Streptococcus suis and *S. parasuis* in the Philippines: Biochemical, molecular, and antimicrobial resistance characterization of the first isolates from local swine

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Streptococcus suis is one of the most economically important pathogens in the swine industry that can also cause diseases in humans. On the other hand, *S. parasuis* still has an unclear pathogenic capacity. In this study, molecular and biochemical methods were employed to detect the presence of *S. suis* and to characterize recovered isolates from local swine. From the swab samples obtained in Laguna, direct detection of the *S. suis*-specific *gdh* gene yielded 87.9% (44/49) positive results, however, only six *gdh* positive isolates were recovered by microbiological plating. An additional *gdh* gene positive isolate recovered from a brain swab of a diseased pig from Batangas was also reported. Biochemical profiling of the seven *gdh* positive isolates identified only one *S. suis* isolate. Using *recN* gene detection, and 16s rRNA and *sodA* gene sequences, the discordant results of *gdh* gene detection and biochemical profiling were resolved. Five isolates were confirmed to belong to the authentic *S. suis* clade while the other two were identified as *S. parasuis*. The clustering observed is highly acceptable because a bootstrap value of 92 was noted. Varied antimicrobial susceptibilities to commonly used antimicrobial agents were observed among the recovered isolates through the disk diffusion method. Notably, 100% (7/7) were observed to be resistant against streptomycin and oxytetracycline, while 57.1% (4/7) and 28.6% (2/7) were resistant to gentamicin and penicillin, respectively. Intermediate resistance against penicillin was also observed for 42.9% (3/7) of the isolates. This paper reports the first published isolates of *S. suis* and *S. parasuis* from local swine. With the plans to further intensify the local swine industry, which is currently ranked 8th worldwide in pork production, this report highlights the need to establish baseline data on the local epidemiology of streptococcal infections in swine, particularly of *S. suis*.

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INTRODUCTION

Swine production is an important component of global food security. The target of all swine producers is to increase production. Pork accounts for 47% to 60% of the animal meat consumption of the Filipinos (Organisation for Economic Co-operation and Development - OECD 2020; Department of Science and Technology – Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development DOST-PCAARRD 2016). The Philippines is one of the world's largest producers of pork, having been ranked 8th worldwide (United States Department of Agriculture – Foreign Agricultural Services, 2020; DOST-PCAARD, 2016). Based on the Swine Situation Report by the Philippine Statistics Authority (PSA), as of January 1, 2019, the swine population in the country is 12.71 million. With a predicted increase in the demand for meat, increasing the production from 1.7578 to 3.5445 tons per sow per year in ten years (Strak 2017) is one of the challenges being faced by swine industry. This continuously growing and intensifying swine-raising industry in the Philippines had "generated conditions conducive for respiratory disease-causing pathogens" (Corales 2011a). In the Philippines, the Bureau of Animal Industry identified that in 2009, 25% of all mortality in farmed pigs were due to respiratory diseases (Corales 2011a).

Streptococcus suis is a major cause of a plethora of swine diseases which include different forms of infection, most notably septicemia, pneumonia, and meningitis, which may result in sudden deaths and outbreaks significantly contributing to the hundreds of millions of dollars of economic losses of swine raisers and farmers (Staats et al. 1997; Goyette-Desjardins et al. 2014; Ma et al. 2019; Meekhanon et al. 2019; Besung et al. 2019). In the US alone, this pathogen causes an annual loss of $300 million (Marois et al. 2007; Staats et al. 1997). On the other hand, the pathologic characteristic of *S. parasuis* remains unclear, although this has been isolated both in diseased and healthy pigs. This prompted researchers to devise a new detection method that would differentiate and identify *S. parasuis* from *S. suis* (Yamada et al, 2018).

S. suis is a facultative anaerobic, Gram-positive, non-spore forming encapsulated round-shaped bacterium commonly occurring in pairs, sometimes in chains or singly (Hughes et al. 2009; Segura, 2009). There are 35 serotypes (serotype 1-34, and serotype 1/2) of *S. suis* that have been described based on polysaccharide capsular antigen (Perch et al. 1983; Gottschalk et al. 1989; Gottschalk et al. 1991; Higgins et al. 1995; Fittipaldi et al. 2012). A more detailed examinations of the genetic characteristics of several serotypes have led to either a reassignment to a different but previously established species (Hill et al. 2005) or a reclassification into a novel species (Tien et al. 2013; Nomoto et al. 2015; Tohya et al. 2017). In 2015, based on average nucleotide identity of whole genome sequences of *S. suis* serotypes 20, 22, and 26, Nomoto and co-workers proposed that these serotypes be recognized as *S. parasuis,* a novel genomic species. In 2017, Tohya et al. proposed that *S. suis* serotype 33 be taxonomically defined as a separate novel species, *S. ruminantium*.

S. suis is not only a swine pathogen, but it has also been identified as one of the emerging (Segura 2009) or re-emerging (Hughes et al. 2009; Dutkiewicz et al. 2017) human pathogens during the last decade (Goyette-Desjardins et al. 2014). VanderWaal and Deen (2018) mentioned that influenza and *S. suis* are two zoonotic diseases that affected production. In 2014, reported human infections of *S. suis* worldwide has reached more than 1500 cases (Huong et al. 2014), of which more than 90% were recorded in Philippine neighboring countries (Goyette-Desjardins et al. 2014). Two epidemics were recorded in China in 1998 and 2005 (Goyette-Desjardins et al. 2014). In a review by Goyette-Desjardins et al. (2014), a single Philippine case of *S. suis* infection in human was reported, citing the study by Lee et al. (2008). Wongjittraporn (2014), Coner-Nobleza et al. (2016), and Domado and Itable (2017) added five more cases of *S. suis* infections in humans in the Philippines. The patients were reported to be suffering from a combination of some of the following conditions: meningitis, fever, chills, rashes, arthritis, neurological deficits, progressive hearing loss, septicemia and/or meningoencephalitis.

