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Molecular Detection and Characterization of *Streptococcus suis* Isolated from Pneumonic Lungs of Pigs

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ABSTRACT

Background: Among various diseases, respiratory disease is considered a leading cause of mortality in pigs. *Streptococcus suis* affects respiratory tract and causes pneumonia as an opportunistic pathogen, that impairs animal health and lowers individual and herd performance in swine. Therefore, it becomes important to have an insight of bacterial pneumonia with special reference to *Streptococcus suis* in pig, so that control measures could be practiced effectively.

Methods: Detailed post mortem examination was carried out on 180 pigs. Bacterial isolation was carried out from 127 (70.55%) lungs samples with pneumonic lesions. Further, suspected colonies of *S. suis* were subjected to Polymerase Chain Reaction for detection of glutamate dehydrogenase gene. Phylogenetic analysis was carried out for the positive *S. suis* samples.

Result: Bacteria isolated were *Pasteurella multocida* (34.44%), *S. suis* (28.89%), *E. coli* (16.67%), *Staphylococcus* spp. (14.44%), *Klebsiella* spp. (3.33%) and *Salmonella* spp. (2.22%). Out of 26 suspected *S. suis* colonies subjected to polymerase chain reaction, 17 isolates with amplicon size 688 bp were confirmed to be positive for glutamate dehydrogenase gene. The Phylogenetic analysis of glutamate dehydrogenase gene of *S. suis* from pigs of Assam showed percent identity above 99% with Germany, Canada and China strain of *S. suis*.

Key words: Glutamate dehydrogenase gene, Molecular characterization, Pigs, *Streptococcus suis*.

INTRODUCTION

Streptococcus suis (*S. suis*) is one of the most important bacterial pathogen of swine associated with septicemia resulting in meningitis, pneumonia, arthritis, endocarditis and polyserositis (Amass *et al*., 1996). Respiratory disease was reported as the leading cause of mortality in nursery and grower-finisher pigs responsible for significant economic losses (National Animal Health Monitoring System, 1996). Assam possesses highest *i.e.* 2.10 million of the total pig population of India which is 9.06 million (20th Livestock census, 2019). However, microbial infestation of the lower respiratory tract causes pneumonia that impairs animal health and lowers individual and herd performance in swine. *S. suis* causes various disease conditions including pneumonia, particularly bronchopneumonia in the pig herds of Assam (Pegu *et al*., 2020). It is the second cause of poor growth and death at all ages and accounts for 30% of deaths of fatteners (Baskerville, 1981).

Streptococcus suis (Lancefield's group D), which is a gram-positive oval cocci found as diplococci or short chains is considered one of the essential bacterial pathogens in the swine industry in the world (Laber and Swindle, 2002). *S. suis* is also an emerging zoonotic pathogen. It is classified into 33 serotypes, where serotypes 1, 2, 3, 7, 9 and 1/2 are the most prevalent in swine (Sousa and Tiwari, 2020). The natural habitat of *S. suis* is the upper respiratory tract (tonsils and nasal cavities) of pigs. It can also be isolated from genital and digestive tracts. Transmission occurs mainly through the respiratory route (Desjardins *et al*., 2014).

While pig production remains a profitable enterprise, commercial and particularly the small-scale farmers face huge constraints in this husbandry practice due to various diseases in pigs, the most important being bacterial infections and its association with morbidity and mortality (Abubakar *et al*., 2017). Pig farming is predominantly practiced by tribal and other economically backward people across the state as a source of livelihood; in such situation outbreak of bacterial infection particularly pneumonia in pigs causes severe economic losses. In India, so far very little efforts have been made to study the occurrence of various disease conditions in lungs of pigs associated with *Streptococcus suis*. Therefore, it becomes important to have an insight of bacterial pneumonia and in this study, it was carried out with the objectives of isolation, identification of major bacterial pathogens and molecular detection and characterization of *Streptococcus suis* from pneumonic lungs of pigs, so that control measures could be practiced effectively.

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MATERIALS AND METHODS

The materials for the proposed study consisted of tissue samples of lungs and upper respiratory tract collected from various slaughter houses, field mortalities, private piggery farms located in and around Guwahati and from postmortem examinations carried out at Department of Pathology, College of Veterinary Science, A.A.U., Khanapara, Guwahati, Assam for a period of one year *i.e.* from March, 2020 to February, 2021. A total of 180 pigs irrespective of age, sex were subjected to detailed post mortem examination. Based on gross examination, 127 pneumonic lungs samples were collected aseptically and subjected for bacteriological investigation.

