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# Experimental Infection of *Streptococcus suis* Isolate in Pig in Bali – Indonesia Produced Mild Clinical Signs but Severe Multiorgan Lesions

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## ABSTRACT

There has been uncertainty about *Streptococcus suis* (*S. suis*) causing disease in pig. Different inoculation routes might lead to different pathologic courses. Here, we provide data on experimental infection of weaning pigs with *S. suis* isolated from pigs with severe infections through intranasal (IN) and intravenous (IV) inoculation. Nine weaning landrace piglets were used as experimental animals. Three were inoculated with *S. suis* intranasally and three intravenously, each with 1x10$^9$CFU *S. suis*, while the other three were left uninoculated as control animals. Prior to inoculation, the animals were anesthetized using ketamine hydrochloride (10mg/BW) delivered intramuscularly. All control animals remained healthy throughout the study. Fever was observed in all IN and IV animals from 3 to 9 days post infection (dpi). Appetite loss was observed at 3 to 9dpi in the IN group and at 3 to 7dpi in the IV group. Diarrhea occurred in one animal in each group from 3-5dpi. Lameness was observed for one animal in the IN group. Body weights (bwt) on Day 14 of the IN, IV, and control group piglets were 11.93±0.83, 9.80±1.83, and 14.70±0.53kg, respectively. After 14 days, only four animals, three from the IN group and one from the IV group, showed pathological lesions of pneumonia and hemorrhage in the myocardium. Necropsied animals from both inoculation route groups showed inflammation in various organs. Using polymerase chain reaction, we identified *S. suis* from the culture of heart samples taken from animals with hemorrhage in the myocardium. We concluded that although producing a mild clinical course, *S. suis* isolated from severely diseased pigs causes multiorgan histological lesion development and slower weight gain. Appropriate vaccines against *S. suis* should reduce its financial and zoonotic impact.

**Key words:** *Streptococcus suis*, Intranasal, Intravenous, Multiorgan, Mild Sign.

## INTRODUCTION

*Streptococcus suis* (*S. suis*) is a zoonotic pathogen that causes septicemia and meningitis with life-threatening sequelae in people. Its natural reservoir is pigs, and people may be infected after contact with pigs or pork (Lun et al. 2007). Cases of *S. suis* meningitis occur worldwide (Huong et al. 2014a), particularly in patients having occupational contact with pigs or pig products (Arends and Zanen 1988). Due to high pork consumption and traditional pork consumption customs based on uncooked pork delicacies, human *S. suis* infection is endemic in Asia (Wang et al. 2007; Mai et al. 2008; Nghia et al. 2011; Huong et al. 2014b; Takeuchi et al. 2017). The bacteria can cause significant economic losses in the pig industry (Goyette-Desjardins et al. 2014; Ma et al. 2018; Rayanakorn et al. 2021; Piccinini et al. 2022).

The epidemiological pattern of *S. suis* infections in pigs and humans are usually sporadic. Our data from Bali, Indonesia, shows that there have been 44 confirmed cases

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during August 2014 and December 2017 (Susilawathi et al. 2019), although the bacteria has been confirmed to be widely spread in pigs in that province (Besung et al. 2019). A large cluster of zoonotic transmission of *S. suis* in the province that has been reported (Tarini et al. 2022) is considered unusual trough sharing common source of infected traditional food. Veterinary investigation in that cluster cases revealed that around 10% of pig tonsil specimens from private slaughtered houses were positive for *S. suis* (Tarini et al. 2022). All animals were without clinical symptoms.

