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Molecular confirmation and detection of virulence genes of *Streptococcus suis* from Pigs in Assam

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Abstract
The study was designed to determine the prevalence as well as virulence genes of *Streptococcus suis* in pigs maintained in organized and unorganized farming systems in and around Guwahati, Assam. A total of 497 samples were collected from clinically healthy (n=267) and diseased (n=230) pigs of varying age and either sex. Samples were processed for isolation of *S. suis* and their identification based on biochemical characteristics and further confirmation through PCR amplification of the housekeeping gene *gdh* (glutamate dehydrogenase) encoding *S. suis* capsular biosynthesis. Important virulence genes of *S. suis* namely, *epf, mrp*, *sly*, and *arc A* were detected by using multiplex PCR. All together seven isolates were confirmed as *S. suis*. But none of the investigated virulence genes could be demonstrated in the present study.

Keywords: *S. suis*, polymerase chain reaction, *gdh gene,* virulence genes

Introduction
Streptococcus suis is an important pathogen of swine responsible for a wide range of diseases, such as meningitis, arthritis, septicemia, endocarditis, encephalitis, abortions, polyserositis, and bronchopneumonia. Outbreaks were reported worldwide in countries with well developed swine industry. At present, there are 35 recognized serotypes of *S. suis* based on capsular polysaccharides, of which serotype 2 is highly prevalent and virulent and recognized as one of the most important emerging zoonotic pathogens frequently isolated from a wide range of mammalian species including humans. All reported human cases were associated with slaughterhouse workers. Subclinical carrier animals are the most important source of transmission to susceptible pigs [1-5].

The pathogenic mechanism(s) of *S. suis* is not well defined. Most studies on virulence factors and the pathogenesis of the infection were focused on capsular type 2 strains [6] and many virulence associated proteins were identified including muramidase-released protein (MRP) [7], extracellular factor (EF) [6] suilysin [8], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [9], fibronectin-binding proteins (FBPS) [10] and hyaluronatelyase (HYL) [11].

The 110 kDa extracellular factor (EF) and the 136 kDa muramidase-released protein (MRP) expressed by some *S. suis* strains are encoded by the genes *epf* [12] and *mrp* [7], respectively. Both EF and MRP are frequently associated with highly virulent serotype 2 strains, but their functions are still unknown. Muramidase-released protein is often present in virulent strains and is absent in the avirulent strain [7, 13]. Extracellular factor is also considered an important virulence factor, and the EF deletion strain has lower virulence than the wild type [6]. Suilysin is a thiol-activated haemolysin encoded by the *sly* gene, which has a cytotoxic effect and might allow penetration into deeper tissues [14, 15]. Another virulence factor *i.e.* enzyme arginine deiminase in *S. suis* (encoded by the *arcA* gene of the arginine deiminase operon) was recently described which may play a role in survival of the bacterium under stress conditions [16, 17].

Many pathogenic streptococci contain *gapdh* gene encoding GAPDH, a gene shown to play an important role in *S. suis* 2 adhesion to host cells [9]. An adherence protein FBPS (*fbps* gene) that binds to fibronectin in host cells [10] is found in a soluble form in the bodily fluids and in an immobilized form in both the extracellular matrix and on the surface of host cells. Some bacteria bind fibronectin in order to colonize or invade host cells. The hyaluronate lyase (gene *hyl*) of *S. suis* 2 is a secreted protein that degrades hyaluronic acid (HA) into an unsaturated

Disaccharide which in turn increases the permeability of tissues and facilitates the spread of the bacteria.

Attempts to control *S. suis* related diseases are still hampered by the lack of knowledge about the epidemiology of the disease and the lack of reliable, rapid, sensitive and specific diagnostics and effective vaccines. Ambiguous or inconclusive results may be obtained by conventional methods [1]. Thus, detection and control of *S. suis* infection depends increasingly on the availability of rapid and precise diagnostic tests.

The objective of the present study was to determine the prevalence of *S. suis* in pigs as well as their molecular confirmation and detection of four important virulence associated genes namely, *epf*, *mrp*, *sly*, and *arcA* through PCR based techniques (multiplex PCR).

Materials and methods

1. Ethical approval

The research work was duly permitted by the Institutional Animal Ethics Committee. All samples were collected as per standard procedure without harming or laying stress to the animals.

2. Sample collection and processing

The study was conducted during October 2012 to April 2014. A total of 497 samples comprising clinically healthy (n=267) and diseased pigs (n=230) of various age groups of either sex were collected from both organized (n=2) and unorganized farms (n=51) located around greater Guwahati. Samples including nasal swabs, joint fluid, heart blood from live pigs and heart fluid, and pieces of lungs from dead pigs were collected aseptically from clinically healthy pigs as well as pigs exhibiting respiratory tract involvement and died of respiratory disease. The nasal samples were collected with the help of sterile cotton-tipped applicator sticks (Hiculture transport swab, HiMedia Laboratories Pvt. Ltd., Mumbai - 400 086). All the samples were transported to the laboratory at 4-8°C in ice-boxes within 24-48 h of collection for further processing.

