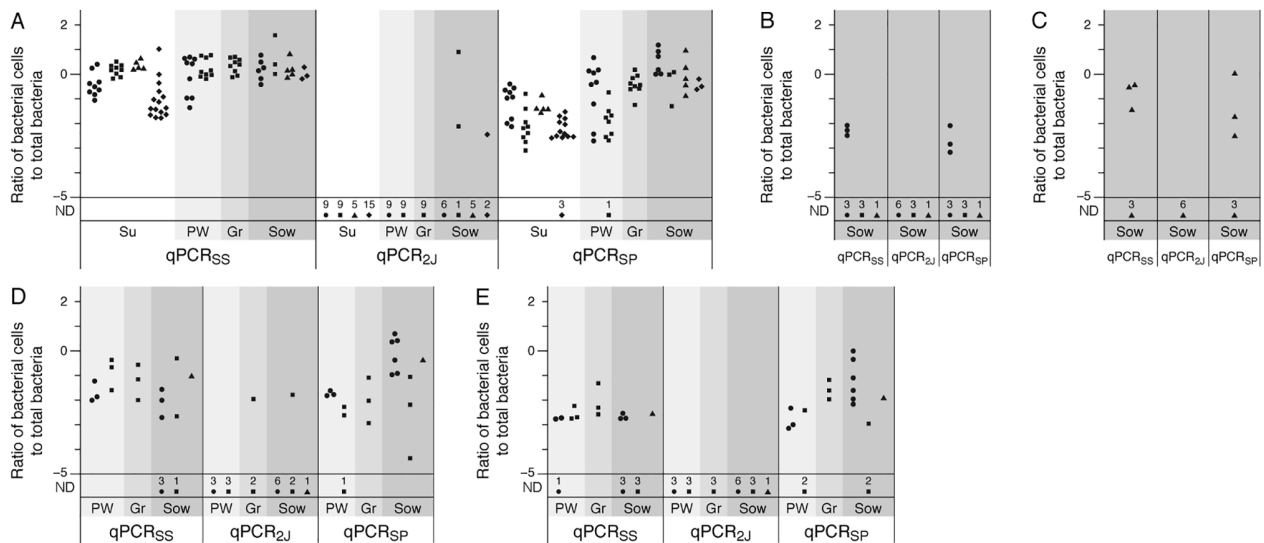


Figure 2—Ratio of the number of a specific bacterium to the number of total bacteria for samples of saliva (A) and feces (B) and vaginal swab specimens (C) collected from pigs on 4 farms and swab specimens of feed troughs (D) and water dispensers (E) collected on those same 4 farms. Results were determined by use of a qPCR assay for *S suis* (designated as the qPCR_{SS} assay), a qPCR assay for *S suis* serotype 2 or 1/2 (designated as the qPCR_{2J} assay), a qPCR assay for *S parasuis* (designated as the qPCR_{SP} assay), and a qPCR assay for total bacteria (designated as the qPCR_{TB} assay); ratios represent the log₁₀ value for the number of a specific bacterium to the number of total bacteria. Each symbol indicates 1 value for samples obtained at a particular farm (farm 1, circle; farm 2, square; farm 3, triangle; and farm 4, diamond) and are plotted for piglets of various growth stages and sows. Numbers represent the number of samples that had negative results, which were defined as values below detection limits (not detected; ND) of the qPCR assays. See Figure 1 for remainder of key.

use of the qPCR_{SP} assay increased slightly with increasing age.

Ratios of numbers of *S suis*, *S suis* serotype 2 or 1/2, and *S parasuis* to the number of total bacteria

The exact amounts of samples could not be measured precisely; thus, it was difficult to directly compare cell numbers. However, the data were converted to ratios of the number of a specific bacterium (*S suis*, *S suis* serotype 2 or 1/2, and *S parasuis*) to the number of total bacteria (Figure 2). Most of the ratios were < 1% (reported as log₁₀ values < 0). For many saliva samples, ratios of the number of *S suis* and *S parasuis* to the number of total bacteria were from 1% to 15%. For a few swab specimen samples of feed troughs, the ratio of the number of *S parasuis* to the number of total bacteria ranged from 2% to 5%. A ratio > 1% for the number of *S suis* serotype 2 or 1/2 to the number of total bacteria was recorded for only the saliva sample from 1 sow. The ratios of the number of *S suis* to the number of total bacteria in saliva samples appeared to increase with increasing age of pigs on all 4 farms, although the farms were geographically distant from each other and differed with regard to the use of antimicrobials. Similarly, ratios of the number of *S parasuis* to the number of total bacteria in saliva samples and feed trough swab specimens appeared to increase with increasing age of pigs. Ratios of the number of *S suis* to the number of total bacteria were lower on the farm where β-lactams were administered, compared with the ratios for the other 3 farms.