Experimental infection of an isolate of *S. suis* in Bali needs to be conducted. There has been uncertainty about *S. suis* causing disease in pigs and humans, as the agent is a commensal in the upper respiratory tract. Sub clinically infected pigs are carriers of *S. suis* mainly in the tonsils (Goyette-Desjardins et al. 2014). Different isolates can cause infections ranging from lethal systemic disease to asymptomatic disease, and there is no correlation between the capacity to cause disease in swine and genotype or serotype of an isolate (Nicholson et al. 2020). From another perspective, *S. suis* is considered a secondary pathogen within the porcine respiratory disease complex, and coinfection with other pathogens can significantly influence the severity of the disease (Obradovic et al. 2021). In this regard, the term *S. suis* refers to the "two faces" of the pathobiont in the respiratory tract (Votsch et al. 2018). Colonization in the mucosa seems to lead to a maturation process of *S. suis* to a more invasive state, as has been indicated (Gottschalk and Segura 2000; Doran et al. 2016; Dutkiewicz et al. 2018). In other words, different routes might lead to different clinical and pathological courses of *S. suis* infection.

Here, we provide data on experimental infection of weaning pigs with *S. suis* isolated from severely diseased pigs through intranasal and intravenous inoculation.

## MATERIALS AND METHODS

Ethical approval for this experiment has been granted by the Ethics Committee of Animal Experiments of the Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia, on June 6, 2019, number B/79/UN14.2.9/ PT.01.04/2019.

A *S. suis* isolate from field cases of sick pigs and confirmed to be serotype 2 or ½ designated PPB5 (Besung et al. 2019) were used in this study. The isolate was aliquoted and preserved in a deep freezer (-80°C) with 10% glycerol. The bacteria were sampled using an inoculation loop, spread on 5% sheep blood agar and incubated in a 37°C incubator with 5% $CO_2$ for 24 hours. One colony was picked using a micropipette tip, inoculated into 2mL medium tryptic soy broth (TSB) and incubated for an additional 6h. Glutamate dehydrogenase (GDH), recombination/repair protein (recN) gene fragments, and cps2I for serotype 2 or ½ were reconfirmed as previously published (Susilawathi et al. 2019). One hundred microliters of culture were added to 50mL of TSB and cultured overnight. The bacterial culture was then washed using PBS through centrifugation at 4°C for 10min, which was repeated three times. The bacteria were resuspended in PBS in a final volume of 10mL. Serial dilution cultures of $10^6$-, $10^7$-, $10^8$-, and $10^9$-fold were made in a volume of one milliliter each. One hundred microliters of the dilution were spread on 5% blood agar in two agar plates. After overnight incubation, the number of colonies was counted, and the content of the culture in colony-forming units (CFUs)/mL was calculated.

Nine weaning landrace piglets were used as experimental animals. The animals were collected from an isolated village in Bali, Indonesia, where no sign of *S. suis* has been reported. Three were inoculated with *S. suis* intranasally and three intravenously, and the other three were left uninoculated as control animals. The body weight of all animals was between 9-10kg. The animals were kept in separate cages with no direct contact between groups at the animal facility of the Disease Investigation Center Denpasar, Indonesia. All animals tested negative for *S. suis* in tonsil swab samples, that were assessed using the GDH primer set, and were free of antibody against *S. suis*, as detected using an ELISA with whole extract of a *S. suis* culture as a coating antigen. The animals were acclimatized for one week prior to inoculation. All animals had access to automatically delivered water. Feed was given twice per day with a commercial pig ratio. All staff working in the facility were protected with complete PPE, such as goggles, a head cover, waterproof shoe covers, an N95 mask, a gown, and waterproof boots.

Bacterial inoculation in mice was conducted as the following. For intranasal inoculation, animals were anesthetized using ketamine hydrochloride (10mg/BW) delivered intramuscularly. The nasal cavity was rinsed with 1% acetic acid as previously published (Sun et al. 2018). Three hours after acetic acid application, the animals were anesthetized, and $1x10^9$CFU of *S. suis* was applied to the nasal cavity using a syringe without a needle. For intravenous inoculation, the animals were anesthetized, and one milliliter of bacterial suspension containing $1x10^9$CFU was inoculated into the auricular vein. Measurement of body temperature and observation of clinical signs were conducted twice per day. Body weight was measured every three days. As none of the animals showed severe clinical signs, the animals were euthanized using pentobarbital delivered intravenously (100mg/BW) and necropsied at Day 14. Pathological examination was conducted for the following tissues: brain, trachea, lung, inguinal lymph nodes, esophagus, stomach, small intestine, large intestine, pancreas, liver, spleen, heart, kidney, urinary bladder, and knee joints. Upon necropsy, animals and organs were washed with a chlorine rinse.

