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Characterization of Pneumonia Due to *Streptococcus equi* subsp. *zooepidemicus* in Dogs[▽]

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***Streptococcus equi* subsp. *zooepidemicus* has been linked to cases of acute fatal pneumonia in dogs in several countries. Outbreaks can occur in kenneled dog populations and result in significant levels of morbidity and mortality. This highly contagious disease is characterized by the sudden onset of clinical signs, including pyrexia, dyspnea, and hemorrhagic nasal discharge. The pathogenesis of *S. equi* subsp. *zooepidemicus* infection in dogs is poorly understood. This study systematically characterized the histopathological changes in the lungs of 39 dogs from a large rehoming shelter in London, United Kingdom; the dogs were infected with *S. equi* subsp. *zooepidemicus*. An objective scoring system demonstrated that *S. equi* subsp. *zooepidemicus* caused pneumonia in 26/39 (66.7%) dogs, and most of these dogs (17/26 [65.4%]) were classified as severe fibrino-suppurative, necrotizing, and hemorrhagic. Three recently described superantigen genes (*szeF*, *szeN*, and *szeP*) were detected by PCR in 17/47 (36.2%) of the *S. equi* subsp. *zooepidemicus* isolates; however, there was no association between the presence of these genes and the histopathological score. The lungs of *S. equi* subsp. *zooepidemicus*-infected dogs with severe respiratory signs and lung pathology did however have significantly higher mRNA levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 8 (IL-8) than in uninfected controls, suggesting a role for an exuberant host immune response in the pathogenesis of this disease.**

Infectious respiratory disease in dogs is usually caused by infection with one or more viruses, such as canine parainfluenza virus (CPIV) or canine herpesvirus (CHV), or bacteria, most notably *Bordetella bronchiseptica*, and results in the clinical syndrome termed canine infectious respiratory disease (CIRD) (7). CIRD has a multifactorial etiology and is most prevalent in shelters where stress, due to overcrowding, results in animals becoming more susceptible to infection (8). The disease is often highly contagious with high morbidity but is rarely fatal, and recovery from mild clinical signs usually occurs within a few weeks (8). The list of pathogens associated with CIRD is extensive and continues to grow; however, for many agents, the pathogenesis and specific associated lesions are poorly defined.

Streptococcus equi subsp. *zooepidemicus* has been shown to be associated with respiratory disease in dogs for a number of years (11); however, more recently, its significance has been highlighted by reports from several countries implicating the bacteria in a number of fatal outbreaks in shelter dogs (5, 6, 15, 24). Clinically, *S. equi* subsp. *zooepidemicus* causes severe acute respiratory distress, often with high morbidity and sometimes high mortality. At necropsy, affected dogs are usually diagnosed with severe, acute fibrino-suppurative, necrotizing, and/or hemorrhagic pneumonia.

Between 1999 and 2001, as part of a study into the causes of respiratory disease in a large rehoming kennel in London, United Kingdom, a kennel where respiratory disease was endemic, Chalker et al. (6) isolated *S. equi* subsp. *zooepidemicus* from the lungs of 48/215 dogs (22.3%) and showed that the presence of *S. equi* subsp. *zooepidemicus* was associated with increasing severity of clinical respiratory disease. *Streptococcus canis* was also present in some dogs but was not associated with respiratory disease (6).

The rapid onset of disease and fast deterioration in the clinical condition in many dogs infected with *S. equi* subsp. *zooepidemicus* are similar to human toxic shock syndrome caused by *Streptococcus pyogenes* (18). While the main site of inflammation in toxic shock syndrome is often the subcutaneous tissue, there are common clinical features of an acute illness characterized by pyrexia, hypovolemia, and coagulopathy (18).

Pyrogenic exotoxins produced by some streptococci, including *S. pyogenes*, act as superantigens by binding simultaneously to major histocompatibility complex (MHC) class II receptors on macrophages and T-cell receptors, bypassing conventional antigen presentation, and leading to the activation of a large proportion of T lymphocytes (9). The ensuing cytokine "avalanche" includes the production of proinflammatory cytokines, such as interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) (12, 20).

