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Streptococcus suis infection in slaughtered pigs and its association with pathological lesions in the lungs, brain and tonsils

Anand Prakash, Ram Kumar, Anoopraj R. and G. Saikumar*
Swine Disease Laboratory, Division of Pathology,
Indian Veterinary Research Institute, Izatnagar-243122 (U.P.)

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ABSTRACT

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To study the prevalence of *Streptococcus suis* infection and associated pathological lesions in pigs, representative specimens from lungs, brains and tonsils collected aseptically from 100 pigs from a local abattoir in Bareilly, Uttar Pradesh, India were processed for bacterial isolation and histopathological examination. *S. suis* isolates were confirmed by colony characteristics, biochemical profiles and PCR using primers specific to 16S rRNA gene. *S. suis* was isolated from 9 pigs with pathological lesions in lungs (5), brain (3) and tonsil (1). Pathogenicity testing in mice revealed that 3 of the 9 isolates (2-lungs and 1-brain) were pathogenic to mice. The study revealed that therewas 9% prevalence of *S. suis* infection associated with pathological lesions in slaughtered pigs in Bareilly city.

Keywords: India, prevalence, pathology, slaughtered pigs, *Streptococcus suis*

INTRODUCTION

Streptococcus suis infections in pigsis responsible for high mortality and significant economic loss to pork industry across the world[1]. *S. suis* is a Gram-positive, lancet shaped organism which appears as pin-pointed colonies on nutrient agar and shows alpha-haemolysis on sheep blood agar[2]. Thirty-five serotypes of *S. suis* have been identified based on capsular antigen of which serotype 2 is a major human and pig pathogen[3]. In pigs, *S. suis* is associated with meningitis, septicemia, endocarditis, pneumonia, arthritis, polyserositis and sudden death[4]. *S. suis* serotypes vary in pathogenicity and clinical signs, both between and even within serotypes[3] and different animal models[5,6]. Moreover, it is an emerging zoonotic agent responsible for septicaemia, with or without septic shock; meningitis and other less common infections in humans[7].

Prevalence of *S. suis* infection among Indian pigs was first reported in 19948. Subsequent reports on *S.suis* infection among pigs are limited to pathogenicity testing of *S. suis* serotype 4 in experimentally infected piglets[9] and sporadic reports on pneumonia caused by the organism[10,11]. In some countries, such as Vietnam, *S. suis* infection has been reported among slaughterhouse workers or those intimately associated with pig husbandry[7]. In view of the scarcity of information on prevalence of *S. suis* infection among pigs in India and its zoonotic potential, we sampled lungs, brain and tonsils from slaughtered pigs and tested them by cultural and biochemical methods, PCR assay and examined the pathological lesions in naturally infected swine and tested virulence of *S. suis* isolates in experimentally infected mice.

***Corresponding author:** e-mail: saikumarivri@gmail.com

MATERIALS AND METHODS

Sample collection

Apparently healthy pigs slaughtered at a local abattoir in Bareilly city, Uttar Pradesh (India) constituted the source of animals for material collection. These pigs were mostly reared under free-range system, in which pigs are let loose to feed on garbage in the semi-urban or peripheral regions of the city. From 100 pigs brought for slaughter, lungs, brain and tonsils with or without any apparent lesions were collected and transported to the laboratory within an hour. Representative samples were stored at -20°C for bacterial isolation using Streptococcus Selective (SS) Broth and a duplicate sample of each was preserved in 10% neutral buffered formalin for histopathology.

Bacterial isolation and biochemical identification

Unless mentioned otherwise, all bacteriological media used in the present study were procured from Pronadisa, Spainand all media were prepared as per manufacturer's instructions. The samples stored at -20°C were thawed and a small piece from different organs was homogenized. The homogenate was transferred to SS Broth and incubated at 37°C for 24 h. A loop-full of inoculum from 24 h broth was streaked on Nutrient Agar (Himedia, India) to get single colonies. Six or seven suspected colonies were then selected and sub-cultured on sheep blood agar and Todd-Hewitt (TH) broth. Genus *Streptococcus* was identified on the basis of colony characteristics on nutrient and blood agar plates, catalase

activity and Gram's staining. Isolated Streptococci were subjected to species characterization by following biochemical tests: Growth in TH broth containing 6.5% NaCl, amylase, VogesProskauer (VP), bile esculin hydrolysis and sugar fermentation tests[2,12].

