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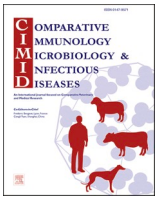

Organ distribution and early pathogenesis of *Streptococcus equi* subsp. *zooepidemicus* in swine

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ABSTRACT

Streptococcus equi subsp. *zooepidemicus* is an emerging pathogen of pigs, resulting in high-mortality outbreaks of septicaemia and abortions. Here, we investigated the early pathogenesis of *S. zooepidemicus* in pigs following oronasal inoculation. Fourteen pigs were inoculated with live cultures of *S. zooepidemicus* ST-194, and monitored at 2,4, 8, and 24 h post-inoculation. Necropsies were performed to assess gross lesions and collect samples for bacterial culture and PCR analysis at each time point. Our findings revealed that *S. zooepidemicus* was detectable in various organs as early as 2 h post-inoculation, including liver and spleen, demonstrating rapid dissemination within the host. Tonsil samples consistently harboured live *S. zooepidemicus* throughout the study period, suggesting their potential for epidemiological sampling and diagnostics. Moreover, the presence of varying bacterial loads in mesenteric lymph nodes indicated persistence, replication, and a potential source for shedding. Further studies are required to determine the initial site of replication.

1. Introduction

Virulent strains of *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) were linked to disease outbreaks that killed over 300,000 pigs in China in the 1970 s. Animals presented with respiratory disease and sudden deaths were also noted [1]. Approximately 50 years later, 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 [2]. Since then, the pathogen was also isolated from outbreaks of septicaemic disease and increased mortality throughout the northern United States of America, Canada, New Zealand (personal communication), South America (personal communication, M. Costa) and the Netherlands [3–5]. *S. zooepidemicus* is a pathogen with a wide range of warm-blooded hosts, including humans, equidae, camelidae, caninae and suidae [6–10]. It is classified as Gram-positive, β-hemolytic coccus belonging to the Lancefield group C. *S. zooepidemicus* is suggested as part of the indigenous microbiota of the palatine tonsils of pigs, detectedable by culture or high-throughput sequencing from healthy animals [10]. A slaughterhouse survey conducted in Canada in 2011 showed that approximately 1% of healthy marketed pigs harboured *S. zooepidemicus* in their tonsils[11].

An initial study described the experimental infection of finisher pigs and sows with *S. zooepidemicus* ST-194 isolates obtained from different hosts [12]. 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. A second infection model study documented pathogen shedding via feces and nasal discharge, as well as subclinical carrier pigs [13]. Both studies reported disease onset within 8 h of inoculation, regardless of the inoculum dose used (ranging from 10^8 CFU/mL to 10^6 CFU/mL), and the use of either oral or nasal inoculation routes.

Currently, there are no commercial vaccines available for this pathogen. Until recently, specific control and prevention methods were not applied given its assumed 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 investigate the number

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and distribution of *S. zooepidemicus* in pigs at different times within the first 24 h of infection. Understanding of the disease mechanism and early pathogenesis of *S. zooepidemicus* infection in pigs should be useful in the development and application of preventative methods.

2. Methods

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).

2.1. Inoculum preparation

A clinical *Streptococcus equi* subsp. *zooepidemicus* ST-194 isolate was recovered from a finisher pig presenting with septicaemia and disseminated intravascular coagulation. Isolate identification was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer and whole genome sequencing [2]. Inoculum was prepared by culturing the isolate in brain-heart infusion (BHI) broth at 37 °C in a 5% CO_2 atmosphere overnight. The resulting inoculum contained 2.5×10^9 CFU/mL.