3. Bacteria isolation and identification

Samples were directly inoculated into Todd Hewitt broth (HiMedia Laboratories Pvt. Ltd., Mumbai - 400 086) and incubated overnight at 37°C aerobically. Subsequently, isolates were grown on nutrient agar (NA) plates to obtain pure colonies. The isolates were screened for haemolytic reaction on Blood Agar plates containing 5% sheep blood and subjected to different conventional biochemical tests for provisional identification [19]. The isolates were finally confirmed through detection of glutamate dehydrogenase gene (*gdh*) [20].

4. Molecular detection of *Streptococcus Suis* by polymerase chain reaction (PCR)

4.1: Detection of *gdh* gene of *Streptococcus suis*

The detection of *S. suis* by polymerase chain reaction (multiplex PCR) was performed following previously described method [20] using primers specific to *gdh* gene. Extraction of DNA from *S. suis* was done using DNEasy blood and tissue kit (QIAGEN) following the manufacturer's instruction. Base sequence of the primers (5′-3′) used are GCAGCGTATTCTGTCAAACG (JP4) and CCATGGACAGATAAAGATGG (JP5) and predicted size of amplified product was 688 bp.

Amplification of the target gene was carried out by multiplex PCR [20]. Amplification reactions of bacterial DNA for detection of *gdh* were performed in a total volume of 50 µl containing 38 µl of nucleus free water, 5 µl of 10 x PCR Buffer (1.5 mM $MgCl_2$, 150 mM Tris-HCL PCR buffer, MBI Fermentas), 1 µl dNTP mixture, 1µl Taq DNA polymerase, 1 µl primers and 3 µl of the template DNA. The PCR assay was carried out in Thermocycler (Applied Biosystem, Veriti, 96 well Thermal Cycler), comprising 5 min of pre-incubation period at 94 ^{0}C, followed by 35 cycles of 1 min at 94 ^{0}C, 1 min at 55 ^{0}C, and 1 min at 72 ^{0}C. Final extension was performed for 7 min at 72 ^{0}C. Amplified PCR products were analyzed by gel electrophoresis in 1.5% agarose containing ethidium bromide (0.5 µg/ml). The products were visualized with UV illumination and imaged with Gel documentation system (Alpha Innotech Corporation, Multi Image System, CA, USA).

For confirmation, *S. suis* strain (positive for *gdh* gene) maintained at the Animal Health Laboratory of NRC on Pig (ICAR), Rani, Guwahati was used as positive control. Negative control had all the ingredients of PCR except the template DNA.

4.2. Detection of different virulence genes of *Streptococcus suis*

Different virulence associated genes (*sly, mrp, epf* and *arcA*) of *S. suis* were detected by multiplex PCR [21].

The strains were characterized based on the presence of virulence-associated markers namely, suilysin (*sly*), arginine deiminase (*arcA),* muramidase-released protein (*mrp*), and extracellular factor (*epf*) [21] by using multiplex PCR. Initially each target gene was confirmed using individual set of primer by using simplex PCR. The multiplex PCR reaction conditions were optimized with the primers designed as per literature followed [21] targeting the genes *sly, arcA, mrp* and *epf*. Details of all oligonucleotide primers (5′-3′) used are GCTTGACTTACGAGCCACA (slyF) and CCGCGCAATACTGATAAGC (slyR) for amplification of the sly gene, TGATATGGTTGCTGCTGGTC (epfF) and GGACTCGAGGATAGCATTGG (epfR) for *epf* gene, ATTGCTCCACAAGAGGATGG (mrpF) and TGAGCTTTACCTGAAGCGGT (mrpR) for *mrp* gene and TGATATGGTTGCTGCTGGTC (arcAF) and GGACTCGAGGATAGCATTGG (arcAR) for *arcA* gene.

Amplification reactions of bacterial DNA for detection of the genes were performed in a total volume of 50 µl containing 38 µl of nuclease free water, 5 µl of 10 x PCR Buffer, 1 µl dNTP mixture, 1µl Taq DNA polymerase, 1 µl primers and 3 µl of the template DNA. DNA amplification was performed in a thermal cycler (Veriti, 96 well Thermal Cycler), the programme consisted of initial denaturation of 2 min at 94 ^{0}C, 30 cycles of 1 min at 94 ^{0}C, 1 min at 58 ^{0}C, 1.30 min at 72 ^{0}C, and final extension of 2 min at 72 ^{0}C.

Ten µl of the PCR products were separated by electrophoresis on 1.5 % Agarose gels with Ethidium Bromide (0.5 mg/ml) and analyzed by a gel documentation system (Alpha Innotech Corporation, Multi Image System, CA, USA).