Genomic DNA preparation and Polymerase Chain Reaction (PCR)

The template for PCR was extracted from biochemically characterized *S. suis* isolates by heat lysis procedure from purified bacterial pellets. The extracted templates were subjected to PCR using previously reported primers for 16S rRNA gene[13]. Amplified products were electrophoresed in 1.5% agarose gel and visualized by ethidium bromide using UV transilluminator. Specificity of the PCR was checked by performing PCR on standard bacterial cultures of *Pasteurellamultocida, Staphylococcus aureus* and *Listeria monocytogenes* and by digesting the PCR products with restriction endonucleases such as Alu1,Hae III and Taq I.

Pathogenecity testing in mice

Four weeks old, 40 Swiss albino mice were procured from "Laboratory Animal Resource Section" of Indian Veterinary Research Institute and were maintained in plastic cages under strict hygienic conditions. These were divided into ten groups of four mice each. After acclimatization for 7 days, nine groups were inoculated intraperitoneally with 0.2 ml ($1.0X10^8$cfu/ml) of seven hour culture of PCR confirmed *S. suis* isolates and the control group was mock infected with 0.2 ml of sterile TH broth. The isolates used for inoculation were grown aerobically at 37°C in TH broth which was sub-cultured from 14-16 h culture of the same organism. Infected mice were observed twice daily for clinical signs such as respiratory distress, depression, lameness and other nervous signs for a period of 10 days. Post-mortem examination was done immediately on severely diseased and dead mice. Surviving mice were sacrificed and necropsied at 10-days post infection. *S. suis* isolate causing death of more than 50% of mice was considered pathogenic. Re-isolation of *S. suis* was attempted from lungs, brain, liver and thoracic and abdominal serosa from infected mice. Internal organs like lungs, liver and brain were thoroughly examined and the gross lesions, if any, were recorded. Representative samples from lungs, brain and liver were collected and processed for histopathology as per standard procedure.

Gross and microscopic pathology

At the slaughterhouse, carcasses were inspected for gross pathological lesions and observed changes were recorded. The samples fixed in 10% neutral formalin were processed conventionally for histopathological examination. Tissue sections (4-5 μm) were stained with Haematoxylinand Eosin (H&E) for routine histopathology and with MacCallum Good Pasture's (MGP) stain for demonstration of bacteria in tissue sections[14].

RESULTS

Bacterial isolation and biochemical identification

After enriching in SS broth and inoculation onto nutrient agar plates, two types of Gram-positive and Catalase negative colonies, indicative of genus Streptococci, were isolated: Broad yellowish-creamy (BYC) colonies were isolated from all samples and Whitish-grey pointed (WGP) colonies were isolated from 83 samples (40 lungs, 40 brains, and 3 tonsils). Gram's staining of BYC colonies revealed thick, plump, round to ovoid Gram-positive colonies in short to long chains while WGP colonies revealed Gram-positive lancet shape organism that occurred singly, in pairs or short chains. BYC colonies exhibited gamma (no)haemolysis on sheep blood agar. Majority of the WGP colonies (55%) produced alpha (incomplete) haemolysison sheep blood agar at 24 h incubation which became wider with prolonged incubation, while 33% isolates displayed no zone of haemolysis surrounding the colony but upon removal of thecolony, alphahaemolysis was observed. Interestingly, one WGP isolate showed beta (complete) haemolysis. BYC colonies grew in TH broth with 6.5% NaCl but WGP didn't. On the basis of Gram staining characteristics, gamma haemolysis on sheep blood agar and growth in TH broth with 6.5% NaCl, BYC were identified as Enterococci and these were excluded from further investigation.

Out of 83 WGP colonies tested, 14 isolates were found Amylase positive and VP negative. These 14 isolates when

Table1. Results of bile esculin hydrolysis, mannitol fermentation and PCR conducted on Amylase positive and VP negative isolates.

Isolate/Tests	P1L#	P1B	P2L	P2B	P7L	P8L	P8B	P33L	P54L*	P55L	P59T	P81B	P83L*	P90B*
Bile Esculin Hydrolysis	+	+	+	+	+	+	+	-	-	-	+	-	-	-
MannitolFermentation	+	+	+	-	+	+	+	-	-	-	-	-	-	-
PCR	-	+	+	-	-	-	-	+	+	+	+	+	+	+

*Pathogenic to mice

The samples were marked with P (Pig) No. followed by type of tissue, lungs (L), brain (B) or tonsils (T). For example P1L stood for Pig No.1 and the type of tissue collected was lung tissue.