2.2. Inoculation trial and sampling

Fourteen healthy pigs were 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* and *Streptococcus equi* subsp. *zooepidemicus*. At 9 weeks of age, pigs were transported to a level 2 biocontainment facility to acclimate for 14 days prior to inoculation. During the entire experimental period, pigs had ad libitum access to unmedicated diet and water. On 0 h post-inoculation (hpi), all animals received live cultures of *S. zooepidemicus* ST-194 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 at 2, 4, 8, and 24 hpi. The following signs were scored: respiratory rate (0 = normal rate, 25–35/min; 1 = increased rate, >35/min; 2 = increased rate, >35/min, dyspnea and/or coughing; 3 = increased rate, >35/min, marked dyspnea and/or persistent paroxysmal coughing), and 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). Rectal temperature measurement was also performed at the above mentioned time points. At each time point, 3 randomly selected pigs were euthanized. At 24 hpi, 2 pigs were euthanized. Following euthanasia, a complete necropsy was performed to characterize gross lesions and collect samples for bacterial culture and PCR. Any visibly affected organs, heart, submandibular lymph nodes (LN), spleen, liver, tonsil, and cranial lung were harvested for pathogen detection.

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

Necropsy samples were refrigerated until processed for bacterial culture. Under asseptical conditions, organ samples were dissected and swabbed 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 environment for 24 h. A sample was designated positive if beta-haemolytic, mucoid colonies were visible and confirmed to be *S. zooepidemicus* by PCR, using a previously developed protocol [13]. DNA from all sample types was extracted using DNEasy blood and tissue kit (Qiagen, Mississauga, ON). A ST194-specific primer-probe set was used: forward MC102: 5′-GGCAAGGTTAGC CCCAATCA-3′, reverse MC103: 5′-TCTTGAGCATGTGGTGAGGG-3′, probe MC104: 5′-FAM TCTACCAAGCCCACACATCAC BHQ1 – 3′. PCR reactions were carried using SSO Advanced Probe master mix (Bio-rad, Ottawa, ON). All reaction plates included a no template, blank, bacterial positive control, and a standard curve prepared with a target-containig plasmid at concentration of 10^0-10^8 copies/reaction. Samples were analysed 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-analysed. A sample was considered positive if a Ct < 35 was detected.

2.4. Statistical analysis

Differences in bacterial load between time points were investigated using one-way ANOVA followed by Tukey's post-hoc using SPSS v24 (IBM Corp., Armonk, USA). The data was tested for normality with Shapiro-Wilk's test.

3. Results

All clinical parameters from all pigs were within normal range prior to inoculation. Within 8 h of inoculation, all pigs showed reduced responsiveness (score 1). At 24 hpi, all remaining pigs were reluctant to move but would do so following stimulation (score 2). Rectal temperatures remained below the 40 °C fever threshold until 24 hpi. Individual rectal temperatures are presented in Fig. 1. Following necropsy, all pigs after 2 and 4 hpi did not have any visible gross lesions, only 1 pig had lesions at 8 hpi and all 3 pigs had lesions at 24 hpi. A summary of the post-mortem findings is presented in Table 1.

Viable *S. zooepidemicus* was detected at all time points, from tonsils samples of all pigs. Tonsil bacterial load was significantly higher at 2 hpi than at any other time point investigated. Live *S. zooepidemicus* was only detected in 66% (2/3) of the submandibular lymph node samples at 2 and 4 hpi, and all samples at 8 and 24 hpi. Bacterial load decreased over time. Bacterial loadwas remarkably similar and not statistically different between submandibular lymph node and tonsil samples. Mesenteric lymph nodes from all pigs harboured live bacteria at 2 and 4 hpi, with the lowest number of *S. zooepidemicus* detected at 4 hpi. Live bacteria were detected in only 1/3 cranial lung samples at 2 hpi, despite *S. zooepidemicus* DNA being detected at higher levels than in mesenteric lymph node samples. Surprisingly, liver and spleen samples contained viable bacteria at 2 hpi. *S. zooepidemicus* hepatic load was numerically higher at 2 hpi than 24 hpi, although this difference was not statistically significant. Viable bacteria were not detected liver samples at 24 hpi. In

