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ORIGINAL ARTICLE

Streptococcus equi subsp. *zooepidemicus* infection of pigs leads to shedding in faeces and a carrier state

Matheus de Oliveira Costa[1,2] | **John Clare Samuel Harding**[1] | **Yanyun Huang**[3] | **Roman Nosach**[1]

[1]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

[2]Department of Population Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

[3]Prairie Diagnostic Services Inc., Saskatoon, Canada

Correspondence
Matheus de Oliveira Costa, Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada.
Email: matheus.costa@usask.ca

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Abstract

In 2019, *Streptococcus equi* subsp. *zooepidemicus* was recognized as an emerging pathogen of swine, associated with sudden deaths, increased abortion rates and septicaemia. Limited data are available regarding this disease in pigs. The objectives of this study were to clarify clinical progression, pathogen shedding, transmission, gross and microscopic lesions following infection in pigs. Six weeks old pigs were inoculated with either *S. zooepidemicus* sequence type 194 (inoculated, $n = 6$) or sham inoculated with sterile culture broth (sentinels, $n = 4$). Animals were housed in the same room, in two pens 2 m apart. Pigs were monitored twice daily for clinical signs, and rectal, nasal and oral swabs were collected once daily. A full necropsy was performed if welfare was a concern or at 5 days post-inoculation (dpi). All sentinels remained disease free and their samples tested negative for the pathogen of interest. All inoculated pigs developed fever within 8 h of inoculation, and severe disease was observed after 2 dpi. A total of 4/6 inoculated pigs developed clinical signs that compromised animal welfare and were euthanized. Nasal swabs (15/23), followed by rectal swabs (9/23) yield the highest number of positive *ante-mortem* samples. Clinically healthy, inoculated pigs had detectable levels of *S. zooepidemicus* in rectal and nasal swabs. Reactive submandibular lymph nodes, kidney petechiae and splenomegaly were found in six of six inoculated pigs. These data suggest that subclinically infected pigs may spread the pathogen through nasal secretions and faeces. Direct contact seems to be required for transmission.

KEYWORDS
disease, infection, pig, streptococcus, swine, zooepidemicus

1 | INTRODUCTION

Streptococcus equi subsp. *zooepidemicus* (*S. zooepidemicus*) is considered an opportunistic pathogen of several warm-blooded hosts, including humans, equidae, camelidae, caninae, and suidae (Cebra et al., 2000; Corpa et al., 2018; Kernaghan et al., 2012; Li et al., 2021; Priestnall & Erles, 2011). It is a Gram-positive, β-haemolytic coccus belonging to the Lancefield group C. Severe disease characterized by pneumonia, septicaemia and meningitis has been associated with *S. zooepidemicus* (FitzGerald et al., 2017; Pelkonen et al., 2013). Historically, this

bacterium has been suggested as a normal inhabitant of the palatine tonsils of pigs, being detected by both culture and high-throughput sequencing in samples collected from healthy animals (Kernaghan et al., 2012). However, virulent strains of *S. zooepidemicus* reportedly killed over 300,000 pigs in China in the 1970s, associated with high-mortality outbreaks of sudden death and respiratory disease (Feng & Hu, 1977). Early in 2019, the first outbreaks of sudden deaths, increased mortality and increased abortion rates associated with *S. zooepidemicus* in pigs housed in commercial facilities in North America were reported (Costa & Lage, 2020). Since then, the pathogen was also isolated from outbreaks of septicaemic disease and increased mortality throughout the northern United States, Canada, New Zealand (D. Lawton, personal communication) and the Netherlands (Chen et al., 2020; Houben et al., 2021; Sitthicharoenchai et al., 2020). Interestingly, *S. zooepidemicus* sequence type 194 (ST-194) was associated with all these outbreaks, except for the one in the Netherlands where a new sequence type was identified (Houben et al., 2021).

