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Research Article
Phenotypic and Genotypic Characterization of *Streptococcus suis* Isolated from Pigs in Papua

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Abstract

Background and Objective: *Streptococcus suis* (*S. suis*) is one of the infectious agents that can cause a variety of symptoms of the disease in pigs. The Papua people live closely with pigs and the pigs raise extensively around their Honai (traditional home). The pigs are part of their way of life and part of their family. Pigs also play an important role in traditional ceremonies. Most of these laboratories would probably misidentify an isolate of *S. suis*. The aimed of this study was to identify of *S. suis* isolated from pigs in Papua. **Methodology:** This study was designed to isolate, identify and characterize of *S. suis* isolated from pig in various districts in Papua. The presence of 16s rRNA, glutamate dehydrogenase (*gdh* gene), muramidase released protein (*mrp* gene), capsular polysaccharide (*cps* gene) were performed by the Polymerase Chain Reaction (PCR) assay. Samples were swabbed from the tonsil of pigs and cultured an aerobically in sheep blood agar, Gram staining, biochemical tests and the growth properties in liquid media as weel as Serum Soft Agar (SSA). **Results:** According to results, it have been identified that 54 isolates were positive of *S. suis* (100%), but only 30% of isolates fermented trehalose and 74% of salicin. Most of *S. suis* isolates showed α-haemolytic of 88.9% on sheep blood agar and only 11.1% formed β-haemolytic. About 40.7% isolates grew with clear supernatant and 59.3% isolates with turbid supernatant. In the serum soft agar, more then half isolates grew diffuse and less than 37% isolates with compact characteristic. Based on genotype characteristics, all isolates were positive for 16s rRNA and *gdh* gene, no more 28% isolates were positive for *mrp* and *cps2j* gene. **Conclusion:** This study indicated that all isolates had been correctly identified by the biochemical methods and that PCR was more definitive and revealed the inherent problem associated with the interpretation of biochemical results.

Key words: *Streptococcus suis*, pig, zoonotic, biochemical tests, PCR

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Over the last 20 years, *Streptococcus suis* has been regarded as one of the main pathogens that cause serious economic losses in countries with progressive pig industry. Clinically healthy pigs is a main reservoir of infection and the most important relation in the epidemiology of human infections by the caused by *S. suis*[1].

Among the 35 serotypes, serotype 2 is a well-known cause of meningitis, septicemia, pneumonia, polyarthritis and polyserositis in pigs[2]. *Streptococcus suis* might also cause meningitis and other diseases in humans[3]. Meningitis is the most common clinical manifestations. In 1968, infections in humans was first reported in Denmark. Since then, more than 100 cases had been occurred in Europe, North America, East and Southeast Asia. The numbers of reported human cases, particularly in Southeast Asian region have rised dramatically in the past few years[4].

The use of antibiotics in food and drinking water has been quite positive results in controlling the disease. Nevertheless, the higher the incidence of antibiotic resistance so that it becomes less effective for the treatment of *S. suis* isolates. In addition, the lack of public awareness and knowledge of the residue as a result of the use of antibiotics[5]. In addition, the virulent serotype varying become a serious problem in vaccine development. This is due to lack of knowledge about the virulence factors and the differences in virulence not only among serotypes, but also in serotypes of *S. suis*[6].

The bacteria has a spherical shape, long chain, appear like a dew on solid media, produces α, β and γ hemolysis on blood agar and Gram positive. Clinical signs of infected pig include anorexia, depression, skin redness, lameness, in coordination, fever and followed by marked nervous sign like paralysis, paddling movement, ophystotonus and tetany[7]. Clinical signs of streptococcosis caused by *S. suis* is resemble to the outbreak of thousand pigs and monkeys that caused by *Streptococcus equi* subsp. *zooepidemicus* (group C) in Bali, Indonesia in 1994. Thats why the diagnosis of the outbreak was mistaken with *S. suis* infection[8].