Bacteriological studies

For isolation of *Streptococci*, the samples (n=127) were streaked onto Brain Heart Infusion (BHI) agar plate for primary isolation of bacteria and the inoculated plates were incubated aerobically at 37°C for 24 hours. The morphological, cultural and biochemical characters were studied (Fig 1 and Fig 2) (Cowan and Steel, 1993; Tarradas *et al*., 1994).

The primary isolation of *Pasteurella* spp. was done on Brain Heart Infusion agar plates and Blood Agar Base medium and incubated at 37°C for 24 hours (Jabeen *et al*., 2013). On Gram's staining of suspected colonies, small, non-motile, gram negative coccobacillus was visible. On further methylene blue staining, bipolar characteristics were visible.

For primary isolation of *Staphylococcus* spp., the samples were streaked onto Mannitol Salt Agar (MSA) plates. The plates were then incubated aerobically at 37°C for 24 hours giving yellow colonies. On Gram's staining purplish spherical and grape like clusters were observed.

For isolation of *Escherichia coli*, *Klebsiella* spp and *Salmonella* spp., the samples were directly inoculated onto MacConkey Lactose Agar (MLA) plates for primary isolation. The plates were then incubated aerobically at 37°C for 24 hours. IMViC test was further performed for differentiation.

Molecular detection by polymerase chain reaction (PCR)

Confirmation of *Streptococcus suis* was made on detection of *gdh* by simplex Polymerase Chain Reaction (PCR). The PCR was performed in a thermocycler using oligonucleotide primers (Table 1) as per the reported methods of Okwumabua *et al*. (2003).

For extraction of bacterial DNA BHI Agar plate grown *Streptococci* cultures were enriched in 2 ml Todd Hewitt Broth by incubation at 37°C for 24 hours and then centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 150 µl of tris-EDTA (TE) buffer with pH 8 by centrifuging at 3000 rpm for 5 minutes and discarding the supernatant twice. To the pellet, 100 µl of sterile nuclease free water was added, mixed properly and transferred to 1.5 ml microcentrifuge tubes. The suspension was boiled for 15 to 20 minutes, snap chilled on ice for 10 minutes and then centrifuged at 12,000 rpm for 10 minutes. The supernatant was used as templates and stored at -20°C for further use. The extracted DNA concentration was measured in Nanodrop Spectrophotometer (Thermofisher scientific, USA).

Amplification of the target gene was carried out in 25 µl reaction volume containing 2× Dream Taq master mix (Thermo scientific, K1081) containing 0.05 units/µl Taq DNA polymerase in reaction buffer, 4.0 mM $MgCl_2$, 0.4 mM each of dNTP (dATP, dCTP, dGTP and dTTP), 4.0 µl (100-150ng) of template DNA and 0.5 µl each of the forward and reverse primers (10 pmol conc.) of *gdh* gene. PCR was performed in a Thermal cycler (Applied Biosystems) with previously reported thermo cycling condition (Okwumabua *et al*., 2003). The amplified products were confirmed by agarose gel electrophoresis, using 2% agarose containing ethidium bromide in 1× Tris-Acetic acid- EDTA (TAE) buffer at 80-100 V for 1-2 hrs. The gel was visualized under UV light in Gel Doc System (BioRad, USA) and images were captured by Imagelab software. DNA lysate brought from ICAR-NRC on pig, Rani was taken as positive control. A non-template control (NTC) was considered as negative control in the present PCR based studies.

Molecular characterization

For sequencing, two amplified PCR products were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions. Phylogenetic analysis was carried out using 688 bp region encoding *gdh* gene corresponding to the two *Streptococcus suis* positive sequences along with 10 reference sequences retrieved from the NCBI GenBank nucleotide database. Phylogenetic and molecular evolutionary analyses were conducted using DNASTAR software with the neighbour-joining method.

RESULTS AND DISCUSSION

In the present study, our microbiological data confirmed 26 *Streptococcus suis* isolates from 127 pneumonic lungs of pigs on the basis of Gram's staining and biochemical tests of suspected colonies. The colonies were Gram +ve, coccus form, short chained and positive in catalase, lactose but negative in Voges-Proskauer test. *S. suis* is an encapsulated gram-positive coccus that occurs singly, frequently in pairs, or occasionally in short chains and the VP test is critical in differentiating *S. suis* from other *Streptococcus* spp. (Higgins *et al*., 1990; Goyette *et al*., 2014). In the present study, pneumonia was broadly classified into bronchopneumonia,

Table 1: List of primer sequences used for detection of *gdh* gene of *Streptococcus suis* isolates.