An initial study described the experimental infection of finisher pigs and sows with *S. zooepidemicus* isolates obtained from different hosts, including ST-194 (Hau et al., 2021). Clinical disease progression, cytokine response, gross and microscopic lesions were described, as well as the lack of cross-protection between isolates obtained from horses and ST-194. Currently, there are no commercial vaccines available for this pathogen. Until 2019, control and prevention methods were not applied given its commensal nature, the lack of evidence of disease in North America and the knowledge gap regarding the transmission routes and pathogen shedding patterns in pigs.

The goal of the work described here was to develop a *S. zooepidemicus* infection model using 6 weeks old pigs (a logistically more efficient age), and to clarify clinical progression, pathogen shedding, transmission, and gross and microscopic lesions following infection.

2 | METHODS

2.1 | Inoculum preparation

Streptococcus equi subsp. *zooepidemicus* ST-194 isolate was recovered from a finisher pig presenting with clinical signs and lesions of septicaemia and disseminated intravascular coagulation. Strain identification was confirmed by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer and whole-genome sequencing (Costa & Lage, 2020). The inoculum was prepared by culturing the isolate on brain-heart infusion (BHI) broth at 37°C on a 5% CO_2 atmosphere overnight. The resulting inoculum contained 4.5×10^6 CFU/ml.

2.2 | Inoculation trial and sampling

Ten healthy pigs (n = 5 males and n = 5 females) sourced from a high-health herd, historically free of major swine pathogens including porcine reproductive and respiratory virus (PRRSV), influenza A virus of swine (IAV-S), *Mycoplasma hyopneumoniae*, *Erysipelothrix rhusiopathiae*, *Salmonella enterica* serovar Cholerasuis, *Actinobacillus suis*, *Actinobacillus pleuropneumoniae*, *Lawsonia intracellularis* and *Brachyspira hyodysenteriae* were used in this study. At 5 weeks of age, pigs were transported to a level 2 biocontainment facility to acclimate for 7 days prior to inoculation. Upon arrival, a nasal swab was collected from all pigs and they were randomly allocated to two groups blocked by sex: sentinel (SENT, n = 4) and inoculated (INOC, n = 6). All pigs were housed in the same room with the SENT pen physically separated from INOC pen by 1.8 m. For every entry, researchers performed all activities in the SENT pen first, and then entered INOC pen. In addition, boots were rinsed prior to entering pens and dipped on a foot-bath with 5000 ppm sodium hypochlorite solution upon entering and leaving any pen. During the entire experimental period, pigs had ad libitum access to unmedicated starter diet and water. On 0 days post-inoculation (dpi), all animals received either *S. zooepidemicus* ST-194 (INOC) or sterile BHI broth (SENT) once, intranasally (0.5 ml/nostril) using an atomizer (MAD Nasal, Teleflex Medical, ON) and 1 ml orally using a 1 ml syringe.

Pigs were monitored for clinical signs once daily pre-inoculation and twice daily post-inoculation for body condition (0 = normal body condition and gut fill; 1 = normal body condition, hollow flanks; 2 = loss of body condition, back bone evident, weight loss up to 15% of body weight; 3 = moderate loss of body condition, back bone prominent, weight loss greater than 15% of body weight), respiratory rate (0 = normal rate, 25–35/min; 1 = increased rate, >35/min; 2 = increased rate, >35/min, dyspnoea and/or coughing; 3 = increased rate, >35/min, marked dyspnoea and/or persistent paroxysmal coughing), skin colour (0 = normal; 1 = small area, <50%, of subcutaneous hyperaemia on extremities; 2 = >50% of extremities or abdomen have hyperaemia or cyanosis, but no necrosis; 3 = >50 of extremities or abdomen have hyperaemia, cyanosis or necrosis of the skin), faecal consistency (0 = formed, normal; 1 = loose faeces; 2 = runny or watery diarrhoea; 3 = mucoid diarrhoea; 4 = bloody diarrhoea), responsiveness (0 = alert and active; 1 = alert, but slower than cohorts; 2 = reluctant to move, but moves by stimulation; 3 = does not respond to stimulation or has seizures), and rectal temperature (measured once daily for welfare reasons). Starting on 0 dpi, rectal, oral and nasal swabs were collected daily from all animals. Swabs were frozen at −20°C until processing for PCR analysis.