In mid-2008, an outbreakon pig farms in Papua led to the death of about 600 pigs. Research on this outbreak isolated *S. suis* in pig joint fluid, demonstrating the presence of *S. suis* in Indonesia[9]. Only few *S. suis* infection cases have been reported in Indonesia. This is probably the consequence of a serious diagnostic problem in laboratories working with human or animal medicine. Most of these laboratories would probably misidentify an isolate of *S. suis* and in order to have a proper diagnosis of *S. suis* it is needed to identify *S. suis* isolated from pigs in Papua.

The aim of this study was to identify the cultures of *S. suis* isolated from pigs in various district in Papua. In addition, these cultures were further characterized phenotypically and genotypically.

MATERIALS AND METHODS

Bacterial isolates: A total of 54 of tonsil swab samples were collected from 54 healthy pigs were used in this study. Tonsil swab samples were collected from various local breed in Papua. Swabs were inoculated immediately into individual vials containing 1% pepton water and placed into a cool box with icefor transfer to the Clinical Pathology Laboratory, Faculty of Veterinary, University of Gadjah Mada. Two *S. suis* reference cultures of P171 and P735 were kindly obtained from Prof. Dr. Christoph Lämmler (Justus-Liebig-University Gieβen, Germany).

Bacterial cultures: Standard techniques for culturing and isolation were used to identify *S. suis*[10,11]. The specimens were left to come to room temperature and then mixed thoroughly before being streaked onto selective agar plates containing 0.1% Na azide with 5% (v/v) defibrinated sheep blood. The plates were then incubated overnight at 37°C in 5% CO_2 atmosphere. Two to three colonies with typical *S. suis* growth habit were grown in Todd-Hewitt Broth (THB), cultured at 37°C for 18 h in anaerobic conditions. Phenotypic characteristics from morphological analysis and haemolysis pattern were used for presumptive identification of *S. suis* colonies.

Biochemical identification: Preliminary identification of *S. suis* was performed as recommended previously[12,13]. Growth of the bacteria was determined in THB containing 6.5% NaCl. The Voges-Proskauer reaction was basically performed as described by previous reseachers[9].

Streptococcus suis growth properties in the liquid medium and serum soft agar was done based on Salasia[14]. A Single colony of *S. suis* was grown in 10 mL THB (Oxoid, Germany), incubated in an anaerobic state for 24 h. Bacterial growth was observed by turbidity growth or formation of sediment on the bottom of the tube with a clear supernatant. Growth in soft serum agar media was done by diluting the bacteria solution of 0.1 mL with 0:14 mol L^{-1} NaCl. Bacterial solution diluted in 10 mL of 0.15% in Brain Heart infusion (BHI, Gibsco, Germany) incubated at 37°C for 18 h. Interpretation of the results on soft media was done by looking at the morphology of colonies that grow compact or diffuse. Utilization of trehalose and salicin was determined in

phenol-red broth (Sigma Aldrich, USA). After preliminary identification, the cultures were tested for utilization of glucose, arabinose, sorbitol and raffinose (Sigma Aldrich, USA). All samples were inoculated for 24 h aerobically at 37°C.

DNA isolation and purification: A QIAmp DNA mini kit (Qiagen, Germany) was used to purify the DNA from *S. suis* according to the manufacturer's protocol. The bacterial strains were cultivated on blood agar base (Oxoid, Germany) containing 5% defibrinated sheep blood for 24 h of 37°C in anaerobic condition. A total of 3-5 *S. suis* colonies were suspended with 180 µL TE buffer (10 mM tris-HCl and 1 mM EDTA [pH 8]) containing 5 µL mutanolysin (Sigma, USA) in 2 mL micro tubes. The suspension was incubated for 1 h at 37°C and 25 µL of proteinase K (14.8 mg mL^{-1}, Sigma, USA) and 200 µL of AL buffer (containing reagents AL1 and AL2, Qiagen, Germany) were then added. The suspensions were incubated for 30 min at 56°C and then for 10 min at 95°C before being spun at 6,000×g for a few seconds. A total of 420 µL ethanol was added toeach sample and placed in a spin QIAmp column. After centrifugation at 6,000×g for 1 min, the spin columns were placed in a clean collection tube and the sample was washed twice with 500 µL of AW buffer (Qiagen, Germany). After the second wash and a centrifugation at 6,000×g for 3 min, the QIAamp spin columns were placed in a clean 2 mL microfuge tube and the DNA was eluted twice with 200 and 100 µL of AE buffer (Qiagen, Germany). The DNA was stored at 20°C until further analysis.