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The presence of superantigen genes in strains of *S. pyogenes* has been linked to increased virulence and has also been suggested to contribute to the pathogenesis of "strangles," a severe disease of horses caused by *S. equi* subsp. *equi* (4, 19, 21, 23). Superantigen genes have also been detected in some isolates of *S. equi* subsp. *zooepidemicus* (2, 13, 17). One canine isolate of *S. equi* subsp. *zooepidemicus* (isolate *Sz*BHS5) was recently shown to encode three novel superantigen genes, *szeF*, *szeN*, and *szeP*, the products of which share 59%, 34%, and 49% amino acid sequence identity with the superantigens SpeH, SpeL, and SpeM of *S. pyogenes*, respectively (23a).

The aims of this study were (i) to characterize systematically the microscopic changes in canine lungs following infection with *S. equi* subsp. *zooepidemicus*, (ii) to test for possible associations between the severity of clinical signs, lung pathology, and the presence of *S. equi* subsp. *zooepidemicus*, and (iii) to investigate whether severe lung pathology is linked to the presence of superantigen genes in *S. equi* subsp. *zooepidemicus* isolates and/or the upregulation of the proinflammatory cytokines TNF-α, IL-8, and IL-6.

We demonstrate the following. (i) *S. equi* subsp. *zooepidemicus* in dogs most often causes severe fibrino-suppurative, necrotizing, and hemorrhagic pneumonia. (ii) Marked elevation of the mRNA of proinflammatory cytokines TNF-α, IL-8, and IL-6 is observed in dogs with *S. equi* subsp. *zooepidemicus*-induced pneumonia. (iii) As many as three superantigen genes are prevalent in canine isolates of the bacterium.

MATERIALS AND METHODS

Overview. In this study, 39 dogs with *Streptococcus equi* subsp. *zooepidemicus* isolated from a postmortem lung wash and 16 control dogs with a sterile necropsy lung wash were studied. The *S. equi* subsp. *zooepidemicus* isolates were further analyzed by PCR for superantigen genes. Postmortem lung tissue samples of all dogs were examined microscopically and investigated by quantitative reverse transcription-PCR (RT-PCR) for mRNA levels of the proinflammatory cytokines TNF-α, IL-6, and IL-8.

Study population. Previously, during a large study of respiratory disease in dogs at a well-established rehoming kennel in London, United Kingdom, a kennel where respiratory disease was endemic, necropsies were carried out on dogs of different ages, breeds, and sexes, euthanized for reasons ranging from behavioral problems to severe respiratory disease (8). During the postmortem examination, lung washes were performed, and the bronchoalveolar fluid obtained was used for bacterial culture by the method of Chalker et al. (6). In addition, standardized tissue samples of apical and diaphragmatic lung lobes were collected and stored frozen and as formalin-fixed paraffin-embedded tissue.

For this study, 39 dogs with positive *S. equi* subsp. *zooepidemicus* culture from the necropsy lung wash were selected. A further 16 dogs from the same study with no clinical signs of respiratory disease and no *S. equi* subsp. *zooepidemicus* cultured from their lung washes were included as controls. All dogs were screened for other potential respiratory pathogens, known to occur at the premises, including viruses (canine parainfluenza virus [CPIV], canine herpesvirus [CHV], canine respiratory coronavirus [CRCoV], canine adenovirus [CAV], and canine distemper virus [CDV]) and bacteria (*Mycoplasma* spp. and *Bordetella bronchiseptica*) by PCR on lung tissue according to previously published methods (7, 8, 10, 16).

Study groups and histopathological scoring. The dogs within this study (n = 55) were assigned to four groups (groups 0 to 3) based on the clinical respiratory signs, which had been recorded by veterinarians immediately antemortem, and the presence or absence of *S. equi* subsp. *zooepidemicus* in the postmortem lung wash (Table 1). For each dog within the study groups, sections of apical and diaphragmatic lung lobes were scored blindly by a veterinary pathologist for the presence and relative abundance of the following parameters: hemorrhage, neutrophils, fibrin exudation, histiocytes, and tissue necrosis (Table 2). The presence of bacteria and vascular thrombosis was also noted. Individual histological scores for each lung lobe were added together to give a total histopathological score for each dog. The parameters of the grading scheme (Table 3) enabled the objective diagnosis of the presence or absence of pneumonia and type of pneumonia. If the apical and diaphragmatic lung lobes differed in regard to certain scoring parameters, the lobe with the highest individual score was used for pneumonia classification.