Animals were euthanized when welfare became compromised (following a clinical score at any category greater than 2 and clinical assessment by a board-certified swine health specialist), or at 5 dpi (INOC) and 7 dpi (SENT). Two (530, 534) INOC pigs that had clinical signs but recovered were euthanized prior to 5 dpi to verify the presence of viable *S. zooepidemicus* in post-mortem samples. Following euthanasia, a complete necropsy was performed to characterize gross lesions. Any visibly affected organs, heart, submandibular lymph nodes (LN), spleen, liver, tonsil, cranial lung, and serum (collected directly from the brachial artery following euthanasia to avoid contamination) were harvested for histopathology (when applicable) and bacterial culture. Terminal sampling also included central nervous system swabs collected through the foramen magnum followed by PCR testing as described below.

2.3 | Histopathologic assessment

Formalin-fixed tissue samples were routinely processed, sectioned, and stained using haematoxylin and eosin (H&E). A board-certified pathologist, blinded to slide identity, characterized the microscopic lesions.

2.4 | *Streptococcus equi* subsp. *zooepidemicus* real-time PCR and culture procedures

Post-mortem samples were refrigerated until processed. Samples were plated on Columbia Nalidixic Acid agar supplemented with 5% sheep blood (Thermo Fisher Scientific, Burlington, ON) and cultured at 37°C in a 5% CO_2 medium for 24 h. Beta-haemolytic mucoid colonies were further speciated by MALDI-TOF (bioMérieux VITEK M, Saint-Laurent, QC). A hydrolysis probe-based real-time PCR protocol was developed for detection of *S. zooepidemicus* and *S. zooepidemicus* strain ST194 using previously described methods (Rozen & Skaletsky, 2000). Two sets of primers and probes were developed and used in this study: a *S. zooepidemicus* specific primer-probe set: forward MC099: 5′-GGTAATGGTCCACAGGTTGG-3, reverse MC100: 5′-GCTGCCACTTCCTTTGTGAT-3′, probe MC101: 5′-FAM AGACAATGAGCTRCAAGCCCAAGGCA BHQ1-3′; and a ST194-specific set: forward MC102: 5′-GGCAAGGTTAGCCCCAATCA-3′, reverse MC103: 5′-TCTTGAGCATGTGGTGAGGG-3′, probe MC104: 5′-FAM TCTACCAAGCCCACACATCAC BHQ1 −3′. A cocktail containing both primer/probe sets was tested against *Streptococcus equi* subsp. *equi* isolated from horses ($n = 6$), and *S. zooepidemicus* isolated from pigs ($n = 4$) and horses ($n = 1$), as well as *Streptococcus canis*, *Streptococcus suis*, *Streptococcus equisimilis* and *Streptococcus pyogenes* ($n = 1$ each). *Streptococcus equi* subsp. *equi*, *S. canis*, *S. suis*, *S. equisimilis* and *S. pyogenes* were not detected by either probe, while the ST-194 was positive for the *S. zooepidemicus* isolated pigs and negative for the horse isolate (data not shown). DNA from swabs was extracted using the MagMax Core Nucleic Acid extraction kit (Applied Byosystems, ThermoFisher Scientific, Ottawa, ON). PCR reactions were carried out using AgPath-ID One-step RT-PCR (Applied Byosystems, ThermoFisher Scientific, Ottawa, ON), and all reaction plates included a no template, blank and bacterial positive control. Samples were analyzed in duplicates, and cycling conditions included 120 s at 95°C, followed by 40 cycles of 5 s at 95°C and 33 s at 60°C. Duplicates with a *Ct* variation greater than 1 were re-analyzed. A sample was considered positive if $Ct < 35$.

2.5 | Statistical analyses

All analyses were performed on SPSS v24 (IBM Corp., Armonk, USA).