Discussion

It is believed that *S suis* does not colonize all pigs.³² However, in the study reported here, *S suis* was detected in all saliva samples, and a ratio > 1% for the number of *S suis* to the number of total bacteria was detected for many of the saliva samples. However, a few of the remaining saliva samples had positive results when tested by use of the qPCR_{SS} assay, and the ratio of the number of *S suis* to the number of total bacteria was low in these samples. These observations suggested that pig saliva may be an important reservoir of *S suis* on swine farms and may lead to infection among pigs. Previously proposed sources of infection (ie, respiratory secretions, vaginal secretions, and fecal contents^{1,4}) may still be important. However, the possibility of spreading an infection from the saliva of sows to piglets is not negligible, as determined on the basis of the increase in the ratios of the number of *S suis* to the number of total bacteria with increasing age of pigs for the saliva samples. This finding indicated the importance of preventing piglets from contacting sow saliva, which thereby would prevent endemic *S suis* infection. In this manner, it may be possible to prevent infections of piglets. We can only speculate on the reservoir and source of *S suis* infection on the basis of results of the present study, although these results should be an initial step in finding the route of *S suis* transmission among pigs.

The ratio of the number of *S suis* to the number of total bacteria reached 5% to 10%, even for saliva

samples of piglets. However, *S suis* serotype 2 or 1/2 was detected in only a few samples from farms where *S suis* infections were prevalent, and it was not detected in the remaining samples. Furthermore, ratios of the number of *S suis* in saliva samples were lower for 1 farm where β -lactams were administered than ratios for the other 3 farms. Statistical analysis could not be conducted because the number of samples was inappropriate and exact amounts of samples could not be measured. The β -lactams are effective against *S suis*,³³⁻³⁵ which would suggest that the amount of *S suis* in pig saliva can be managed by use of these drugs. On the basis of these observations and the aforementioned 100% prevalence of *S suis* in saliva samples, several procedures for sows with positive results for the qPCR_{SS} assay may be effective for preventing the transmission of *S suis* infections from sows to piglets. Sows infected with prevalent serotypes of *S suis* (eg, serotype 2 in Japan) should be replaced with uninfected sows. A partition should be placed to separate a sow's head from the area where her piglets suckle. Antimicrobials such as β -lactams should be used prudently, with careful consideration for the emergence of antimicrobial-resistant bacteria. Administration of β -lactams in drinking water may be a strategy that can be used on pig farms to effectively reduce the amount of *S suis* in the oral cavity and prevent diseases attributable to *S suis* infection. However, β -lactams must be used in a prudent manner and under veterinary supervision to prevent *S suis* infections on farms, with careful consideration for the emergence of antimicrobial-resistant bacteria.

Most saliva samples contained *S parasuis*, although ratios of the number of *S parasuis* to the number of total bacteria were low in saliva samples of piglets. This finding indicated that *S parasuis* has a low ability to colonize pigs, compared with the ability of *S suis* to colonize pigs. Ratio of the number of *S parasuis* to the number of total bacteria was 1% to 2% in some feed trough and water dispenser swab specimens, whereas the ratio of the number of *S suis* to the number of total bacteria in these samples was < 1%. These observations indicated that the colonization preference of *S suis* on pig farms differed from the colonization preference of *S parasuis* and that *S parasuis* colonized the environment and may not have been involved as a cause of disease.

Results for the study reported here were obtained by use of qPCR assays (particularly by the modification of the DNA extraction method), rather than by use of culture-based detection methods.^{7-9,25,36} Use of a homogenizer with 2 types of zirconia beads can efficiently crush the cell walls of gram-positive bacteria,³⁷ including *S suis* and *S parasuis*. In addition, we confirmed that inhibitory activity of PCR assays was not evident for all sample categories used in the study (data not shown). Therefore, the method we used had high sensitivity and specificity.

A qPCR assay can be used to detect DNA of dead and live bacteria; however, the method used in

the present study quantitatively detected bacterial colonization, as determined in a previous study²⁰ in which intact *S suis* represented up to 13% of the estimated amount for a qPCR assay. The method used in the present study was important for providing the ability to detect *S suis* in samples obtained from live pigs as an alternative to detecting it in organs of slaughtered pigs. Detection of *S suis* serotype 2 or 1/2 from piglets will enable farmers to replace infected piglets before they mature into sows. This may reduce labor and costs, compared with labor and costs for postmortem examination of sows, and may prevent colonization of *S suis* serotype 2 or 1/2 in sows and thus prevent transmission of infection via saliva on pig farms. The design or use of other PCR assay primers for other *S suis* serotypes (eg, serotypes 3, 7, 9, and 14)³⁸⁻⁴⁰ as well as use of the qPCR_{2j} assay to test the saliva of pigs will enable clinicians to detect and monitor colonization of specific serotypes of *S suis*. Incorporation of these methods with use of the qPCR_{SS} assay, which provided reliable results and detected background amounts of *S suis* colonization, may help in the successful control of *S suis* infections on pig farms. Therefore, we propose that health evaluations conducted with saliva samples obtained from live pigs will help reduce *S suis* infections and provide substantial benefits for pig farms.