Distribution of isolated *S. suis* serotypes vary significantly in different geographic locations. In Japan and Indonesia, cases of diseased swine were dominated by serotype 2 isolates (Ichikawa et al. 2020; Besung et al. 2019). On the other hand, reported isolates from slaughterhouse pigs were observed to be dominated by serotypes 2, 9, 31, 32 and 3 in Vietnam (Hoa et al. 2011), serotypes 23, 9, 7 and 2 in Northern Thailand (Thongkamkoon et al. 2017), and serotypes 16, 8, 9 and 3 in Central Thailand (Meekhanon et al. 2017). There were also observed changes in capsular serotype prevalences within a period of time in a given geographic area as in the cases of Canada, Korea, China, and Spain (Oh et al. 2017; Gurung et al. 2015; Goyette-Desjardins et al. 2014).

In 2011, DOST-PCAARRD and the Australian Center for International Agricultural Research embarked in a project to improve investigation, diagnoses, and control of respiratory diseases in swine. Notably, the project involved bacteriological training on identification and antibiotic sensitivity testing of *Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Bordetella*, *S. suis* and *Pasteurella*, and proceeded on to develop loop mediated isothermal amplification (LAMP) assays for the first three only (Corales 2011b).

Previous attempts to isolate and document existence of *S. suis* in the Philippines have not been successful. Yee (1998) attempted to isolate nasal and tonsillar *S. suis* from weaned pigs in some swine farms in the provinces of Cavite, Rizal, and Bulacan, but the biochemical tests used for its identification indicated that no *S. suis* was present. Another attempt at Benguet State University similarly yielded no isolate (Bagcigil et al. 2013).

Considering the advances in molecular techniques, *gdh*, *recN*, and *sodA* genes were amplified, the 16s rRNA and *sodA* genes were sequenced, biochemical tests were performed, and antimicrobial susceptibility tests were conducted to identify and characterize local *S. suis* isolates from swine.

MATERIALS AND METHODS

Bacterial Strains

S. suis serotype 2 (DSM 28762) was used as the positive control for Polymerase Chain Reaction (PCR) detection of *gdh* and *recN* genes. The additional isolate reported in this paper, SS-BAT16S3A, was recovered from a brain swab of a diseased pig from a farm in Batangas, which was submitted to the Vaccine Laboratory, BIOTECH-UPLB for further characterization and subsequent identification. All bacterial isolates were cultured in brain heart infusion broth or brain heart infusion agar supplemented with 5% sheep blood incubated at 37°C in a microaerophilic condition using the candle jar method.

Sample Location and Collection

The samples were collected from a backyard farm located in Laguna. The pigs, sourced locally from swine breeders in the area, were fed with commercial swine feeds or with discarded human food—washed and boiled to remove spoilage—as cheaper alternative. Historical signs of fever, depression, and

colds, or other respiratory related symptoms were reported. Herbal medicines (e.g. feeding sick pigs with guava tree bark) were used to treat these symptoms. The only known use of antibiotics were for cholera treatment administered several months before the collection. The specific antimicrobial agent used was not determined. No disease or clinical symptoms were observed during sampling.

The pigs were divided according to age group: pre-weaner (<1 month old), weaner (1-2 mo.), grower (3-4 mo.), finisher (4-6 months old), gilt (5-6 months old), and sow (>8 mo.). Forty-nine (49) oral and nasal swabs were collected following the guidelines of the UPLB Institutional Animal Care and Use Committee (UPLB-IACUC) under the Approval # BIOTECH-2018-001 (August 21, 2018), and conducted under the supervision of a veterinarian. Pigs were properly restrained with the head positioned upward. Sterile single-headed cotton swabs with a long and thin wooden handle were gently and aseptically inserted into the nasal or oral cavity. The samples were preserved for transport according to the protocol of Rosendal et al. (1986) by placing the swabs into a test tube containing 3.0 mL of sterile tryptone broth as transport medium. Samples maintained at 4ºC were transported back to the Vaccine Laboratory (BIOTECH-UPLB) and immediately processed for the isolation of *S. suis*.

Detection of *S. suis* from swab samples by species specific *gdh*-gene PCR primers

From each of the transport medium, 1.5mL sample was taken and centrifuged at 10,000×g for in order for the suspended cells to form a pellet. The supernatant was carefully pipetted out and discarded. Ninety µL of HPLC grade water and 10 µL of 0.1 N NaOH was added to the pellet. After vortexing, the suspension was incubated in a boiling water bath (100°C) for 20min to lyse the cells. This crude DNA extract was used as template for amplification of the *S. suis*-specific *gdh* gene. PCR amplification of *gdh* gene was done according to the recommendation of Kerdsin et al. (2014). The PCR primers used consists of the forward primer (5′-TTCTGCAGCGTATTCTGTCAAACG-3′) and the reverse primer (5′-TGTTCCATGGACAGATAAGATGG-3′) which resulted in the amplification of a 695bp fragment. The PCR mixture consisted of 2.0 µl 10x ViBuffer S, 0.2 mM dNTPs, 0.1 µM of each primer, 0.10 U of DNA Taq Polymerase (Vivantis, Malaysia) and 1.0-2.0uL of DNA template in a final volume of 20 uL. The following PCR thermal profile was used: initial activation of DNA polymerase for 3 minutes at 95°C, then 30 cycles of denaturation at 95°C for 20 seconds, primer annealing at 58°C for 30 seconds, extension at 72°C for 40 seconds, and final extension for 5 minutes at 72°C.

DNA staining solution (Biotium GelRed, Fremont, California, USA) was added to the PCR products to a final concentration of 10X (Huang et al. 2010) and were finally visualized by electrophoresis (Mini Gel II, Select Bioproducts, United States) at 100V for 35 min on a 1.5% agarose gel. The gel was then subjected under UV light using Gel Documentation System (Syngene G:BOX, United Kingdom). The sizes of the PCR products were determined by comparison with a molecular size standard (SiZer^{TM}-100 DNA Marker, iNtRON Biotechnology, Inc., South Korea).

Bacterial isolation of putative *S. suis* from swab samples

A sample of each individual transport medium was serially diluted in 0.85% normal saline solution (NSS). One hundred µL of the 10^{-4} to 10^{-5} dilutions were spread on brain heart infusion blood agar plates (BAP) containing 5.0% sheep blood. The lids of each plates were sealed with parafilm and the plates were incubated under a microaerophilic condition (candle jar) for 24-48 hours at 37°C.