Genotype characterization: Molecular identification was conducted using polymerase chain reaction (Mastercycler PCR, Eppendorf, Germany) for molecular detection of gene coding for 16s rRNA, glutamate dehydrogenase (*gdh* gene) and muramidase released protein (*mrp* gene) of *S. suis*. The reaction mixture (25 µL) contained 1 µL primer (10 pmol), 1 µL primer 2 (10 pmol; Invitrogen, USA), 12.5 µL PCR mix containing taq DNA polymerase, MgCl and dNTPs (Roche, Germany), 3 µL of DNA template and 7.5 µL distillated water. The oligonucleotide primers were used as described by some riseachers[14-16]. The PCR product was purified by using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The PCR products were visualized by electrophoresison 1% agarose gel following by standard procedures. The oligonucleotide primers and the thermal cycler programs are shown in Table 1.

RESULTS

Streptococcus suis in this study showed results that vary widely in most tests. However, using the PCR method showed interesting results with a high percentage all the samples tested were *S. suis*. The biochemical properties, molecular identification and the phenotypic characteristic of the *S. suis* cultures are summarized in Table 2 and 3.

Similar results to the reference cultures (P171 and P735), all *S. suis* were Gram positive and no growth could be observed in fluid media containing 6.5% NaCl. Most of the *S. suis* isolates gave a positive salicin and trehalose reaction

Table 1: Oligonucleotide primers sequences used in this study

Primer	Sequence (5-3)	Programe*	PCR product size (bp)
16s rRNA	CAGTATTTACCGCATGGTAGATAT	1	294
	GTAAGATACCGTCAAGTGAGAA		
gdh gene	GCAGCGTATTCTGTCAAACG	2	688
	CCATGGACAGATAAAGATGG		
cps2j gene	TTTGTCGGGAGGGTTACTTG	3	498
	TTTGGAAGCGATTCATCTCC		
mrp gene	ATTGCTCCACAAGAGGATGG	4	188
	TGAGCTTTACCTGAAGCGGT		

*1: 40 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec, 2: 35 cycle at 94°C for 60 sec, 55°C for 60 sec, 72°C for 60 sec, 3: 30 cycle at 94°C for 60 sec, 55°C for 60 sec, 72°C for 90 sec and 4: 30 cycle at 94°C for 60 sec, 58°C for 60 sec, 72°C for 90 sec

Table 2: Biochemical properties and molecular identification of *Streptococcus suis* isolated from pigs in Papua

	Biochemical assay										
Gram staining	NaCl (6.5%)	VP	Tre	Sal	Glu	Arab	Sorbit	Raff	16s rRNA	*gdh* gene	*mrp* gene
+	-	-	+	+	+	+	+	+	+	+	+
(100%)	(100%)	(100%)	(30%)	(74%)	(96.3%)	(62.96%)	(88.89%)	(96.3%)	(100%)	(100%)	(27.85%)

VP: Voges-Proskauer, Tre: Trehalose, Sal: Salicin, Glu: Glucose, Arab: Arabinose, Sorbit: Sorbitol and Raff: Raffinose

Table 3: Characterization of selected *Streptococcus suis* isolated from pigs in Papua