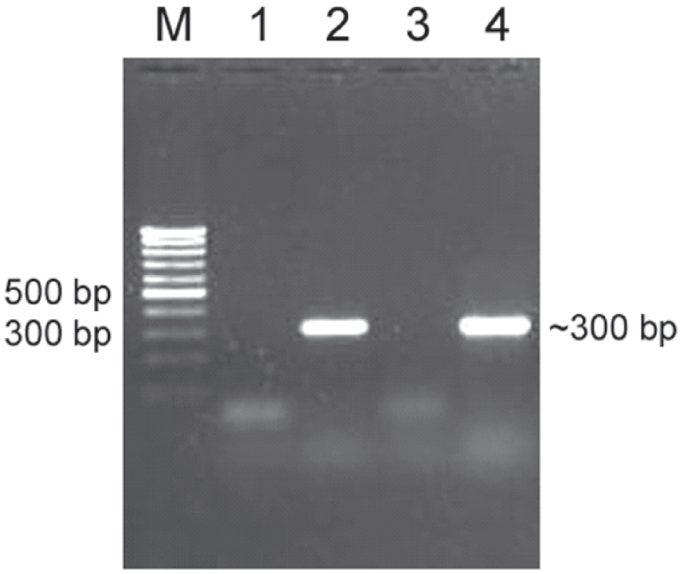

Fig.1. Polymerase Chain Reaction assay for *S. suis* identification. Lane M: 100 bp DNA ladder; Lane 1 and 3: Negative control; Lane 2 and 4: PCR product of *S. suis* (~300bp).

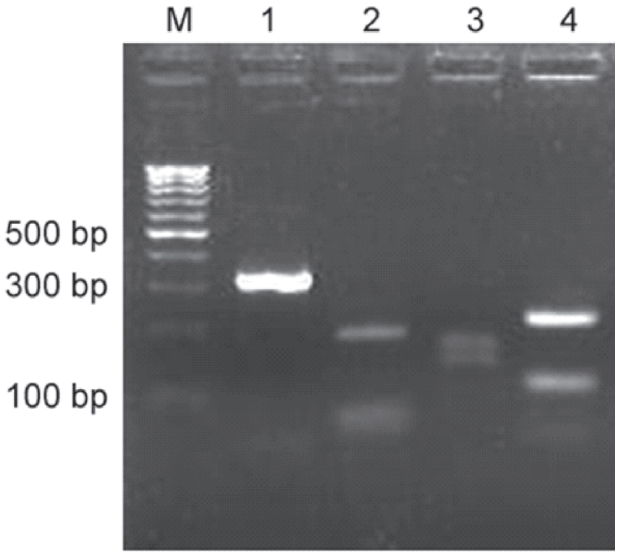

Fig.2. Gel electrophoresis of PCR amplicons digested with different restriction endonucleases. Lane M: 100 bp DNA ladder; Lane 1: undigested PCR product; Lane 2: Alu I digest (<200 and <100 bp); Lane 3: HaeIII digest (135 and 157 bp); Lane 4: TaqI digest (~200 and 104 bp).