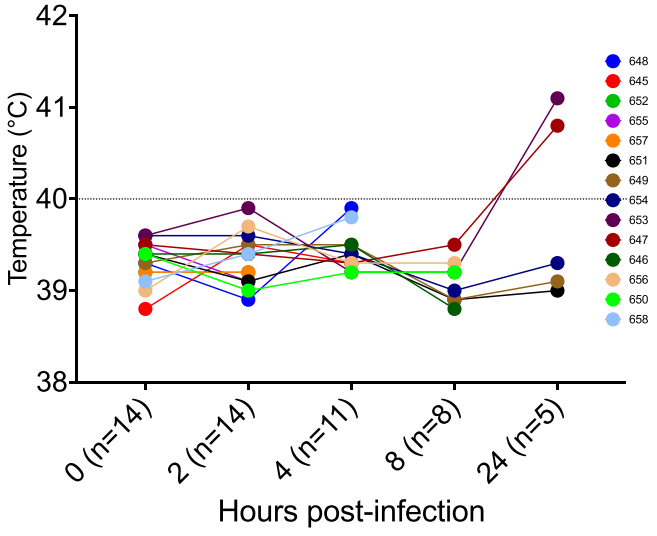

Fig. 1. – Individual rectal temperatures of pigs following inoculation with *S. equi* subsp. *zooepidemicus*.

Table 1 –
Summary of gross lesions identified in pigs following inoculation with *Streptococcus equi* subsp. *zooepidemicus*. Pigs not included did not have any gross lesions identified.

ID	Time-point	SM-LN	Tonsil	Thoracic cavity	Cranial lung	Abdominal cavity	Liver	Spleen	Mesenteric LN
650	8 h	Yes: Mild haemorrhage	No	Yes: pericardium - serous exsudate	No	Yes: serous abdominal fluid	Yes: mild local fibrinosum exsudate	Yes: mild, focal, necrotic areas	No
652	24 h	Yes: Mild haemorrhage	No	No	No	No	No	Yes: mild, focal, necrotic areas	No
655	24 h	Yes: moderate edematous, reactive, enlarged	No	No	No	No	No	No	No
657	24 h	Yes: moderate hyperemic	No	No	No	No	No	No	Yes: moderate presence of white, flat, round, firm, non-purulent masses.

contrast, viable bacteria were present in all spleen samples from 2 to 8 hpi, and in 75% of the samples at 24 hpi. Highest splenic *S. zooepidemicus* load was observed at 24 hpi, followed by 2 hpi. These data are presented in Fig. 2.

4. Discussion

Given its wide range of hosts, including humans, the recent emergence of *Streptococcus zooepidemicus* as a pig pathogen is a concern in the western hemisphere. However, very little is known regarding its parasitic relationship with pigs, including early disease mechanisms. Here we described the temporal infection progression of *S. zooepidemicus* across different organs following inoculation. Surprisingly, *S. zooepidemicus* DNA and live cells were detected as early as 2 h post inoculation in every sampled organ. Tonsil samples contained live bacteria at all sampling times, and may hold potential for epidemiological sampling and diagnostic. Mesenteric lymph nodes harboured different loads of the bacterium, suggesting persistence, replication in that site, and a potential source for shedding.

S. zooepidemicus disease in pigs was described over 50 years ago [1,14]. Since 2019, its emergence in the western world resulted in outbreaks linked to severe economic losses, limiting production in affected farms [2,4,5]. Recently, several cases of human disease linked to the consumption or handling of pigs were reported in southeast Asia [15]. However, very little information is available regarding early pathogenesis and infection progression in pigs and other animal species. One study investigated the entry and subsequent multiplication of *S. equi* subsp. *equi* in horses [16]. This study used a similar inoculum dose as used here (10^9 CFU/mL), and found that oro- and nasopharynx tonsil served as port of entry for the pathogen. Bacterial cells were cultured from tonsillar tissue as early as 1 h following inoculation. Adjacent lymph nodes were colonized at 3 hpi. In contrast, we found that 100% of the tonsils, 66% of the submandibular and 100% of the mesenteric lymph nodes sampled were positive by culture as early as 2 hpi. In our study, all tonsils samples harboured live bacteria at all sampling times, although *S. zooepidemicus* load decreased over time. Submandibular lymph nodes also showed a reduction in bacterial load over time, but an increasing number pigs with viable bacteria detectable over time. In vitro, *S. zooepidemicus* has been shown to adhere to epithelial cells as early as 1.5 h following exposure [17]. Epithelial cell invasion occurred through 3 morphologically different mechanisms: cytoskeletal rearrangement, membrane invagination, and bacterial engulfment. Heat-inactivated *S. zooepidemicus* did not induce any of these changes. Given that off-feed episodes are one of the earliest signs of *S. zooepidemicus* infection noted in barns[2,4], and it is usually noted by farm staff at intervals of 4–6 h between chores, and that 100% of the tonsils samples contained viable *S. zooepidemicus*, we suggest that tonsil swabs could be used as diagnostic samples early on during infection. In parallel, submandibular lymph nodes may be used for sudden death pigs, especially those deceased overnight (or if pigs have not been observed for at least 8 h).