All tissue samples with or without macroscopic lesions were collected. All tissues were paraffin embedded following a standard protocol and stained with hematoxylin-eosin (HE) (Kiernan 2015). Tissue examination was conducted using a stereomicroscope at 400x magnification.

Heart samples were also taken from animals with hemorrhage in the myocardium before disinfection. The tissue was homogenized in PBS, cultured on 5% sheep blood agar and incubated in a 37°C incubator with 5% $CO_2$ for 24h. Five colonies were grown separately in TSB

and incubated overnight. The bacterium was identified using PCR of GDH.

## RESULTS

The daily number of pigs showing clinical signs of fever, appetite loss, diarrhea, and lameness following intranasal (IN) and intravenous (IV) infection with *S. suis* as well as those of control pigs is presented in Table 1. All control animals remained healthy throughout the study. Fever was observed in all intranasally and intravenously inoculated animals from 3 to 9days post infection (dpi). Appetite loss was observed at 3 to 9dpi in the IN group and at 3 to 7dpi in the IV group. Diarrhea occurred in one animal in each group from 3-5dpi. Lameness was observed for one animal in the IN group.

Body weights of the intranasal, intravenous, and control group pigs at Day 14 were 11.93±0.83, 9.80±1.83, and 14.70±0.53kg, respectively. The macroscopic lesions of animals harboring lesions are presented in Fig. 1. After 14 days, only four animals, three from the IN group and one from the IV group, showed pathological lesions of pneumonia and hemorrhage in the myocardium.

Histopathological findings of various tissues of two pigs from the *S. suis* intranasal and intravenous infection groups are presented in Table 2. Both necropsied animals from both inoculation route groups exhibited meningoencephalitis, tracheitis, interstitial pneumonia, gastritis, pancreatitis, hepatitis, perifollicular hemorrhage in the spleen, follicle activation in lymph nodes, interstitial glomerulonephritis, and cystitis. No sign of arthritis was observed. Microscopic lesions found in one animal of each group were esophagitis, colitis, and enteritis. Lesions found in the cerebellum were hemorrhage and meningitis in the IV group only in both necropsied animals.

Using PCR of GDH, we identified *S. suis* from the culture of heart samples taken from animals with hemorrhage in the myocardium (data not shown).

## DISCUSSION

The pathogenesis of *S. suis* infection seems very complex and involves the host, the infectious agent, and environmental factors. Although the most common serotype isolated from severe infections in pigs and humans is serotype 2 (Gottschalk et al. 2007; Feng et al. 2014; Goyette-Desjardins et al. 2014; Kerdsin et al. 2018), and the serotype of the isolate under our research has been identified as serotype 2 or ½ and isolated from severely diseased pigs (Besung et al. 2019), in general, we produced only mild disease in our experiment. Different isolates can cause a spectrum of diseases from lethal systemic disease to an asymptomatic course, which is unrelated to genome size, serotype, sequence type, or *in vitro* virulence-associated phenotypes (Nicholson et al. 2020). Therefore, *S. suis* can be considered a secondary pathogen within the porcine respiratory disease complex, and coinfection with other pathogens can influence the disease outcome (Obradovic et al. 2021). The term "two faces" for this pathobiont in the respiratory tract (Votsch et al. 2018) seems valid. In our study, the route of inoculation seemed to produce a slight difference in the clinical signs, as intranasal infection resulted in longer durations of fever, appetite loss, and lameness. Colonization in the mucosa seems to be needed toward a more invasive phenotype (Gottschalk and Segura 2000; Doran et al. 2016; Dutkiewicz et al. 2018).