Colonies with typical *S. suis* morphology were then selected and restreaked on BAP to ensure purity. Well isolated colonies were tested for Gram-reaction and catalase production. Gram-positive catalase-negative isolates were further tested for selected biochemical parameters as prescribed by Gottschalk et al. (2012), and Higgins and Gottschalk (1990). Inability to grow in 6.5% NaCl, positive result in amylase test, negative result in Voges-Proskauer test were used to identify putative *S. suis* isolates. Concurrently, crude extract of a single colony of each isolate was prepared, and the *S. suis*-specific *gdh*-gene amplification assay was also conducted, as described above.

Biochemical Profiling

Putative *S. suis* isolates identified by the simplified biochemical tests described above, and by detection of *gdh* gene were subjected to full biochemical profiling by Vitek 2 Systems Version 08.01 (Biomérieux,USA) at the Philippine National Collection of Microorganisms (PNCM, BIOTECH-UPLB).

Identification of "authentic" *S. suis* by PCR detection of *recN*-gene

Genomic DNA of all the isolates were extracted using Genomic DNA Prep kit (BIOFACT, South Korea) following manufacturer's instructions. DNA concentration and purity were determined by spectrophotometry, and the quality was evaluated by visualization of bands following electrophoresis.

PCR amplification of the *recN* gene was performed as indicated by Ishida and colleagues (2014). Primers used were SSrecN forward primer (5'-CTACAAACAGCTCTCTTCT-3') and SsrecN reverse primer (5'-ACAACAGCCAATTCATGGCGTGATT-3') which amplifies a 336bp sized amplicon. PCR was performed using a thermal gradient cycler (Techne Prime Large-Format Thermal Cycler, Cole Parmer, United States) in a final volume of 20 µl containing 0.4 mM dNTPs, 0.2 µM of each primer, 2 µl of 10× ViBuffer A, 2 U of DNA Taq Polymerase (Vivantis, Malaysia) and 25ng of purified genomic DNA as template. The cycling conditions used for the PCR method targeting *recN* were: an initial denaturation for 2 minutes at 94 °C, 30 cycles of denaturation for 30 seconds at 94 °C, annealing for 10 seconds at 58 °C, extension for 30 seconds at 68 °C, and a final incubation of 5 minutes at 68 °C. PCR products were visualized as previously mentioned.

Sequencing of 16s rRNA region and the *sodA* gene

PCR amplification of the 16s rRNA region was performed using the primers F1 (5'-GAGTTTGATCCTGGCTCAG-3') and R13 (5'-AGAAAGGAGGTGATCCAGCC-3'). These primers amplify a 1500bp sized amplicon (Dorsch and Stackebrandt 1992) in a final volume of 100 µl, containing 1.6 mM dNTPs, 0.7 µM of each primer, 10.0 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 2.5 U of DNA Taq Polymerase (Vivantis, Malaysia) and 50 ng of purified genomic DNA as template. The cycling conditions used were as follows: an initial denaturation for 2.5 m at 95 °C, 30 cycles of denaturation for 1.0 m at 94 °C, annealing for 45s at 50 °C, extension for 1.5 m at 72 °C, and a final incubation of 10 minutes at 72 °C. PCR products were visualized as previously mentioned, and they were sent to Integrated DNA Technologies IDT (Singapore) for sequencing.

The amplification of *sodA* gene was performed using the primers by Poyart et al. (1998), d1 (5'-CCITAYICITAYGAYGCIYTIGARCC-3') and d2 (5'-ARRTARTAIGCRTGYTCCCAIACRTC-3'). The PCR mixture, in a final volume of 100 µl, contained 0.8 mM dNTPs, 0.25 µM of each primer, 10.0 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, 2.0 U of DNA Taq Polymerase (Vivantis,

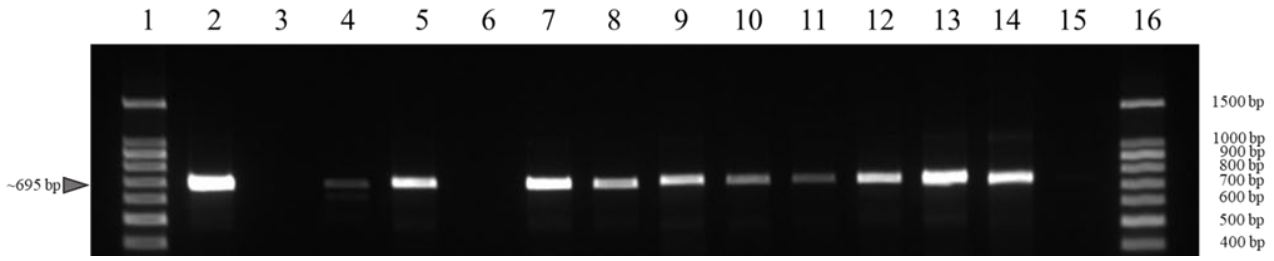


Figure 1: Representative agarose gel electrophoretic profiles showing positive detection of *gdh* gene from swab samples from swine. (Lane 1, 16 – Ladder; Lane 2 – positive control *S. suis* DSM 28762; Lane 3 – negative control No Template; Lanes 4-15 – swab samples)

Malaysia) and 50 ng of purified genomic DNA as template. The cycling conditions used were as follows: an initial denaturation for 2.5 m at 95 °C, 35 cycles of denaturation for 1.0 m at 94 °C, annealing for 45s at 50 °C, extension for 1.5 m at 72 °C, and a final incubation of 10 minutes at 72 °C. PCR products were visualized as previously mentioned, and they were sent to Macrogen (Korea) for sequencing. The PCR products were sequenced using the primers by Meekhanon et al. (2019), sodAfs (5'-TAAGCACCATGCGACTTATG-3') and sodArs (5'-AAGAAAGCCCAACCTGAACC-3').