Primer	Sequence (5′-3′)	Target gene	Product size	Reference
JP4 F	GCAGCGTATTCTGTCAAACG	*gdh*	688 bp	Okwumabua *et al.* (2003)
JP5 R	CCATGGACAGATAAAGATGG			

interstitial pneumonia, haemorrhagic pneumonia, suppurative pneumonia and fibrinous pneumonia based on distribution, texture, color, appearance and exudation of the affected lungs. *S. suis* could be isolated from cases of bronchopneumonia (11), interstitial pneumonia (5), suppurative pneumonia (8) and fibrinous pneumonia (2). Other bacteria isolated along with *Streptococcus suis* were *Pasteurella multocida* (34.44%), *E. coli* (16.67%), *Staphylococcus* spp. (14.44%), *Klebsiella* spp. (3.33%) and *Salmonella* spp. (2.22%). Several workers (Dosen *et al*., 2007; Lavanya *et al*., 2011; Fablet *et al*., 2012) have also detected one or more of these pathogens in pigs died of pneumonia in different proportions. Mixed bacterial infection indicated etiological complexity involved in the pathogenesis of pneumonia as one or more bacteria were isolated from the pneumonic lungs of pigs.

Fig 1: *Streptococcus suis* colonies on nutrient agar.

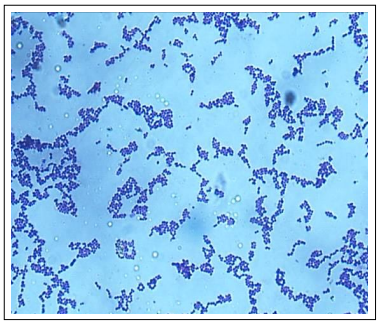

Fig 2: *Streptococcus suis* organism in gram's staining showing short chains (100×).

On screening by simplex Polymerase Chain Reaction (PCR), the primers JP4 and JP5 specific for *S. suis gdh* gene, amplified the DNA from 17 *S. suis* out of 26 isolates with amplicon size 688 bp (Fig 3). In the remaining 9 isolates, *gdh* gene could not be detected which may be due to presence of other *Streptococcus* spp. Amplification of *gdh* gene of *S. suis* by PCR in the present study was in conformity with the previous workers who reported that *gdh*-based PCR assay is highly specific and sensitive and can be used successfully for the detection of *S. suis* isolates regardless of serotype or geographic origin (Okwumabua *et al*., 2001; Sonowal *et al*., 2013; Pegu *et al*., 2020). *Gdhs* are of interest because they are highly conserved and exhibits an extremely low rate of point mutation relative to many other genes as reported by Creighton (1984). Thus, *gdh* gene is of great importance from diagnostic point of view.

Among the 17 PCR positive samples, two samples of *S. suis* were sequenced and used for phylogenetic analysis. Using the neighbour-joining method a phylogenetic tree was constructed (Fig 4). Multiple aligned distances are shown in Supplementary Table (Fig 5). The sequences of *gdh* gene of two isolates reported in the present study were compared with 10 reference isolates reported worldwide from pigs. The Phylogenetic analysis of *gdh* gene of *S. suis* from pigs of Assam *viz.* Assam1 in the present study showed highest percent identity of 99.1% with Canada followed by Germany 98.8% and Assam2 sequence showed 100% similarity with Canada strain followed by 99.7% identity with the Germany strain. Strains of *Streptococcus oralis and Streptococcus pneumoniae* sequences were used as outgroup revealing that the pigs were infected with *Streptococcus suis* only.

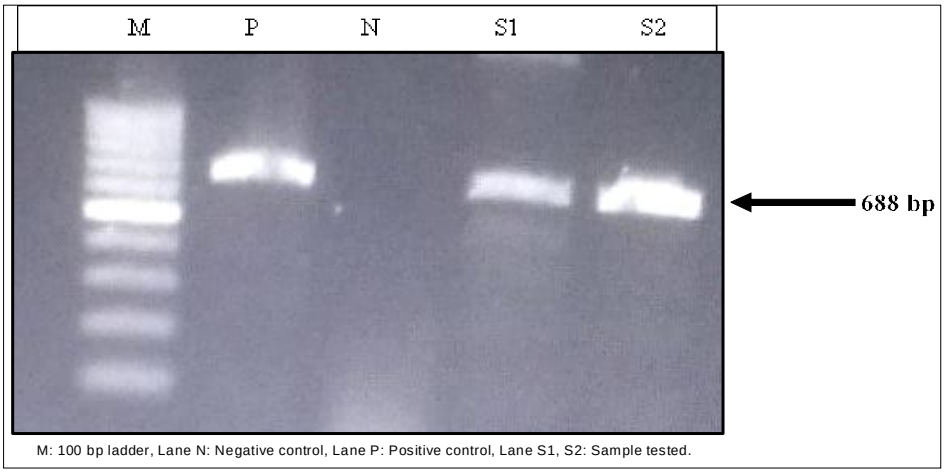

Fig 3: Detection of *gdh* gene (688 bp) of *Streptococcus suis* by PCR.