Mild clinical signs with no fatality, despite multiorgan histological involvement, were notable in our study. Clinically, all animals recovered after 9dpi without any treatment. Only one animal from each inoculation group exhibited diarrhea, and only one animal from the intranasal group showed lameness. Localized macroscopic lesions in the lung and heart were also observed in our study. Sun et al. (2018) managed to induce meningoencephalitis signs in pigs intranasally with an isolate from a pig with meningitis signs, while that from a pig with arthritis did not. However, Seele et al. (2018) produced severe clinical signs with intravenous inoculation in one-third of experimental pigs. The inflammatory response of individual animals seems responsible for this observation (Seele et al. 2018; Salogni et al. 2022). From an infectious agent point of view, other groups have reported that there is a strong association between serotype and the production of extracellular factors (EFs) (Wisselink et al. 2000).

**Table 1:** Daily Number of pigs showing clinical sign of fever, appetite loss, diarrhea, and lameness following intranasal (IN) and Intravenous (IV) infection of *S. suis* isolated from pig in Bali – Indonesia.

| | Clinical Signs | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fever | | | Appetite loss | | | Diarrhea | | | Lameness | | |
| Observation day | IN | IV | Control | IN | IV | Control | IN | IV | Control | IN | IV | Control |
| 1 | 0/3 | 1/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 2 | 0/3 | 1/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 3 | 1/3 | 2/3 | 0/3 | 2/3 | 2/3 | 0/3 | 1/3 | 1/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 4 | 3/3 | 3/3 | 0/3 | 3/3 | 3/3 | 0/3 | 1/3 | 1/3 | 0/3 | 1/3 | 0/3 | 0/3 |
| 5 | 3/3 | 3/3 | 0/3 | 3/3 | 3/3 | 0/3 | 1/3 | 1/3 | 0/3 | 1/3 | 0/3 | 0/3 |
| 6 | 3/3 | 3/3 | 0/3 | 3/3 | 3/3 | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 0/3 | 0/3 |
| 7 | 3/3 | 3/3 | 0/3 | 3/3 | 3/3 | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 0/3 | 0/3 |
| 8 | 3/3 | 3/3 | 0/3 | 1/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 0/3 | 0/3 |
| 9 | 3/3 | 3/3 | 0/3 | 1/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 10 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 11 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 12 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 14 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |

The number of animals per group was three.