	Growth characteristics of *S. suis*					
	Sheep blood agar		Liquid media (THB)		Serum Soft Agar (SSA)	
Colonies	α-hemolysis	β-hemolysis	Turbid	Clear	Diffuse	Compact
Small and transparent	48 (48/54, 88.9%)	6 (6/54, 11.1%)	32 (32/54, 59.3%)	22 (22/54, 40.7%)	34 (34/54, 63%)	20 (20/54, 37%)

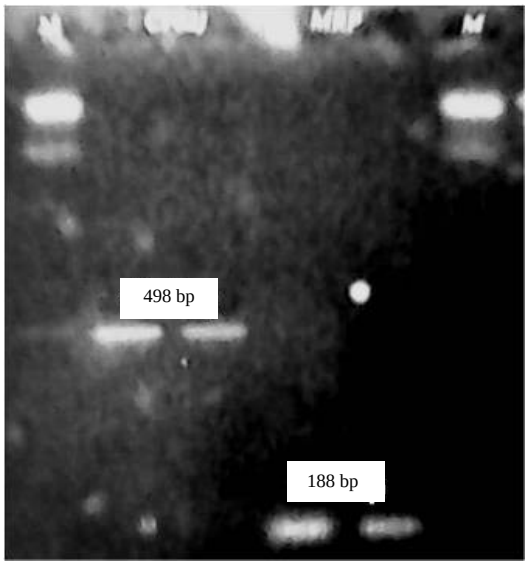

Fig. 1: PCR-amplified gene encoding MRP (*mrp* gene) and serotype 2 (*cps2j* gene) of selected *Streptococcus suis* isolated from pigs, Lane M: 100 bp marker DNA Lane 1-2: Amplicon of 498 bp (*cps2j* gene), Lane 3-4: Amplicon 188 bp (*mrp* gene)

and it were negative in the Voges-Proskauer test. Additionally, most of the strains utilized glucose, raffinose, arabinose and sorbitol. The characteristic of *S. suis* from different areas in Papua may indicate the of genetic relatedness and association between the source (pig) and human cases.

Colonies of *S. suis* were small, transparent, appeared like a dew on solid media. Cultivation of *S. suis* isolates on sheep blood agar, 48 (88.9%) were α-hemolytic and only 6 (11.1%) of the *S. suis* cultures were β-hemolytic. The growth properties of *S. suis* in liquid media were 32 (59.3%) isolates grew with turbid supernatants and 22 (40.7%) isolates with clear supernatants, 34 (63%) isolates grew diffuse and 20 (37%) isolates compact and in the SSA (Table 2).

Molecular characterization of *S. suis* strains were determined by PCR analysis (Fig. 1). Overall, a hundred percent of the strains were isolated from tonsils typically affected by *S. suis*. Furthermore, a high percentage (100%) of isolates were obtained for *gdh* gene. No false negatives were encountered. More than half of these strains had a *mrp* positive (27.85%), suggesting that also in these isolates expression of *mrp* and *gdh* seemed to be associated with virulence.

These results showed that all isolates were identified properly by biochemical test and PCR method was more definitive and revealed the inherent problems associated with the interpretation of the results of biochemistry test.

DISCUSSION

Identification of *S. suis* isolates are easily by using a minimum of biochemical tests, especially when isolates are recovered from diseased pigs. As proposed by Estoepangesti and Lämmler and Salasia[10,14], *Streptococcus suis* that fermented salicin and trehalose, negative acetoin and NaCl 6.5% may be considered *S. suis*. Acetoin (VP) test could be used to differentiate between *S. suis* and *S. bovis*[13,15]. Although some isolates, trehalose or salicin test gave a negative test result, it is rare that a negative for both tests. *S. suis* can be identified by using only a few tests[15]. No growth in broth with 6.5% NaCl and trehalose reactions and negative VP test. The reseachers[17,13] argued the following indicators as specific for *S. suis*: The VP negativity, absence in the presence of 6.5% NaCl, salicin and trehalose positivity. Acid production from raffinose is worth testing to differentiate of group R and S streptococci. In addition, this test shows that either raffinose fermentation tests or other tests may be used to differentiate of one type from another capsule.