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The authors declare that there were no conflicts of interest.

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Footnotes

- a. BD BBV CultureSwab EZ II, Becton Dickinson, Milano, Italy.
- b. Fisherbrand disposable sterile spoons, Thermo Fisher Scientific, Waltham, Mass.
- c. RNALater stabilizing solution, Thermo Fisher Scientific, Waltham, Mass.
- d. Statens Serum Institut, Copenhagen, Denmark.
- e. PowerBiofilm DNA isolation kit, Mo Bio Laboratories Inc, Carlsbad, Calif.
- f. Toray Industries Inc, Tokyo, Japan.
- g. Bead crusher μ T-12, Taitec Corp, Saitama, Japan.
- h. Quantus fluorometer, Promega Corp, Fitchburg, Wis.
- i. QuantiFluor dsDNA system, Promega Corp, Fitchburg, Wis.
- j. NanoDrop 1000 spectrophotometer, Thermo Fisher Scientific, Waltham, Mass.
- k. GENETYX, version 13.0.4, Genetyx Corp, Tokyo, Japan.
- l. BLASTN, National Center for Biotechnology Information, Rockville, Md. Available at: blast.ncbi.nlm.nih.gov/. Accessed Feb 20, 2017.

- m. FAM, Integrated DNA Technologies, Tokyo, Japan.
- n. ZEN, Integrated DNA Technologies, Tokyo, Japan.
- o. IBFQ, Integrated DNA Technologies, Tokyo, Japan.
- p. ROX, Toyobo Co Ltd, Osaka, Japan.
- q. THUNDERBIRD probe qPCR mix, Toyobo Co Ltd, Osaka, Japan.
- r. Thermo Fisher Scientific, Waltham, Mass. Available at: www6.appliedbiosystems.com/support/techtools/calc/. Accessed Sep 20, 2017.
- s. StepOnePlus real-time PCR system, Thermo Fisher Scientific, Waltham, Mass.
- t. Water for molecular biology (RT-PCR tested), MilliporeSigma, Bedford, Mass.
- u. R, version 3.1.1, R Foundation for Statistical Computing, Vienna, Austria.

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Appendix

Primers and probes used to detect bacteria in samples collected from pigs and the environment on 4 pig farms in Japan.

qPCR assay*	Primer or probe	Nucleotide sequence (5'-3')	T _m (°C)	Product size (bp)	Reference
qPCR _{SS}	qPCR _{SS} -F	CAGGCAATGATTTATCTGGAGATG	60.6	95	NR
	qPCR _{SS} -R	GCGTGATTGGCTGAGCTGACCT	66.8	—	—
	qPCR _{SS} -P	(FR) GAAAGAAT (IQ) TGGTTGAACGAGC (DQ)	57.4	—	—
qPCR _{SP}	qPCR _{SP} -F	CGGGAAATACTGTTTCTGATGAAG	59.8	83	NR
	qPCR _{SP} -R	TAATTGGTTGGCCAAGGAA	57.4	—	—
	qPCR _{SP} -P	(FR) TGAAAATCA (IQ) CGTCAAGTTATTGG (DQ)	57.7	—	—
qPCR _{2j}	cps2j-F	GGTACTTGCTACTTTTATGATGGAAATT	60.0	88	29
	cps2j-R	CGCACCTCTTTTATCTCTTCCAA	60.5	—	—
	cps2j-P	(FR) TCAAGAATC (IQ) TGAGCTGCAAAAAGTGCAAAATTGA (DQ)	70.8	—	—
qPCR _{TB}	qPCR _{TB} -F	TCCTACGGGAGGCAGCAGT	61.7	467	28
	qPCR _{TB} -R	GGACTACCAGGGTATCTAATCCTGTT	59.9	—	—
	qPCR _{TB} -P	(FR) CGTATTAC (IQ) CGCGGCTGCTGGCAC (DQ)	71.8	—	—

*Represents a qPCR assay for *Streptococcus suis* (designated as the qPCR_{SS} assay), a qPCR assay for *S suis* serotype 2 or 1/2 (designated as the qPCR_{2j} assay), a qPCR assay for *Streptococcus parasuis* (designated as the qPCR_{SP} assay), and a qPCR assay for total bacteria (designated as the qPCR_{TB} assay).

cps = Capsular polysaccharide synthesis. DQ = Dark quencher.^o F = Forward. FR = Fluorescent reporter.^m IQ = Internal quencher.ⁿ NR = No reference; developed for the study reported here. P = Probe. R = Reverse. T_m = Melting temperature.

— = Not applicable.