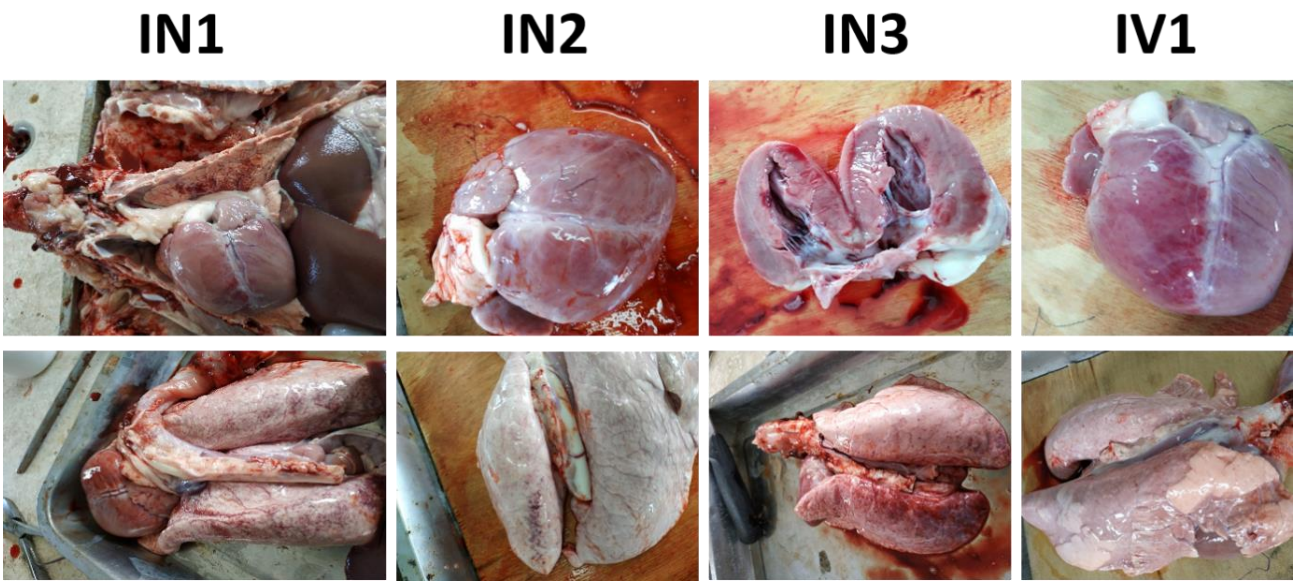


**Fig. 1:** Macroscopic lesions in the heart (above) and lung (bottom) of experimental pigs inoculated intranasally (IN) and intravenously (IV) with *S. suis* isolated from severe field cases. All intranasal infected animals (IN1-3) showed hemorrhage in both organs while only one out of three intravenously infected animal (IV1) show picture of hemorrhage in hearts as well as sign of emphysema in the lung.