5. Results And Discussion

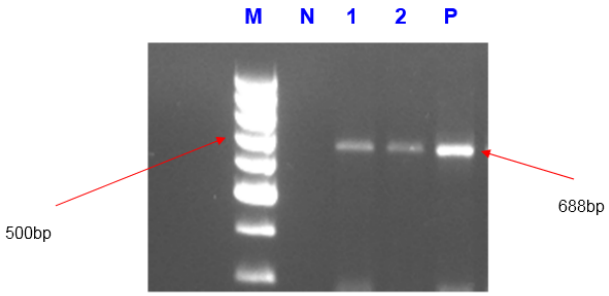
[i] Isolation, identification and molecular confirmation of *Streptococcus* spp.

Out of 497 samples screened, 35 (7.04%) isolates were obtained and identified as *Streptococcus* species based on biochemical parameters of which seven isolates (20.0%) were confirmed as *S. suis* by PCR (TABLE 1 and Fig. 1). Of these

seven *S. suis* isolates, five isolates originated from clinically healthy pigs (two isolates from organized farms; three from unorganized farms) and the remaining two were from diseased pigs belonging to an organized farm.

Table 1: Detection Of *gdh* of *Streptococcus suis* By Multiplex-PCR

Total isolates tested	No of isolates positive for *GDH* gene	Percentage
35	7	20.00



Lane M: Molecular marker (100bp DNA ladder), Lane N: Negative control, Lane 1& 2: Test samples and lane P: Positive control

Fig 1: Detection of *gdh* gene of *Streptococcus suis* by PCR.

For epidemiological studies as well as for eradication purposes, the detection of specific serotypes or strains of *S. suis* in live animals was attempted by using PCR [22]. Identification of *S. suis* infection based on clinical criteria and biochemical and serological tests were time consuming, laborious, and could not unequivocally distinguish *S. suis* from other related organisms. The development of a sensitive DNA-based assay for the identification of *S. suis* isolated from clinical specimens improved the rapidity and accuracy of the diagnosis [1]. Determination of *gdh* gene of *S. suis* by PCR in the present study was in consonance with previous report [22] who identified an *S. suis*-specific 16S RNA region for specific detection of *S. suis*. However, it was also reported that the targeted DNA regions were not conserved across capsular types or pathogenic strains and observed that geographical locations influenced the presence or absence of the targeted genes in several isolates of *S. suis* [23].

Okwumabua *et al.* [24] cloned the house-keeping gene encoding the glutamate dehydrogenase (GDH) of *S. suis* type 2 and had showed that *gdh* gene appeared to be conserved in *S. suis* like in other bacteria. Glutamate dehydrogenase was also used in the diagnosis of certain other bacterial infections [25] and was found to be highly conserved and exhibited an extremely low rate of point mutation relative to other genes [26].

Streptococcus suis type 2 was identified based on morphological, cultural and biochemical characteristics and PCR assay from slaughtered healthy pigs in China (). Similarly, *S. suis* was also detected in pigs and pig farmers in east and west Thailand by targeting 16S rRNA along with bacteriological examination and biochemical identification [28].

[ii] Detection of virulence associated genes of *Streptococcus suis* by multiplex- PCR

All *S. suis* isolates (n=7) were further tested for presence of four virulence-associated genes viz, *sly*, *epf*, *mrp* and *arcA*. None of the virulence associated genes could be detected in the isolates in the present study.

The main virulence-related genes of *S. suis* were glutamate dehydrogenase (*gdh*), capsular polysaccharide (*cps*), lysozyme released protein (*mrp*), extracellular factor (*ef*), hemolysin (*sly*) and virulence-associated sequences orf 2. Most of the studies on virulence factors and the pathogenesis of the infection were focused on capsular type 2 strains. Several workers reported different virulence associated genes from various diseased conditions of pigs from several parts of the world [6, 21, 29-32].

Different serotypes and virulence genes of *Streptococcus suis* from fattening and slaughtered pigs were detected in Chiang Mai city using multiplex PCR [33]. *Streptococcus suis* was detected by PCR targeting the *sly* gene encoding for suilysin, isolated from different clinical conditions like pneumonia and from joints and tonsils of asymptomatic animals [34]. Six virulence genes of *S. suis* serotype 2 were detected by PCR from pigs in Hebei Province [Rui *et al.*, 2012, 35] and those were glutamate dehydrogenase gene (*gdh*), capsular polysaccharides gene (*cps*), muramidate-released protein gene (*mrp*), extracellular factor (*ef*), suilysin gene (*sly*) and virculence-associated sequence (*orf2*).

In the present study, none of the four virulence-associated genes could be detected. However, many workers reported different virulence genes of *S. suis* from different part of the world [10, 21, 28, 33, 35]. Most studies on virulence factors and the pathogenesis of the infection was focused on capsular type 2 strains.

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Competing Interests

The authors declare that they have no competing interests

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