3 | RESULTS

All clinical parameters from all pigs were within the normal range prior to inoculation, and nasal swabs collected at arrival yielded negative PCR results for *S. zooepidemicus*. SENT pigs remained free of clinical signs throughout the experimental period. Select animals in the INOC group were euthanized due to severe clinical signs or welfare concerns on 2 ($n = 1$), 3 ($n = 1$), 4 ($n = 2$) and 5 ($n = 2$) dpi. All INOC pigs developed fever within 8 h of inoculation (Figure 1). INOC pigs developed lethargy, were reluctant to move unless physically stimulated (4/6) as early as 1 dpi and as late as day 5. The other main clinical abnormality identified was increased respiratory rate (3/6), associated with dyspnoea (2/6) and marked abdominal breathing (1/6). One INOC pig developed focal skin hyperaemia on 5 dpi (#533). Neither lameness nor diarrhoea was observed. Interestingly, two INOC pigs developed only mild clinical signs and transient fever (#527, #534). A summary of clinical scores is presented in Table 1.

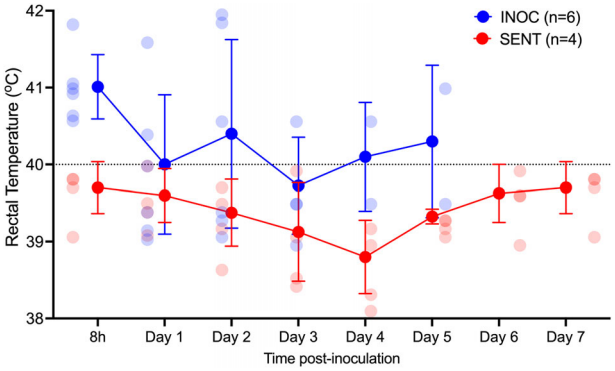

FIGURE 1 Daily rectal temperature measurements following inoculation. INOC: Pigs inoculated with *S. zooepidemicus* ST-194. SENT: animals inoculated with sterile broth

A summary of *S. zooepidemicus* shedding findings, based on PCR data, is presented in Table 1. SENT pigs did not have detectable levels of *S. zooepidemicus* in any of the oral, nasal or rectal swabs collected. Nasal swabs yield numerically more positive samples ($n = 15/23$) than rectal ($n = 9/23$) or oral ($n = 2/23$) swabs. All sampling sites resulted in positive samples by 1 dpi.

At termination, all SENT samples tested negative for *S. zooepidemicus* by culture. A summary of post-mortem samples culture results is shown in Table 2. While submandibular lymph nodes had the highest proportion of positive samples (4/6), mesenteric lymph nodes also had culturable levels of *S. zooepidemicus* in three of six pigs.

A summary of all the gross lesions identified can be found in Table S1. The most prevalent gross lesions observed in INOC pigs were enlarged submandibular LN (6/6), kidney petechiae (6/6) and splenomegaly (6/6). Reactive mesenteric LN was observed in three of six INOC pigs. Colonic and gall bladder haemorrhage were seen in two of six animals. Representative photos of the lesions described above are shown in Figure 2. INOC pigs had microscopic changes suggestive of septicaemia. Notably, four of six INOC animals' lung sections displayed congestion of the capillaries and haemorrhage in the alveoli. The alveoli also contained oedema and multifocal fibrin thrombi were noted in the capillaries. Two animals without lung lesions were #527 and #534, which also had mild clinical signs. The sinuses of the lymph nodes were congested and hemorrhagic (3/5 INOC). There were clusters of mature neutrophils infiltrating in the red pulp of the

TABLE 1 Individual *S. zooepidemicus* shedding (by PCR), clinical disease progression and CNS invasion