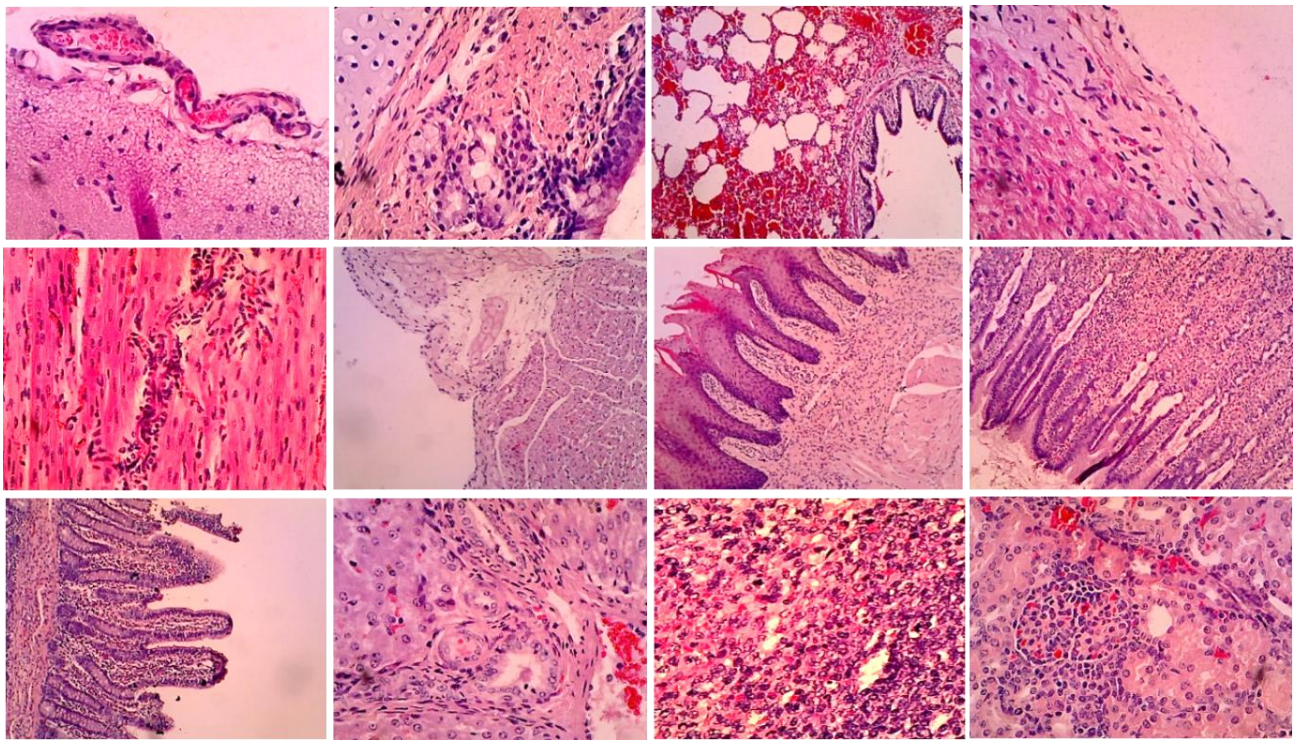

**Fig. 2:** Examples of histopathological pictures of various organs of experimental pigs inoculated intranasally and intravenously with *S. suis* isolated from severe field cases. Pictures is upper row are meningitis, tracheitis, bronchopneumonia, and pericarditis. Pictures in middle row are myocarditis, endocarditis, esophagitis, and gastritis. Pictures in bottom row are enteritis, hepatitis, perifollicular hemorrhage in spleen, and glomerulonephritis. Hematoxylin-Eosin; 400X.

How to induce an inflammatory response and stimulate the culture to produce EFs that can lead to severe disease prior to inoculation remains to be elucidated.

The route of inoculation seems to result in slightly different lesions. Different pathological findings were also seen in necropsy. All three animals from the intranasal group exhibited pneumonia and hemorrhage in the myocardium, while only one from the intravenous group showed the same lesions. Other animals showed no pathological lesions upon necropsy in any tissues examined. The organs outside of the lung and heart of all four animals were also clean without any remarkable changes. These results indicate again that our experiment tended to produce a mild lesion. As discussed above, colonization in the mucosa seems to result in a maturation process of *S. suis.*

**Table 2:** Histopathological impression of various tissues of two pigs from intranasal and intravenous infection of *S. suis* isolated from pig in Bali-Indonesia

| Organ | Animal No. | Intranasal | Intravenous |
|---|---|---|---|
| Brain | 1 | Congestion and inflammation predominantly neutrophil in meningen; Cerebellum normal | Congestion and inflammation predominantly neutrophil in meningen and necrosis neuron; Congestion and inflammation with predominantly neutrophil in cerebellar meningen |
| | 2 | Severe congestion, haemorrhage, and inflammation predominantly neutrophil in meningen; Cerebellum normal | Severe congestion, haemorrhage, and inflammation predominantly neutrophil in meningen as well as microglia proliferation; Congestion and inflammation with predominantly neutrophil in cerebellar meningen |
| Trachea | 1 | Inflammation | Necrosis epithelia and sub-epithelial gland |
| | 2 | Inflammation | Focal necrosis in epithelial cell and inflammation in subepithelial area |
| Lung | 1 | Inflammation with predominant neutrophil from septa alveoli to bronchioles | Inflammation with predominant neutrophil from septa alveoli to bronchioles; Haemorrhage in the bronchiole lumen; Macrophage in septa alveoli |
| | 2 | Inflammation with predominant neutrophil and haemorrhage from septa alveoli to bronchioles | Inflammation with predominant neutrophil from septa alveoli to bronchioles; predominant macrophage |
| Heart | 1 | Fibroblast proliferation with macrophage and neutrophil infiltration in pericardium, myocardium and endocardium | Fibroblast proliferation with macrophage and neutrophil infiltration in pericardium, myocardium and endocardium |
| | 2 | Fibroblast proliferation with macrophage and neutrophil infiltration in pericardium | Macrophage and neutrophil infiltration in myocardium |
| Esophagus | 1 | Follicle activation and surrounding fibroblast activation | Normal |
| | 2 | Normal | Infiltration of macrophage sub-epithelial area |
| Gastric | 1 | Macrophage and neutrophil infiltration in lamina propria | Focal necrosis and inflammation in mucosa as well as inflammation in lamina muscularis |
| | 2 | Macrophage and lymphocyte infiltration in lamina propria | Normal |
| Intestine | 1 | Infiltration of macrophage and neutrophil in lamina propria | Infiltration of macrophage and lymphocyte in the intestinal villi |
| | 2 | Infiltration of macrophage and neutrophil in lamina propria | Congestion, haemorrhage and inflammation with macrophage and lymphocyte |
| Colon | 1 | Neutrophil and macrophage infiltration around gland | Lymphocyte and macrophage infiltration around gland |
| | 2 | Normal | Normal |
| Pancreas | 1 | Congestion between acinar cells | Normal |
| | 2 | Necrosis of acinar cells | Necrosis of acinar cells |
| Liver | 1 | Congestion and inflammation in portal area | Congestion and inflammation in portal area |
| | 2 | Congestion and inflammation in portal area | Congestion and inflammation in portal area with swollen hepatocytes |
| Spleen | 1 | Haemorrhage perifollicular | Haemorrhage perifollicular |
| | 2 | Congestion in medulla | Haemorrhage perifollicular |
| Inguinal Lymph Node | 1 | Lymphoid follicle activation in cortex and medulla | Lymphoid follicle activation in cortex and medulla |
| | 2 | Lymphoid follicle activation in cortex and medulla | Lymphoid follicle activation in cortex and medulla |
| Kidney | 1 | Swollen cells and inflammation in glomerulus and interstitial tissue. Congestion in glomerulus and intratubular tissue | Swollen and necrotic cells and inflammation in glomerulus and intratubular tissue |
| | 2 | Necrotic in glomerulus and macrophage and neutrophil infiltration | Swollen cells and inflammation in glomerulus. Macrophage infiltration in interstitial spaces |
| Urinary Bladder | 1 | Swollen cells and proliferation of epithelia and proliferation of fibroblast in sub-epithelial area | Thickening of epithelia cells |
| | 2 | Swollen cells and proliferation of epithelia and proliferation of fibroblast in sub-epithelial area | Normal |