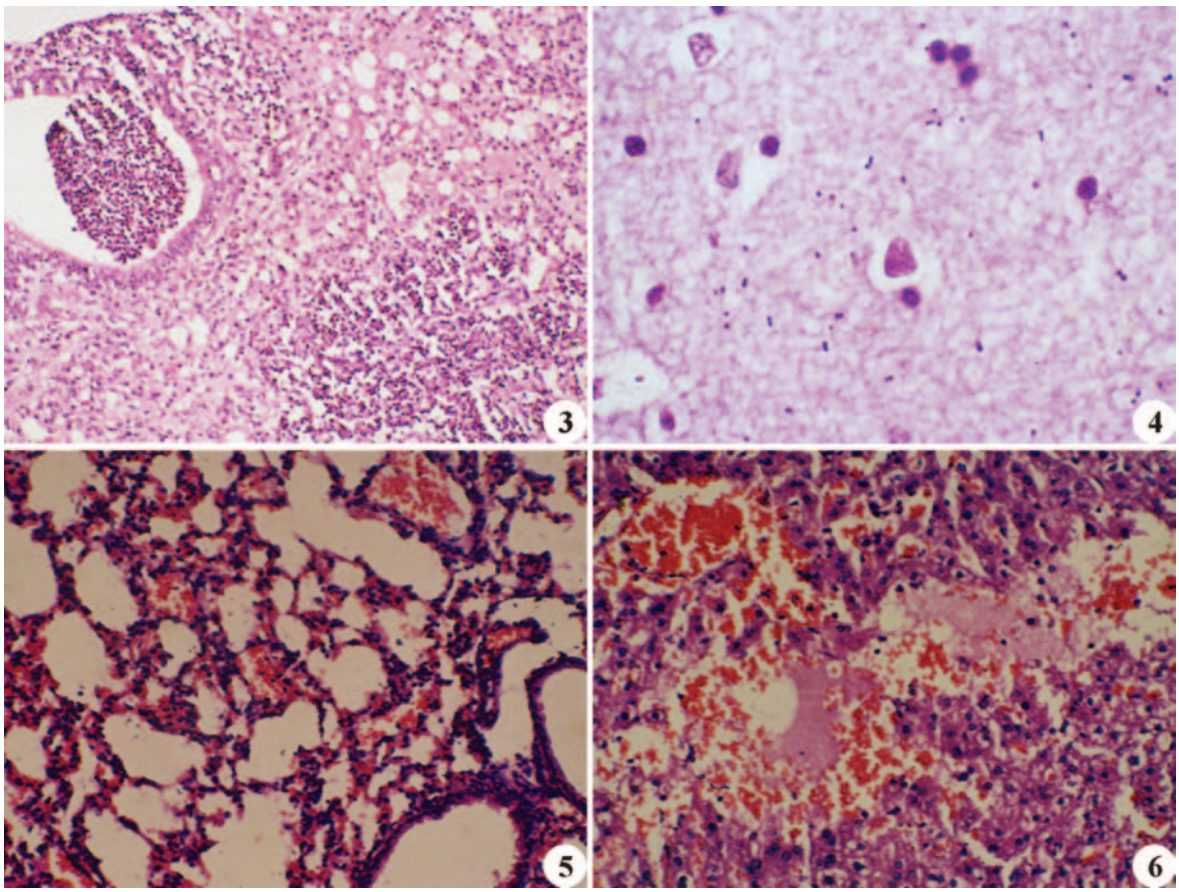

Fig.3. Lungs of a pig with acute bronchopneumonia: Bronchiole and adjacent alveoli containing neutrophils and cellular debris. Alveolar spaces filled with serous exudate. H&E x100. **Fig.4.** Pig brain showing Gram-positive cocci indistinguishable from Streptococci in the cerebral parenchyma.MGP x650. **Fig.5.** Lung of mice infected with *S. suis* showing lesions of acute haemorrhagic pneumonia with alveoli containing RBCs. H&E x100. **Fig.6.** Liver of mice infected with *S. suis* showing haemorrhages and degenerative changes. H&E x100.

subjected to bile esculin hydrolysis test, 8 isolates were found positive for esculin hydrolysis. All 14 isolates (Amylase positive and VP negative) were tested for sugar fermentation and all of them fermented raffinose, salicin and inulin but were unable to ferment sorbitol. Variable results were observed for mannitol sugar fermentation (Table 1).

Polymerase Chain Reaction (PCR)

When 14 isolates tentatively identified as *S. suis* on the basis of biochemical tests were subjected to PCR assay with primers specific for 16s rRNA gene, 9 isolates yielded PCR product which was about 300 bp in size (Table 1; Fig.1). Specificity of the PCRwas verified by testing standard bacterial cultures of *Staphylococcus aureus, Pasteurellamultocida,* and *Listeria monocytogenes* and all of these bacteria returned negative results. Specificity of the PCR products was further confirmed by digesting the PCR amplicons with restriction endonucleases, like single cutter Taq1 (cuts at 104 bp) and double cutters Alu1 (cuts at 72 bp and 258 bp) and HaeIII (cuts at 135 and 157 bp). Expected digestion pattern was observed with Taq1 and HaeIII.Restriction digestion with Alu1 did not yield expected product and provided two fragments of approximately 200 and 100 bp size (Fig. 2).