Percent identity of each isolate was compared to sequences from type materials deposited in the GenBank databases of the National Center for Biotechnology Information (NCBI) GenBank (Bethesda, MD, U.S.A.) using nucleotide Basic Local Alignment Search Tool (BLASTn) available online. To better determine sequence similarity, the 16s rRNA gene of reference isolates of relevant *Streptococcus* species (Yamada et al. 2018) and sequences of *sodA* gene of previously identified serotypes of *S. suis* (Tien et al. 2013) were also retrieved from the GenBank. The identified species names of the deposited sequences were retained, including the serotypes reassigned to a different taxonomic group. Sequences obtained from this study and the corresponding retrieved sequences from NCBI were aligned using ClustalW method in Molecular Evolutionary Genetics Analysis (MEGA-X) software (Kumar et al. 2018). Neighbor-joining method (Saitou and Nei 1987) with bootstrap analysis of 1000 replications were used to construct separate phylogenetic trees based on the 16s rRNA sequences, and the *sodA* sequences.

The sequences of the newly described isolates were deposited to NCBI Genbank with accession numbers MT256094-MT256100 for the 16s rRNA sequences, and MT263691-MT263697 for the *sodA* gene sequences.

Antimicrobial resistance profiling

To assess the antibiotic susceptibility profile of the streptococcal isolates, the antibiotic susceptibility test was carried out by disk diffusion assay according to the standard protocol of Clinical and Laboratory Standards Institute (CLSI 2016) with minor modifications.

Eight commercially available and commonly used antibiotic drugs for veterinary and human uses were used for susceptibility testing of the isolates. These include beta-lactams (amoxicillin AMX – 25 mg, cefazolin CFZ – 30 mg, ceftriaxone CTX – 30 mg and penicillin G PEN – 10 units), fluoroquinolone (enrofloxacin ENR – 5 mg), aminoglycosides (gentamicin GEN – 10 mg and streptomycin STR -10 mg), and tetracycline (oxytetracycline OTR – 30 mg). Blank antibiotic disks were prepared by cutting 6 mm diameter circle pieces of Whatman No. 1 filter paper disk (WHATMAN®, Springfield Mill, Maidstone, Kent, England) and sterilizing at 121^{0}C for 20 min. Well isolated colonies from overnight culture plates of each of the isolates were selected and suspended in 0.85% NSS. The bacterial cell suspension was adjusted to a McFarland 1.0. One hundred microliter of the adjusted cell suspension was spread-plated on 4-mm depth Mueller Hinton agar supplemented with 5% sheep blood. Two to three sterile blank disks were placed per plate on the agar surface using sterile forceps. The aforementioned amounts of antibiotics were loaded onto the disks. The diameters of the inhibition zone were measured and interpreted as susceptible, intermediate, or resistant, according to CLSI 2016 - *Streptococcus* viridans group (for CTX), CLSI 2014 for CFZ, PEN, GEN and STR (Zhao et al. 2019), CLSI 2008 for AMX, and ENR (Lakkitjaroen et al. 2011). The diameter of inhibition zone for OTR was interpreted using the values for tetracycline indicated in CLSI 2016 – *S.* viridans group. Alternatively, the use of Antimicrobial Disk Diffusion Zone Interpretation Guide by VetLab (n.d.) for oxytetracycline did not change interpreted results.

RESULTS AND DISCUSSION

Almost all swab samples were *gdh* positive. Of the 49 swine individuals sampled, 44 (89.7%) showed a positive PCR detection of *gdh* gene directly from the transport medium (Figure 1). By age group, results showed 100% detection in sow (n=2), 90% in pre-weaners (n=10), 100% in weaners (n=14), 85.7% in growers (n=14), 71.4% in finishers (n=7), and 100% in gilts (n=2).

S. suis is part of the normal microbiota of the respiratory tract of pigs, colonizing the pig nasal and tonsil cavities in their early life stages and successfully adapting to its reservoir host (Votsch et al. 2018). Murase et al. (2019) reported that *S. suis* has the largest share (5.7% - 9.4%) of the total bacteria in pig saliva. However, reported carriage rates vary from 0% to 100% (Dutkiewicz et al. 2017) depending on the age group sample, herd health status, geographic location, and method used.

While presence of *S. suis* from nasal and tonsil cavities does not equate to disease detection (Gottschalk and Segura 2019), disease manifestation may arise from these latent asymptomatic infections when triggered by different biotic and abiotic factors (Dutkiewicz et al. 2017). Additionally, these clinically healthy swines are not only significant in *S. suis* spread inside and among herds, but they are also important sources of human infections (Lun et al. 2007; Meekhanon et al. 2017).

Several previous reports failed to detect and recover isolates of *S. suis* from local swine (Bagcigil et al. 2013; Yee 1998). This seemingly led to the relegation of *S. suis* as an unimportant causative agent of swine diseases in the country as evidenced by the absence of further studies on this pathogen despite reported cases of human infections. In fact, Huong et al. (2014) identified Philippines as the geographic gap in *S. suis* research in Asia. Additionally, government-led efforts to investigate and develop detection kits for respiratory swine pathogens excluded *S. suis* (Corales 2011b).