TABLE 1. Scoring scheme for clinical respiratory signs

Study group (no. of dogs)	Score for clinical signs	*S. equi* subsp. *zooepidemicus* isolated	Clinical sign(s)
0 (16)	0	No	None
1 (7)	1	Yes	None
2 (17)	2	Yes	Cough and nasal discharge
3 (15)	3	Yes	Cough, nasal discharge, and pyrexia

Statistical analysis of histopathological scoring. The total histopathological scores for apical and diaphragmatic lung lobes were compared by the Wilcoxon test. The total histopathological scores for *S. equi* subsp. *zooepidemicus*-infected and uninfected dogs were compared using a Mann-Whitney independent sample test, and this was repeated for other respiratory pathogens (CPIV, CHV, CRCoV, *Mycoplasma* spp., and *B. bronchiseptica*). In addition, histopathological scores for study groups were compared by the Kruskal-Wallis test, and posthoc pairwise comparisons were made by using the Mann-Whitney test with Bonferroni's correction for multiple comparisons. The histopathological score was also compared to the length of time in the kennel (days from admission to death) using the Spearman rank correlation coefficient. All statistical tests were performed using SPSS Statistics 17.0 (SPSS Inc.), and for all analyses, a P value of <0.05 was considered significant.

PCR for superantigen genes in *S. equi* subsp. *zooepidemicus*. *S. equi* subsp. *zooepidemicus* isolates, obtained from necropsy lung washes, were available from 38/39 dogs scored microscopically. A further 9 isolates were available from additional dogs within the same population which did not have sections available for histopathological evaluation. Each isolate was recultured from cryopreserved stock (−70°C) on blood agar. After 48 h of culture, individual colonies were selected, placed in Todd-Hewitt broth (Oxoid, Basingstoke, United Kingdom), and further incubated overnight in a shaking incubator (200 rpm) at 37°C. Overnight cultures were centrifuged (800 × g for 10 min), the supernatant was removed, and the bacteria in the pellet were lysed using the DNeasy blood and tissue kit according to the manufacturer's protocol (Qiagen, Crawley, United Kingdom). To confirm adequate DNA quality and quantity, a superoxidase dismutase A (*sodA*) gene PCR was performed on the extracted DNA (3).

A single canine isolate of *S. equi* subsp. *zooepidemicus* (*Sz*BHS5) from one of the necropsied dogs was previously sequenced at the Centre for Genomic Research, University of Liverpool, United Kingdom (23a). PCR primers were designed to amplify the *szeF*, *szeN*, and *szeP* genes (Table 4). Two sets of primers were designed for each gene: one pair to amplify the full-length gene (primers 1 and 2) and another pair to amplify a shorter internal sequence (primers 3 and 4). PCR amplification was performed in a 50-μl reaction mixture containing 1× *Taq* amplification buffer (Promega, Southampton, United Kingdom), 0.8 mM deoxynucleoside triphosphate (dNTP) mixture (Bioline, London, United Kingdom), 2.0 mM $MgCl_2$ (Promega), 0.5 μM internal primers, and 1.25 units GoTaq DNA polymerase (Promega). Cycling conditions were as follows: (i) denaturation for 5 min at 95°C; (ii) 30 cycles of PCR amplification, with 1 cycle consisting of denaturation for 1 min at 95°C, annealing for 30 s at 50°C, and extension for 45 s at 72°C; (iii) a final extension for 5 min at 72°C. Detection of the amplified products was carried out by electrophoresis in an agarose gel stained with SafeView (NBS Biologicals, Huntingdon, United Kingdom). Each experiment included two negative controls (a blank sample consisting of reaction mixture without DNA and another with *S. canis* DNA) and a positive control (isolate *Sz*BHS5).