Gross and microscopic pathology

On the basis of biochemical tests and PCR assay, 5 lungs, 3 brains, and 1 tonsil yielded *S. suis* isolates. Collected slaughterhouse lungs infected with *S. suis* infected lungs revealed lesions of acute bronchopneumonia, acute serofibrinous bronchopneumonia and necrotising pneumonia, one case each, and acute interstitial pneumonia in 2 cases. In acute bronchopneumonia, grossly irregular areas of consolidation and congestion were observed primarily in the apical lobes. The cut surface was moist and bronchioles contained mucus. Histopathologically, bronchioles and immediately adjacent alveoli contained neutrophils and variable amounts of cell debris, mucus, fibrin and macrophages (Fig. 3). In acute serofibrinous pneumonia, lungs appeared red to reddish brown with interlobular septa distended with serofibrinous exudates. Histopathology revealed serofibrinous exudates admixed with various amounts of erythrocytes, a few macrophages and neutrophils in the alveoli and bronchioles. The bronchioles showed denudation of epithelium and mild peribronchiolar infiltration. In necrotizing pneumonia, dark red, irregularly shaped, firm, slightly raised necrotic areas were scattered in various lobes. Microscopically, irregularly shaped necrotic areas were composed of lymphocytes and macrophages and alveolar spaces were filled with necrotic debris. Duplicate sections stained with MGP stain revealed typical Gram-positive cocci to ovoid structures indistinguishable from *S. suis.* In acute interstitial pneumonia, affected areas were highly congested and haemorrhagic on gross examination. Also, the interlobular septae were thickened and areas of consolidation interspersed with emphysema were observed in various lobes. Histologically, the thickened alveolar septa showed infiltration of lymphocytes, mononuclear cells and mild connective tissue proliferation. Occasionally, presence of syncytial cells and mild to moderate peribronchiolar and perivascular lymphoid cell proliferation was also observed.

Of the 3 brains which yielded *S. suis*, 2 cases showed meningeal congestion and one case revealed lesions of acute non-suppurative meningoencephalitis. Meningeal and brain vessels showed dilation and congestion. Mononuclear cell infiltration in brain parenchyma and rarely, perivascular cuffing with lymphocyteswas also observed. The duplicate sections of tissues revealed presence of Gram-positive cocci (Fig. 4).

The tonsil sample which yielded *S.suis* was grossly reddish brown in color with lesions of necrosis and ulceration. Histopathologically, extensive haemorrhages and necrotic debris was observed in the tonsilar crypts.

Pathogenicity testing in mice

Mice infected with PCR confirmed *S. suis* isolates showed nervous signs such as depression and circling. Out of 9 isolates inoculated, 3 (2-lungs and 1-brain) caused death of more than 50% of infected mice. A total of 12 mice out of 40 died. Grossly, hyperemic lesions were observed in lungs, brain, liver, and heart. Microscopically, lungs were characterized by acute haemorrhagic pneumonia (Fig. 5). Brain revealed haemorrhages and oedema and liver showed haemorrhages and degenerative changes (Fig. 6). The organism could be re-isolated from lungs and/or brain tissues of dead mice.

DISCUSSION

In the present study conducted on slaughtered pigs, *S. suis* infection was detected in 9% of the animals examined which is close to the earlier reports of 9% and 9.5% from Thailand and India, respectively[8,15]. According to some previous reports, the prevalence rate varies between 0 to 100%in samples collected from healthy and diseased pigs worldwide[16]. The variation in prevalence rate could be attributed to a number of factors: the sampling size-small versus large samples[17]; age of animal-young versus adults[18]; rearing system: intensive versus extensive system of pig rearing[19] and screening methods: bacterial isolation, immunohistochemistry and PCR[20]. The prevalence rate of infection does not correlate with clinical disease in pigs[18], but the carrier pig could spread the disease to uninfected pigs[17]. Although the present investigation was restricted to a single place in a North Indian State (Uttar Pradesh), but considering previous reports on occurrence of disease caused by *S. suis*in pigs

of Central India[8,9], South India[10] and the Northeast India[11], it is expected that *S. suis* would be present wherever pig farming is done though prevalence may vary from place to place depending on type of farming and management practices followed.

Strangely, we were able to isolate *S. suis* from only one tonsil specimen even though it is the primary site of localization[12]. Often we observed that tonsillar specimens yielded heavy growth of bacteria, almost flooding the entire culture dish, so possibly *S. suis* was overshadowed by growth of other bacteria though SS broth was used. Similar observation has been reported recently[7]. Previously, *S. suis* was obtained in pure culture or as a mixed culture with other bacterial agents such as *Pasteurellamultocida, Escherichia coli, Actinobacillus-pleuropneumoniae, Streptococci,* and mycoplasmalor viral agents from diseased pigs[4]. The role of *S. suis* in these cases as a primary etiological agent is debatable.