Pig ID		1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	CNS
524	Shedding	N	R	N			Neg
	Responsiveness	1	1	2			
	Respiration	0	0	2			
527	Shedding	N	R	Neg	N/O/R	N	Neg
	Responsiveness	1	1	0	0	0	
	Respiration	1	1	0	0	0	
529	Shedding	N	Neg				Pos
	Responsiveness	1	2				
	Respiration	0	2				
530	Shedding	N	N/R	Neg	N		Neg
	Responsiveness	2	1	0	0		
	Respiration	1	1	1	0		
533	Shedding	N/O	N/R	N/R	N/R	N/R	Pos
	Responsiveness	0	0	0	0	2	
	Respiration	0	1	1	1	3	
534	Shedding	R	Neg	Neg	N		Neg
	Responsiveness	1	0	0	0		
	Respiration	1	0	0	0		

Note: Letters N, R and O represent nasal, rectal or oral samples positive at a given time for a given animal. All SENT group samples tested negative for shedding and had no clinical signs (data not shown). Black cells: euthanized, no sample. Neg: negative PCR. Pos: Positive PCR. CNS: central nervous system terminal swab. Respiration: 0 = normal rate, 25–35/min; 1 = increased rate, >35/min; 2 = increased rate, >35/min, dyspnoea and/or coughing; 3 = increased rate, >35/min, marked dyspnoea and/or persistent paroxysmal coughing. Responsiveness: 0 = alert and active; 1 = alert, but slower than cohorts; 2 = reluctant to move, but moves by stimulation; 3 = does not respond to stimulation or has seizures.
Abbreviations: dpi, days post-inoculation

TABLE 2 Post-mortem *S. zooepidemicus* culture results. All SENT group samples tested negative and are not shown

Pig ID	Blood	Heart	Kidney	Liver	Lung	LN - Mesenteric	LN - Submandibular	LN - Tracheobronchial	Spleen	Tonsil
524	1+	1+	1+	1+	1+	2+	1+	–	3+	2+
527	–	N/A	–	–	–	–	1+	–	–	–
529	–	N/A	–	4+	1+	–	–	–	3+	1+
530	–	N/A	–	–	–	–	–	–	–	–
533	2+	N/A	–	3+	4+	2+	1+	1+	4+	–
534	–	N/A	–	–	–	1+	1+	–	–	3+

Abbreviation: LN, lymph node. "–": No growth detected.

spleens (6/6 INOC). However, small numbers of neutrophils could be also found in all the spleens of the SENT pigs.

4 | DISCUSSION

Streptococcus zooepidemicus is an emerging pathogen of pigs in the western hemisphere, and disease is particularly associated with ST-194. In this study, we have described an infection model where 6 weeks old pigs were found susceptible to infection, developed clinical signs, lesions, and shed the bacterium in the environment following inoculation. Surprisingly, *S. zooepidemicus* DNA was detected in faecal samples and mesenteric lymph nodes of diseased pigs were reactive and culture-positive for the pathogen. These findings are suggestive of faecal shedding, and faecal-oral may be a new route of transmission between pigs. Two out of six inoculated pigs developed transient fever but did not succumb to severe disease while still shedding the pathogen, evidencing a carrier state. Indirect transmission was