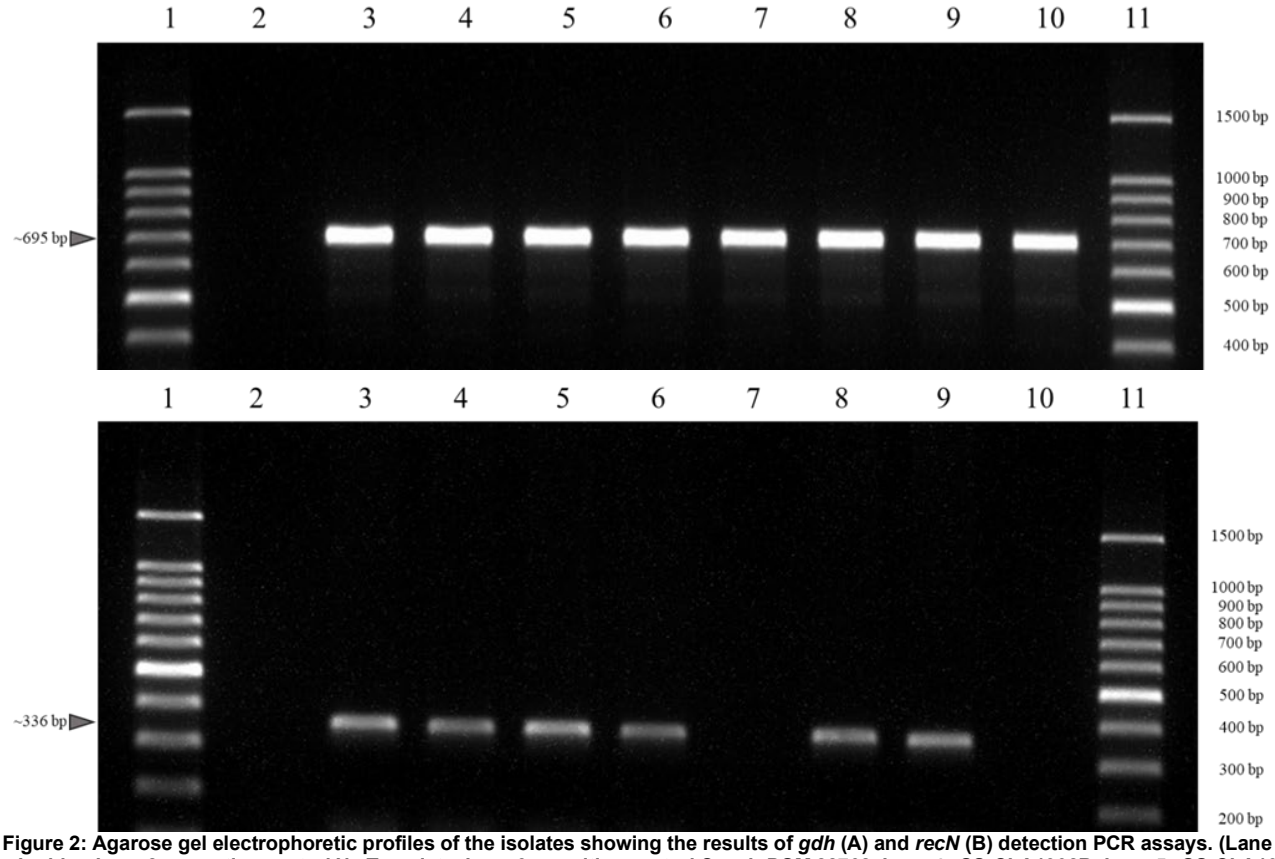


Figure 2: Agarose gel electrophoretic profiles of the isolates showing the results of *gdh* (A) and *recN* (B) detection PCR assays. (Lane 1, 11 – Ladder; Lane 2 – negative control No Template; Lane 3 – positive control *S. suis* DSM 28762; Lane 4 - SS-CLA1902B; Lane 5 - SS-CLA1902D; Lane 6 - SS-CLA1904A; Lane 7 - SS-CLA1913C; Lane 8 - SS-CLA1926A; Lane 9 - SS-CLA1926B; Lane 10 - SS-BAT16S3A)

Our result demonstrated high detection of *S. suis*, even from asymptomatic swine, which opens a possible link to the occurrence of the reported cases of the zoonotic infections in the archipelago.

Few putative *S. suis* were isolated by plating. Isolation by spread plating from the transport medium only yielded six putative *S. suis* isolates based on the simplified biochemical tests (Gottschalk et al. 2012; Higgins and Gottschalk 1990) and by detection of *gdh* gene (Kerdsin et al. 2014). The six isolates were from four swine individuals (4/44 – 9.09% recovery rate). All of the six isolates were obtained from oral swabs, close to the palatine tonsil, indicating more opportunity for isolation of *S. suis* from the oral cavity. Of the six isolates, three of which were from 2-week-old pre-weaners and another three from 4-month-old finishers. The additional isolate, SS-BAT16S3A, also passed the simplified biochemical tests, and tested positive for *gdh* gene (Fig. 2A).

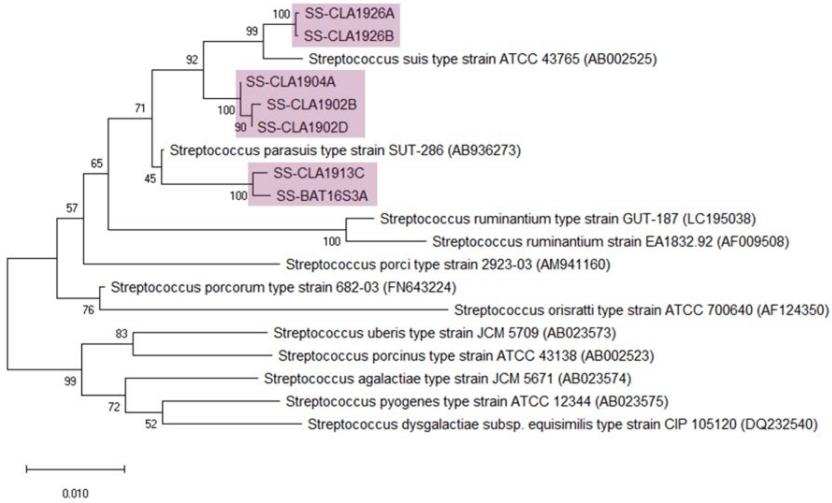
The observed low recovery rate or sensitivity employing traditional microbiological techniques in *S. suis* detection can be attributed to the similarity in colony morphologies and the presence of higher numbers of other streptococcal and related bacterial species in swab samples (Gottschalk et al. 1999).

Vitek® 2 Systems identification results by biochemical profiling are discordant with that of the simplified biochemical tests and PCR detection of *gdh* gene. The results of the Vitek® 2 Systems biochemical profiling of the putative isolates are shown in Table 1. Consistent positive results to optochin test, assimilation of saccharose, D-mannose, D-maltose, and production of tyrosine-, leucine- and Ala-Phe-Pro arylamidase were observed among the isolates and the positive control *S. suis* (DSM 28762) strain. Table 2 shows the six isolates identified as putative *S. suis* based on the simplified biochemical test (Gottschalk et al. 2012; Higgins and Gottschalk 1990) that were all positive for the presence of the *gdh* gene. However, based on the Vitek® 2 Systems biochemical profiling, only one isolate (SS-CLA1904A) was identified as *S. suis*. The other isolates were identified as *S. alactolyticus* (SS-CLA1902D, SS-CLA1926A, SS-CLA1926B, and SS-BAT16S3A), *S. ovis* (SS-CLA19135c) and *S. gallolyticus* (SSCLA1902B).