In the present investigation, isolates from WGP colonies were tentatively identified as *S. suis* with various biochemical characteristics similar to earlier observations[21]. According to some workers, *S. suis* causes hydrolysis of bile esculin[12] but we observed that only 8 of the 14 isolates were positive for bile esculin hydrolysis and this is in accordance with a previous report which recorded variation among *S. suis* isolates to bile esculin hydrolysis test[22]. Our results confirmed that bile esculin hydrolysis cannot be used as a parameter for identifying *S. suis.* Sugar (inulin, salicin, raffinose and sorbitol) fermentation profile of the isolates in the present study was similar to some previous reports but a wide variation in fermentation pattern was observed, so it is hard to come up with a reliable sugar fermentation test to identify *S. suis*. In the present study, only 9 out of 14 biochemically presumptive isolates could be confirmed as *S. suis* by PCR assay. Since some aerococci and other beta hemolytic streptococci may also be Amylase positive and VP negative[2], biochemical tests alone cannot be relied upon for *S.suis* identification. The PCR assay is more specific and saves considerable time in identification of *S. suis*. On the basis of nucleotide sequence of the 16S rRNA gene, the isolates of present study were identified them as *S. suis* which is not only responsible for serious disease in pigs, but has also been incriminated in severe fatal disease in humans, especially in Southeast Asia. In Vietnam, *S. suis* is the leading cause of bacterial meningitis in adult humans and zoonotic transmission[7]. Chronic osteomyelitis in humans has been recently reported from India but the contribution of *S. suis* to meningitis in human population in the country is still in infancy[23].

S. suis were recovered from 5 lungs, 3 brains and 1 tonsil in the present study. Lesions of bronchopneumonia, necrotizing pneumonia, and interstitial pneumonia were reportedly involved with natural and experimental *S. suis* infection[4,6,24], suggesting a possible etiological role of *S. suis*. However, porcine mycoplasmosis could yield lesions such interstitial pneumonia[25]. We did not assess prevalence of mycoplasma in present study, thus the status of *S. suis* as a primary invader of the lungs is debatable. It might be possible that *S. suis* is an opportunist/secondary invader in lungs, but even then the role of *S. suis* in morbidity and mortality cannot be ignored[1]. *S. suis* was found associated with meningeal congestionand non-suppurative meningoencephalitis and these findings were similar to earlier observations[4]. According to some workers, *S. suis* isolated from brain alone can be considered as a primary etiological agent[1]. The brain isolates were pathogenic to mice and subsequent re-isolation suggested that the isolate was primarily responsible for the aforementioned lesions. Tonsils are considered to be the primary site of *S. suis* localization in healthy and diseased pigs[12]. In the present study, *S. suis* could be isolated from a single case of necrotic tonsillitis and similar observation has been reported[24], but care should be taken while interpreting the lesions as *S. suis* may be a commensal in pigs[2].

In the present investigation, only 3 of the 9 isolates were found pathogenic to mice. This is not unusual as some porcine isolates may not be pathogenic to mice[6], virulence may be lost as a result of prolonged storage at 2°C to 8°C between the isolation and pathogenecity testing[1]. Absence of meningitis in experimentally infected mice corroborates earlier observations that likelihood of mice developing meningitis was related to the size of inoculum and route of administration with chances being high when inoculated intravenously[5].

The prevalence of S.*suis* infection among slaughter pigs in Bareilly city of India was 9%. Biochemical test results, especially for bile esculin hydrolysis and sugar fermentation varied between different isolates. The pathogenic isolates were negative for bile esculin hydrolysis and mannitol fermentation. PCR assay targeting the 16S rRNA gene was useful for confirmation of *S. suis* isolates. In pigs, the organism was found associated with lesions of acute pneumonia, meningitis and tonsillitis. Experimental infection in mice indicated that 2 isolates from lungs and 1 from brain were pathogenic to mice. The prevalence of *S. suis* and its serotypes circulating in pigs in other regions of the country, and virulence profile of the prevailing isolates would lead to a better understanding of *S. suis* and its relationship with disease in pigs and humans.

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