Mesenteric lymph node colonization was an important finding in this study, as it helps confirm previous observations. We reported *S. zooepidemicus* shedding in feces of pigs inoculated experimentally, up to 5 days post inoculation [13]. Other researchers have proposed that *S. zooepidemicus* is shed in the feces of poultry infected with the pathogen [18]. The *S. zooepidemicus* DNA load detected at 2 hpi may be simply inoculum pass-through, whereas the decay at 4 hpi and subsequent increase at 8 hpi suggests true bacterial replication in (or systemic spread to) mesenteric lymph nodes. However, it is unclear if colonization of the mesenteric lymph node results from luminal uptake or systemic spread. Other streptococci, such as *S. suis*, *S. dysgalactiae*, are known to colonize and survive in the gastrointestinal tract of healthy pigs [19]. Given that pigs in this trial were septicaemic at the time of sampling, one cannot rule out the systemic route of mesenteric lymph node colonization. Interestingly, bacterial pathogen fecal shedding following persistence in the mesenteric lymph node has been documented in mice infected with *Salmonella*, and clinically healthy pigs experimentally challenged with *S. zooepidemicus* [13,20]. It is important to note here the difference in virulence strategies observed between *S. zooepidemicus* and *Salmonella*, and the how well the virulence mechanisms of the latter are understood, when compared to the former.

Our data suggests that cranial lungs are an unlikely route of entry and target infection organ, given the reduction in prevalence and bacterial load over time. Similarly, viable *S. zooepidemicus* was detected in the liver of fewer animals as time progressed. Hepatic bacterial DNA load sharply increased at 24 hpi, but none of the sampled animals had viable *S. zooepidemicus* detected at this time-point. This may reflect clearance of bacteria-infected lymphocytes or carry over from other lymphocytic organs rather than true replication in situ[21,22]. Interestingly, spleen samples were positive by culture and PCR at 2 hpi, and the number of bacteria rapidly decreased between 2 and 4 hpi. This suggests an inoculum overload that was rapidly controlled by 4 hpi. However, at 24 hpi, spleen samples had numerically more *S. zooepidemicus* DNA than at 2 hpi, which may have been true pathogen replication, or bacterial drainage from other lymphoid tissues and blood. In pigs, signs of systemic disease are observed as early as 8 hpi, the same as llamas [6,12,13]. The capacity of *S. zooepidemicus* to invade the swine host so quickly is also reflected in clinical presentation, as several animals are found dead without clinical signs during outbreaks. Despite the lack of clinical signs, necropsy of sudden death animals often identifies enlarged and congested spleens, which may be linked to the bacterial overload detected in spleen samples from this study.

Here, we explored the early pathogenesis of *S. zooepidemicus* in pigs, providing evidence of rapid invasion and spread in the host organism. Tonsils were consistently infected by live bacteria, detectable from all animals at all time points. Its diagnostic value during early stages of infection is remarkable, especially given the unspecific clinical signs at