Previous studies using Vitek® 2 Systems and other biochemical methods have also reported misidentification of *S. suis* as enterococci, other species of *Streptococcus*, or even *Listeria monocytogenes* (Gomez-Torres et al. 2017; Goyette-Desjardins et al. 2014; Meekhanon et al. 2019; Tarini et al. 2019). Misdiagnosis and underdiagnosis of *S. suis* infections were mostly probably due to its similar characteristics shared with other causes of human infection with the same disease presentation, and the unawareness of laboratories regarding the pathogen (Chau et al. 1983; Gottschalk 2004; Gomez-Torres et al 2017).

Species-specific PCR assay for detection of *recN* gene, and sequences of the 16s rRNA region and *sodA* gene confirms 5 "authentic" *S. suis* and 2 *S. parasuis* isolates. Using the PCR assay adapted to the recent taxonomic reorganization of the genus *Streptococcus* and the serotypes of *S. suis* (Ishida et al. 2014), five (5) isolates (SS-CLA1902B, SS-CLA1902D, SS-CLA1904A, SS-CLA1926A, and SS-CLA1926B) tested positive for the detection of *recN* gene, while the other two (2) isolates (SS-CLA1913C, SS-BAT16S3A) tested negative (Fig. 2B). Due to the previously mentioned reclassification of several serotypes of *S. suis*, these discrepancies between the *gdh* gene and the *recN* gene detection results were possible with the latter detecting only what are currently considered "authentic" *S. suis*. Also, results of the BLASTn search of the 16s rRNA sequences

A.



B.

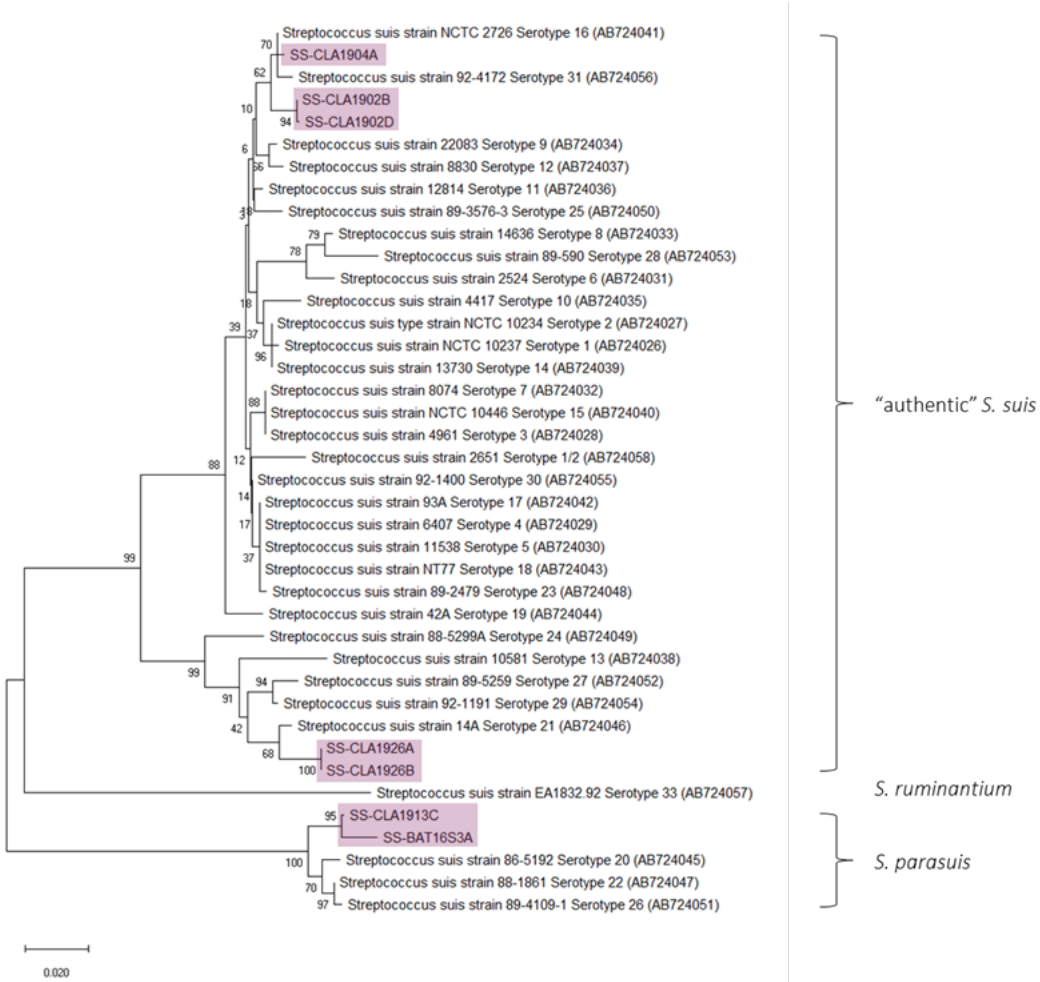


Figure 3: Neighbor-joining phylogenetic consensus tree showing the relationships between the isolates and the reference strains of the genus *Streptococcus,* and the serotypes of *Streptococcus suis* based 16s rRNA (A) and *sodA* (B), respectively.

Table 1: Biochemical profile of each of the isolates as determined by Vitek® 2 Systems.