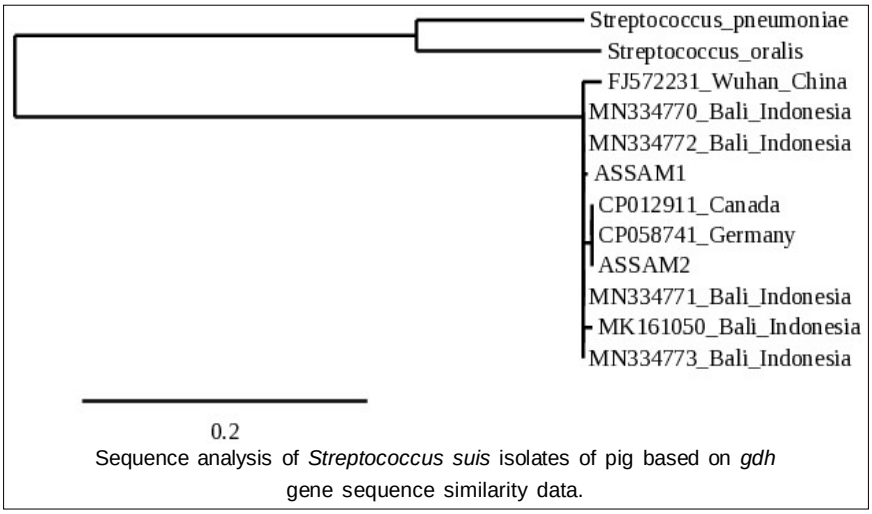

Fig 4: Phylogenetic tree of *Streptococcus suis* from field samples.

	1	2	3	4	5	6	7	8	9	10	11	12		
1		99.1	99.1	98.8	20.9	21.8	21.4	21.4	21.4	21.4	20.3	22.8	1	ASSAM1
2	0.9		100.0	99.7	20.8	21.3	20.8	20.8	20.8	20.8	21.8	24.3	2	ASSAM2
3	0.9	0.0		99.7	20.8	21.3	20.8	20.8	20.8	20.8	21.8	24.3	3	CP012911_Canada
4	1.2	0.3	0.3		20.8	21.6	21.3	21.3	21.3	21.3	21.4	23.7	4	CP058741_Germany
5	120.1	119.7	119.7	119.7		98.2	99.1	99.1	99.1	99.1	24.5	24.1	5	FJ572231_Wuhan_China
6	118.2	117.8	117.8	117.8	1.8		99.1	99.1	99.1	99.1	22.5	23.0	6	MK161050_Bali_Indonesia
7	117.5	117.1	117.1	117.1	0.9	0.9		100.0	100.0	100.0	22.2	22.4	7	MN334770_Bali_Indonesia
8	117.5	117.1	117.1	117.1	0.9	0.9	0.0		100.0	100.0	22.2	22.4	8	MN334771_Bali_Indonesia
9	117.5	117.1	117.1	117.1	0.9	0.9	0.0	0.0		100.0	22.2	22.4	9	MN334772_Bali_Indonesia
10	117.5	117.1	117.1	117.1	0.9	0.9	0.0	0.0	0.0		22.2	22.4	10	MN334773_Bali_Indonesia
11	146.7	147.4	147.4	147.4	90.4	93.3	91.4	91.4	91.4	91.4		85.9	11	*Streptococcus_oralis*
12	141.8	140.4	140.4	140.4	92.6	93.9	93.9	93.9	93.9	93.9	14.4		12	*Streptococcus_pneumoniae*
	1	2	3	4	5	6	7	8	9	10	11	12		

Fig 5: Pairwise distance analysis of *Streptococcus suis* from field samples showing a maximum identity of 100%.

CONCLUSION

Present study was conducted to detect glutamate dehydrogenase gene (*gdh*) in *S. suis* isolated from pneumonic lungs of pigs. The *gdh* gene specific primers amplified the DNA from 17 *S. suis* out of 26 isolates indicating *S. suis* as one of the causative agent in pig pneumonia. The PCR technique by targeting the *gdh* gene was found to be highly specific, sensitive and rapid way of detecting *S. suis* compared to conventional biochemical tests. Further detailed study of the streptococcal infection, particularly, the serotype involved should be re-evaluated to elucidate the risk factors for human infection.

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Conflict of interest: None.

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