Multiorgan involvement was remarkable in the histopathological findings. Both necropsied animals from both inoculation route groups showed the same tissue changes in various organs. A previous publication showed meningitis, pleuritis, peritonitis, synovialitis, splenitis, hepatitis, pneumonia or endocarditis (Seele et al. 2018), while other group (Sun et al. 2018; Wang et al. 2022) reported encephaloma, purulent lung lesions, spleen swelling and infarction, kidney nephremia and hydrops articulation. Histologically, they described meningitis, such as neuronophagia, edema, and hyperemia, inflammatory cell infiltration and purulent lesions in lung tissue, and hyperemia in the kidney and spleen.

Slow body weight gain was evident in our study. Intravenous inoculation affected body weight more than intranasal inoculation. After two weeks of observation, the control group was 2.8kg and 4.1kg heavier than the intranasal and intravenous groups, respectively. Intravenous inoculation seemed to allow the bacterium to colonize various tissues. This indicator has never been observed in scientific publications. This is an important observation for pig production: *S. suis* might contribute to slow weight gain, and appropriate vaccines might affect the income of the industry. Although producing relatively mild clinical signs, Koch's postulate was established in our study. *S. suis* was identified from the culture of heart samples taken from animals with hemorrhage in the myocardium. These findings should eliminate any hesitancy that *S. suis* is only a commensal agent of the upper respiratory tract of pigs.

This study was conducted using conventional non-specific pathogenic-free (non-SPF) pigs. The animals were collected from an isolated village in Bali, Indonesia, where no sign of *S. suis* has been reported, owned by a veterinarian and a coauthor. The animals were kept in separate cages with no direct contact among groups at the animal facility of the Disease Investigation Center Denpasar, Indonesia. All animals tested negative for *S. suis* and were free of antibody against *S. suis*. No direct contact was proven in our study, as control animals remained healthy clinically and pathologically throughout the study. No contamination among animals was identified.

## Conclusion

Infection with *S. suis* isolated from severely diseased pigs causes a mild clinical course with multiorgan histological lesion development and slower weight gain. The presence of the bacterium in the pig industry could cause significant economic loss and a higher risk of human infection. Appropriate vaccines against *S. suis* should reduce its financial and zoonotic impact.

## Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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## Author Contribution

IBO Winaya and NK Suwiti were responsible for pathologic evaluation. GN Mahardika and BK Mahardika were responsible for molecular works. P Wiliantari, NM Susilawathi, KK Agustina, PH Sudipa and INK Besung were conducting the animal experiment. IGK Suarjana and INK Besung were supervising bacteriological work. GN Mahardika and INK Besung drafted the manuscript.

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