BIOCHEMICAL TESTS	ISOLATES							
	DSM 28762	SS-CLA 1902B	SS-CLA 1902D	SS-CLA 1904A	SS-CLA 1913C	SS-CLA 1926A	SS-CLA 1926B	SS-BAT 16S3A
AMY	-	+	+	+	-	+	+	-
PIPLC	-	-	-	-	-	-	-	-
dXLY	+	-	-	-	-	-	-	-
ADH1	+	-	-	-	-	-	-	-
BGAL	-	-	-	-	-	-	-	-
AGLU	+	+	+	+	+	+	-	-
APPA	+	+	+	+	+	+	+	+
CDEX	-	-	-	-	+	-	-	-
AspA	-	-	-	-	-	-	-	-
BGAR	-	-	-	-	-	-	-	-
AMAN	-	-	-	-	-	-	-	-
PHOS	-	-	-	-	-	-	-	-
LeuA	+	+	+	+	+	+	+	+
ProA	-	-	-	-	-	-	-	-
BGURr	-	+	+	+	-	-	-	-
AGAL	+	-	-	+	-	-	-	-
PyrA	-	-	-	-	-	-	-	-
BGUR	-	-	+	+	-	-	-	-
AlaA	+	+	+	+	+	+	+	-
TyrA	+	+	+	+	+	+	+	+
dSOR	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-
POLYB	+	+	+	+	-	+	-	-
dGAL	+	-	+	+	+	+	+	+
dRIB	-	-	-	-	-	-	-	-
ILATk	-	-	-	-	-	-	-	-
LAC	+	+	+	-	+	-	-	-
NAG	+	-	-	+	+	-	-	+
dMAL	+	+	+	+	+	+	+	+
BACI	-	+	+	-	-	+	-	+
NOVO	+	+	+	-	+	+	+	+
NC6.5	-	-	-	-	-	-	-	-
dMAN	-	-	-	-	+	+	+	-
dMNE	+	+	+	+	+	+	+	+
MBdG	+	+	+	+	-	+	+	-
PUL	-	-	-	-	-	-	-	-
dRAF	+	-	+	+	-	-	-	+
O129R	-	-	-	-	-	-	-	+
SAL	+	+	+	+	-	+	+	-
SAC	+	+	+	+	+	+	+	+
dTRE	+	+	-	+	+	+	+	-
ADH2s	-	-	-	-	-	-	-	-
OPTO	+	+	+	+	+	+	+	+

of each of the isolates completely agree with the result of the *recN* gene detection (Table 1).

Shown in the constructed 16s rRNA-based phylogenetic tree (Fig. 3A), isolates that tested positive for *recN* were in the same cluster with the reference *S. suis* strain. This is supported by a bootstrap value of 92 which means we are confident that this grouping is highly significant and acceptable. The isolates that tested negative for *recN* were positioned in the same cluster as the reference *S. parasuis* strain. The same clustering was observed in the *sodA*-based phylogenetic tree (Fig. 3B) differentiating isolates SS-CLA1913C and SS-BAT16S3A from the "authentic" *S. suis* clade. This clustered them with *S. suis* serotypes 20, 22, and 26 with a bootstrap value of 100. These serotypes were proposed and reassigned as *S. parasuis* (Nomoto et al. 2015).

Antimicrobial susceptibility profiles of isolates showed varied but multiple resistances to classes of commonly used antibiotics. The results show that the recovered isolates were

Table 2: Results of bacterial identification based on biochemical and molecular methods.

ISOLATES	BIOCHEMICAL		MOLECULAR		
	Simplified Biochemical Test (Gottschalk et al. 2012; Higgins and Gottschalk 1990)	Vitek® 2 Systems	*S. suis* specific *gdh* gene detection (Kerdsin et al. 2014)	*S. suis* specific *recN* gene detection (Ishida et al. 2014)	16s rRNA sequencing (Dorsch and Stackebrandt, 1992)
SS-CLA1902B	*S. suis (putative)*	*Streptoccus gallolyticus* ssp. *pasteurianus* (99% Probability, Excellent identification)	+	+	*Streptococcus suis* NCTC 10234 (98.64% Id.)
SS-CLA1902D	*S. suis (putative)*	*Streptococcus alactolyticus*/ *Streptococcus salivarius* ssp. *salivarius* (Low discrimination)	+	+	*Streptococcus suis* NCTC 10234 (98.56% Id.)
SS-CLA1904A	*S. suis (putative)*	*Streptococcus suis* I (91% Probability, Good identification)	+	+	*Streptococcus suis* NCTC 10234 (98.26% Id.)
SS-CLA1913C	*S. suis (putative)*	*Streptococcus ovis* (93% Probability, Very good identification)	+	-	*Streptococcus parasuis* SUT-286 (98.83% Id.)
SS-CLA1926A	*S. suis (putative)*	*Streptococcus alactolyticus* (93% Probability, Very good identification)	+	+	*Streptococcus suis* NCTC 10234 (99.72% Id.)
SS-CLA1926B	*S. suis (putative)*	*Streptococcus alactolyticus* (89% Probability, Good identification)	+	+	*Streptococcus suis* NCTC 10234 (99.72% Id.)
SS-BAT16S3A	*S. suis (putative)*	*Streptococcus alactolyticus* (94% Probability, Very good identification)	+	-	*Streptococcus parasuis* SUT-286 (98.78% Id.)

highly susceptible to the beta-lactam group of antibiotics (85.7-100%)—excluding PEN—and the fluoroquinone ENR (71.4%) (Table 3). For the aminoglycoside GEN, resistance was observed for 57.4% of the isolates; three (3) of which are "authentic" *S. suis* while the other two (2) are the *S. parasuis* isolates. All 7 recovered isolates (100%) were resistant to STR and OTR.

The tested antimicrobial agents are of particular concern in the Philippine setting because of their wide commercial availability and usage frequency. According to the Philippine Action Plan to Combat Antimicrobial Resistance: One Health Approach crafted by the Inter-agency Committee on Antimicrobial Resistance (2015), oxytetracycline was reported to be the leading choice of antibiotic medication for swine. Penicillin comes in third and this is followed by amoxicillin. Barroga and colleagues (2020) also observed that enrofloxacin was the most frequently used antimicrobial active ingredient for 36% of the swine farms they surveyed. Gentamicin, streptomycin, amoxicillin, penicillin, and oxytetracycline were also reported to be used by the swine farms in this study. Use of antimicrobials are mainly for therapeutic treatment of respiratory diseases (Barroga et al. 2020). It was also observed that use of anti-bacterial agents on the animals without proper veterinary advice are fairly common (Huyn et al. 2006; Vergne et al. 2014), and this is made possible by the poor regulation of over-the-counter availability of these antibiotics at local agrovet supply stores (Barroga et al 2020).