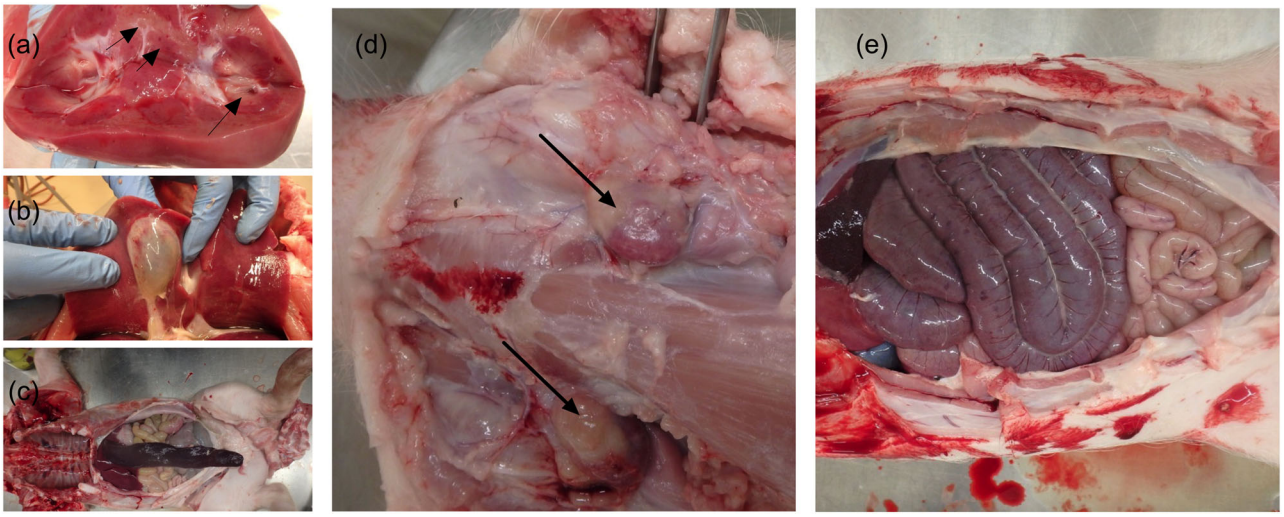

FIGURE 2 Representative post-mortem gross lesions findings in pigs inoculated with *S. zooepidemicus*. (a) Renal petechiae (arrows). (b) Gall bladder haemorrhage. (c) Splenomegaly. (d) Enlarged and haemorrhagic submandibular lymph nodes (arrows). (e) Colonic haemorrhage

not observed, as sentinel pigs kept in the same room with the inoculated pigs remained healthy and tested negative by PCR throughout the studied period and culture following euthanasia.

SStreptococcus equi subsp. *zooepidemicus* infection in pigs is not a new disease, as it has been reported in southeastern Asia for decades (Feng & Hu, 1977; Soedarmanto et al., 1996). However, in the past 3 years, several outbreaks were reported in commercial swine operations throughout North America and Europe (Costa & Lage, 2020; Houben et al., 2021; Sitthicharoenchai et al., 2020). It is not clear why this pathogen is emerging now, especially considering the improved biosecurity measures applied by the swine industry due to the porcine epidemic diarrhoea virus epidemic in North America in 2013, and the recent global threat of African swine fever (Stevenson et al., 2013). Most reports to date suggest that *S. zooepidemicus* require direct contact or fomites for pathogen transmission (Abbott et al., 2010; Pelkonen et al., 2013; Torres et al., 2018). Our findings corroborate this concept, as housing naïve pigs in the same room with acutely sick pigs did not result in colonization or disease of SENT animals under our experimental settings. Bacterial shedding, however, has historically been associated with nasal and oral discharges in dogs and horses, but no data are available for pigs and other species (Priestnall & Erles, 2011). One previous case report of high-mortality events due to *S. zooepidemicus* infection in commercial, free-range chicken flock suggested the potential transmission through the faecal-oral route. The authors reported large numbers of the pathogens detected in caecal samples of affected birds (Garmyn et al., 2020; Timoney & Kumar, 2008). A second report has shown that healthy horses can shed this bacterium in faeces (Pisoni et al., 2009). Here, we provide evidence that *S. zooepidemicus* is shed in swine faeces. This finding has significant implications on how disease control and prevention should be performed, especially in modern swine rearing operations. Biosecurity measures related to *S. zooepidemicus* are suggested to include testing of individual or pooled faecal samples prior to new introductions to a naïve herd, the latter sampling method is likely more efficient *in lieu* of nasal swabs. This is noteworthy as we have shown that inoculated yet clinically healthy pigs also shed the pathogen. Such an approach can be particularly useful in commercial systems that are subclinically infected with *S. zooepidemicus* ST-194, as it may help prevent naïve herds from breaking with the disease.