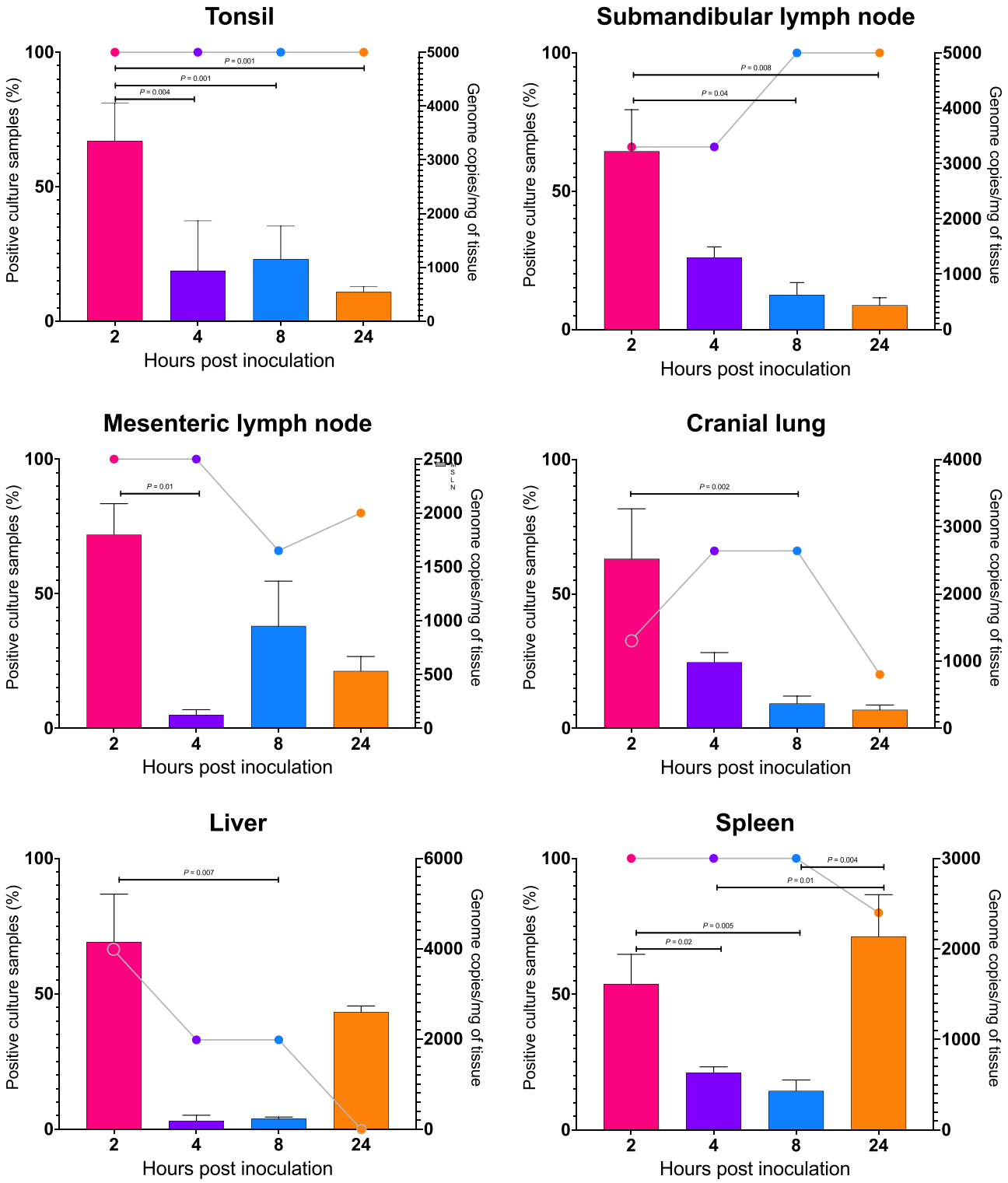

Fig. 2. – Temporal trends over the first 24 h post inoculation of pigs *S. equi* subsp. *zooepidemicus*. Lines and circles denote the percentage of samples (pigs) positive for *S. equi* subsp. *zooepidemicus* by bacterial culture. Bars represent average number of pathogen genome copies per mg of tissue, whiskers denote standard deviation of the mean.

that stage. Lymph nodes appear to remain sites of infection, but not replication. In contrast, the spleen was the only organ to show an increase in viable bacterial load and DNA at 24 h post infection. Future studies are suggested to focus on the very early infection period, up to 2 h post exposure, to help pinpoint the initial site of infection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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