Penicillin G is a broad-spectrum beta-lactam antibiotic that is commonly used in swine production most especially for pre-weaning piglets. It is often combined with STR (commonly referred as PenStrep locally) to reduce pathogenic infections that may result to immediate death. As first-line agents against bacterial infections, the reduced susceptibility of the recovered isolates alerts for a possibly on-going acquisition of PEN resistance by *S. suis*. Beta-lactam antibiotics are one of the frequently used antibiotics against *S. suis* infections (Haas and Grenier 2017). Several studies still report low resistance against this class, especially against AMX, ceftiofur, and PEN. But there had been recent reports of PEN resistance (0-27%) or intermediate susceptibility to PEN (Soares et al. 2015). This now includes this study wherein 42.9% of the recovered isolates were intermediately susceptible and 28.6% were observed resistant to PEN. It is also remarkable to note that between the two isolates of *S. parasuis*, isolate SS-BAT16S3A recovered from the brain of a diseased pig displayed resistance, while isolate SS-CLA1913C recovered from an asymptomatically infected individual showed susceptibility.

Meanwhile, STR resistance of *S. suis* is not commonly observed as only a few studies report on STR susceptibility (Varela et al. 2013). Genes coding for resistance to some aminoglycosides such as kanamycin and streptomycin have been identified for *S. suis* in multidrug resistant strains (Palmieri et al. 2011b). Thus, the observed resistance of all isolates to streptomycin is of particular concern.

Table 3: Antimicrobial susceptibility profiles of the *Streptococcus suis* and *S. parasuis* isolates.

CLASS	MODE OF ACTION	ANTIBIOTIC	µg PER DISK	ISOLATE		
				SUSCEPTIBLE	INTERMEDIATE	RESISTANT
Beta-lactams	Inhibits cell wall synthesis	Amoxicillin (AMX)	25	SS-CLA1902B, SS-CLA1902D, SS-CLA1904A, SS-CLA1913C, SS-CLA1926A SS-CLA1926B (85.7%)	-	SS-BAT16S3A (14.3%)
		Cefazolin (CFZ)	30	all isolates (100%)	-	-
		Ceftriaxone (CTX)	30	all isolates (100%)	-	-
		Penicillin G (PEN)	10 UI	SS-CLA1904A, SS-CLA1913C (28.6%)	SS-CLA1902B, SS-CLA1902D, SS-CLA1926B (42.9%)	SS-CLA1926A, SS-BAT16S3A (28.6%)
Fluoroquinolon	Inhibits DNA gyrase	Enrofloxacin (ENR)	5	SS-CLA1902B, SS-CLA1902D, SS-CLA1904A, SS-CLA1926A SS-CLA1926B (71.4%)	SS-CLA1913C (14.3%)	SS-BAT16S3A (14.3%)
Aminoglycosides	Breaks cell wall formation and disrupts protein synthesis	Gentamicin (GEN)	10	SS-CLA1904A, SS-CLA1926A, SS-CLA1926B (42.9%)	-	SS-CLA1902B, SS-CLA1902D, SS-CLA1913C, SS-BAT16S3A (57.1%)
		Streptomycin (STR)	10	-	-	all isolates (100%)
Tetracycline	Disrupts protein synthesis	Oxytetracycline (OTR)	30	-	-	all isolates (100%)

On the other hand, the other aminoglycoside in this study, gentamicin, can only affect the mortality of 42.9% of the isolates. Gentamicin resistance can be used to immediately infer resistance to all aminoglycoside antibiotics except for streptomycin (EUCAST 2019). As broad-spectrum antibiotics commonly used for streptococcal infections and other veterinary uses, important considerations must be done with the use of PEN, STR, and GEN.

Enrofloxacin susceptibility was observed among "authentic" *S. suis* isolates, while the two *S. parasuis* isolates showed either intermediate susceptibility (SS-CLA1913C) or resistance for the isolate from the brain of a diseased pig(SS-BAT16S3A). Fluoroquinolone resistance is only occasionally described for *S. suis* but genetically, it has been observed that resistance is often associated with single point mutations, as well as with a novel efflux pump (Palmieri et al. 2011b).

Resistance to tetracyclines has been well-documented for *S. suis* even as far as determining and establishing the resistance genes associated with it. A Vietnamese report on antimicrobial resistance spanning 11 years reported a significantly increasing trend for tetracycline resistance (Hoa et al. 2011). Most Asian studies on antimicrobial susceptibility of *S. suis* have in fact observed as much as 92% resistance to tetracycline (Varela et al. 2013). In the Philippines, Castro and co-workers (2018) presented an observation of 94.64% resistance to tetracycline for *S. suis* isolates they have collected in local swine. Tetracycline is usually the drug of choice to show susceptibility to the tetracycline class of antibiotics (CLSI 2016) but resistance to the

whole class may be inferred even with the other derivatives such as oxytetracycline. If both the functional group and its key component tetracycline fails to kill the bacteria, then these are already resistant to tetracycline itself.

S. suis has been identified as a possible reservoir of antimicrobial resistance determinants for streptococcal pathogens (Palmieri et al. 2011b). A number of publications identifying integrative and conjugative elements, transposons, genomic islands, and prophages have been reported (Chen et al. 2007; Du et al. 2019; Hu et al. 2011; Li et al. 2011; Palmieri et al. 2011ab; Ye et al. 2009; Zhu et al. 2019). The natural competence of streptococci for DNA transformation, and the current and on-going discovery of diverse sets of mobile genetic elements in *S. suis*, demonstrate how this pathogen of concern pose questions of risks to livestock industry and human health.

Until now, the Philippines does not have a single published study on the characteristics of the existing and circulating strains of *S. suis* from local swine, which are its natural host and reservoir. This is the first report on the isolation, identification and characterization of *S. suis* and *S. parasuis* in the Philippines. Having one of the world's largest swine raising industry and with the plans to intensify the said industry, compounded with the fact that several cases of human infections of *S. suis* have already been reported locally, there is a need to start paying attention to this pathogen.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Conceived and designed the experiments: S.A.S., B.B.I.S. Performed the experiments: S.A.S., B.B.I.S., A.G.M.S. Analyzed the data: S.A.S., B.B.I.S., A.G.M.S., M.S.M. Manuscript Preparation: S.A.S., B.B.I.S., A.G.M.S., M.S.M. All authors have read and agreed to the submitted version of the manuscript.

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