Healthy carriers of *S. zooepidemicus* have been reported in other species (Abbott et al., 2010; Pelkonen et al., 2013; Pisoni et al., 2009). It was unclear if pigs could survive infection by *S. zooepidemicus* ST-194 based on clinical outbreaks where high mortality rates were described, and if shedding was a feature of such carrier animals (Chen et al., 2020; Costa & Lage, 2020; Ma et al., 2019). A swine infection challenge model did not find evidence of this, as they observed 100% mortality rate when sows and 5 months old pigs were inoculated with 10^9 CFU/ml of *S. zooepidemicus* ST-194 intranasally and orally (Hau et al., 2021). The model described here used 6 weeks old pigs (commercially categorized as nursery animals). We found evidence of carrier pigs, as all inoculated animals developed fever within 8 h of exposure. The period between inoculation and the onset of fever was observed in llamas experimentally inoculated intratracheally with 10^9 CFU/ml of *S. zooepidemicus* (Cebra et al., 2000). The age difference of the animals used between the two swine inoculation studies may have contributed to the reduced mortality rate observed, in conjunction with the decreased bacterial load used in the inoculum. In commercial swine operations, replacement breeding stock may be introduced to a new herd at around 6 weeks of age (nursery), especially when disease eradication procedures are carried out. This raises a potential biosecurity concern if gilts carrying *S. zooepidemicus* are not detected upon entrance. However, it remains unknown for how long viable bacteria are shed by pigs and through which secretions. This is key information missing that will help design effective biosecurity protocols. In the study described here, the inoculum containing 10^6 CFU/ml was enough to induce colonization, shedding and disease. However, others have shown that the pathogen load affects mortality rates in mouse and llama *S. zooepidemicus* infection models (Cebra et al., 2000; Ma et al., 2019).

Further research is required to clarify the *S. zooepidemicus* infectious dose in pigs.

Clinical signs and gross lesions observed in this study were similar to previous reports of *S.* zooepidemicus associated disease in pigs (Costa & Lage, 2020; Hau et al., 2021; Sitthicharoenchai et al., 2020). Overall, animals developed fever, lethargy, signs and lesions suggestive of acute septicaemia. Despite nasal inoculation, lungs remained free of bronchopneumonia, suggesting that the histopathologic lesions observed are due to septicaemia rather than a descending air way infection. The fibrin thrombi observed in the lungs of INOC pigs are further evidence of septicaemia. This is different from what was observed in older animals inoculated with *S. zooepidemicus*, as sows developed areas of consolidation and epistaxis (Hau et al., 2021). Bacterial detection in different organs was not different from what was described previously (Hau et al., 2021). Field veterinarians should consider in their differential diagnosis (prior to laboratory testing) any agents associated with swine systemic infections, such as *Erysipelothrix rhusiopathiae*, *Salmonella enterica* Cholerasuis, *Streptococcus suis*, *Glaesserela parasuis*, *Actinobacillus suis*, African swine fever virus, classical swine fever virus, Aujeszky's virus, highly-pathogenic porcine reproductive and respiratory syndrome virus. This list should be expanded with other agents known to circulate locally, as it is intended to provide an initial general guidance only.

Here, we provided evidence that the inoculation of 6 weeks old pigs with *S. zooepidemicus* ST-194 leads to clinical disease and lesions similar to that observed in clinical outbreaks. The pathogen was consistently shed from nasal and rectal secretions of diseased and healthy but inoculated animals. Furthermore, sentinel pigs reared in the same air space as the inoculated pigs did not develop disease or become colonized, thus suggesting that direct contact of susceptible animals with a contaminated fomite is required for pathogen transmission. Taken together, these findings suggest the possibility of a carrier state in this age of pigs, and that the faecal-oral transmission route may play a role in disease dissemination. Further studies are suggested to elucidate the duration and route(s) of pathogen shedding to aid in the development of efficient biosecurity measures.

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ETHICS STATEMENT

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (permit #20210025).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supporting Information of this article.

ORCID

Matheus de Oliveira Costa https://orcid.org/0000-0001-9621-1977

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SUPPORTING INFORMATION

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