Final identification of strains must be carried out by using *S. suis* species-specific Polymerase Chain Reaction (PCR) tests[17]. Based on the molecular identification by using species-specific primers, all isolates were positive for gene coding 16s rRNA, indicated that all isolates were *S. suis.* Concerning the 54 isolates, the fact that all isolates were positive by PCR at the species level indicated that the PCR assay was compatible with biochemical test on those isolates.

The bacterial surfaces have an important role in the pathogenicity of *S. suis*. Characteristics of *S. suis* that grew with turbid supernatants in the liquid media and diffuse in the SSA are thought to be more pathogen than its growth with clear supernatant and compact[14]. Only encapsulated isolates can be serotyped. These isolates will present a homogeneous growth in Todd-Hewitt broth, in contrast uncapsulated isolates will form a sediment at the bottom of the tube, showing a very clear supernatant. Loss of capsule has been reported among *S. suis*. Around 5% of isolates recovered from diseased animals were not encapsulated[2].

Glutamate dehydrogenase has been widely used to perform diagnosis on a wide range of bacterial infections[18,19] and showed a point mutation is very low compared with other genes[20]. Previous study indicates a successful cloning of the gene encodes *gdh*. The *S. suis* serotype 2 and confirms that genes *gdh* are conserved in *S. suis*. The *gdh* gene contained in *S. suis* could potentially become target gene that is

suitable for the development of diagnostic tools for *S. suis* identification from clinical samples. Identification of *S. suis* based on *gdh* gene using the PCR method is specific, rapid and sensitive regardless of the serotypes or origin sample areas. The primer that has been used in this study is specific for the detection of *gdh* gene of *S. suis*, efficiently amplify *S. suis* of all isolates tested and yielded 688 bp of PCR product without false negative.

Heretofore, based on the characteristic of the capsular polysaccharide, 33 serotypes of *S. suis* were identified. Among them, serotype 2 is one of the stunning serotype in pigs. A 489 bp fragment accordance to a region of the gene encoding for capsule *cps* obtained by only with *S. suis* serotype 2 as expected. Within *S. suis*, *cps* locus covers several genes encoding regulatory proteins, glycosyl transferase, polysaccharide polymerase, lipase and some transferase. Capsular polysaccharides highly diverse molecules that may be different not just by monosaccharide units but also on how these units assemble together. Capsular polysaccharide biosynthesis pathway requires complex and commonly, the genes involved of this process gathered in the *cps* locus[21,22].

Streptococcus suis isa zoonoticagent that can infect humans with 88% of such infections characterized by clinical symptoms of meningitis[15]. Previous reseachers[23] reported that *S. suis* infections in humans are observed frequently in intensive pig farming areas, or where people live in close contact to pigs. This contention is strengthened by the existence of cases of infection with *S. suis* in hunters after contact with wild pigs[24]. *Streptococcus suis* can be isolated from the tonsil area of healthy pigs[25]. Researchers understand that domesticated and wild healthy pigs are a reservoir of *S. suis* infection and that this is transmitted by direct contact with carrier pigs, consumption of contaminated meat or aerosol[26]. The characteristics of *S. suis* in pigs in Papua could be used as a basic control for the disease. *Streptococcus suis* infection in pigs is a remarkable disease in Papua. The way of life with pigs may promote a zoonotic disease in Papua people. Further study of the prevalence of *S. suis* in pigs in Indonesia is important from a public health perspective.

CONCLUSION

In summary, the characteristics of *S. suis* isolated from pigs in our study that be expressed from the bacterial surfaces have an important role in the pathogenicity of *S. suis* and could be used for early screening and base control for the disease. *Streptococcus suis* infection in pigs is a remarkable zoonotic disease in Papua. The Healthy carrier pigs are possible to be essential for the maintenance of virulent strains of *S. suis* on pig farms and control strategies are needed to discover and reduce the carrier state.

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