PCR product cloning and sequencing. PCR amplification of the full-length superantigen genes (*szeF*, *szeN*, and *szeP*) was performed using external primers (Table 4) in a 50-μl reaction mixture using the LongRange PCR system (Qiagen), according to the manufacturer's protocol. Cycling conditions were as follows: (i) denaturation for 3 min at 93°C; (ii) 35 cycles of PCR amplification, with 1 cycle consisting of denaturation for 15 s at 93°C, annealing for 30 s at 50°C, and extension for 1 min at 68°C; (iii) a final extension for 5 min at 68°C. PCR products were purified using the QIAquick gel extraction kit (Qiagen) and then cloned into the pGEM-T Easy plasmid vector (Promega), both following the manufacturer's protocol. The plasmid was used to transform *Escherichia coli* XL1-Blue competent cells (Stratagene, Stockport, United Kingdom), and suc-

TABLE 2. Histopathological scoring of lung parameters according to their presence and severity

Parameter	Definition of histopathological score of:			
	0	1	2	3
Red blood cells (% parenchyma)	None	<10% parenchyma with >3 cells or 1 to 3 cells in <50% parenchyma	10 to 50% parenchyma with >3 cells or 1 to 3 cells in >50% parenchyma	>50% parenchyma with >3 cells
Neutrophils (% parenchyma)	None	<10% parenchyma with >3 cells or 1 to 3 cells in <50% parenchyma	10 to 50% parenchyma with >3 cells or 1 to 3 cells in >50% parenchyma	>50% parenchyma with >3 cells
Fibrin exudation (% parenchyma)	None	<10%	10 to 50%	>50%
No. of histiocytes (per alveolus)	<3 cells	3 to 10 cells	>10 cells	
Tissue necrosis	None	10%	10 to 50%	>50%

cessful transformants were selected on LB agar supplemented with ampicillin (100 μg ml^{-1}) (Sigma) and also containing isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (80 μg ml^{-1}) (Promega). Purified plasmid was obtained from selective (ampicillin [100 μg ml^{-1}]) LB broth cultures of transformed cells using the QIAprep spin miniprep kit (Qiagen) according to the manufacturer's protocol. DNA sequencing was performed by the Sequencing Service, School of Life Sciences, University of Dundee. Sequences were aligned using MegAlign (DNASTAR, Madison, WI), and a sequence similarity search was performed using FASTA.

Quantitative RT-PCR for the detection of proinflammatory cytokine mRNA. The mRNA levels of the proinflammatory cytokines TNF-α, IL-6, and IL-8 were compared in samples of lung tissue from 10 dogs of study group 3 (i.e., severe clinical respiratory signs and lung pathology and *S. equi* subsp. *zooepidemicus* isolated) and 10 dogs of study group 0 (i.e., no clinical respiratory signs or lung pathology and a sterile postmortem lung wash).

Samples of lung tissue (3 mm^3) (stored at −70°C) were homogenized using a ball mill (MM 300; Retsch, Leeds, United Kingdom) for 4 min at 20 Hz, and RNA was extracted using the RNeasy minikit (Qiagen) following the manufacturer's protocol. Genomic DNA was removed using RQ1 RNA-free DNase (Promega) according to the manufacturer's protocol, and cDNA synthesis was performed as follows. One microgram of RNA was added to 1 μl of $pd(N)_6$ random hexamers (0.5 mg ml^{-1}) (GE Healthcare, Little Chalfont, United Kingdom) and incubated at 70°C for 10 min. cDNA was synthesized using the ImProm-II reverse transcription system (Promega) and quantified using Quant-iT PicoGreen double-stranded RNA reagent (Invitrogen, Paisley, United Kingdom) according to the manufacturer's protocol. Quantification of IL-6, TNF-α, and IL-8 mRNA was performed by previously published methods using the GoTaq quantitative PCR master mix (Promega) according to the manufacturer's protocol (25). cDNA samples and standards were processed in duplicate, and a negative control and two internal controls (previously quantified cDNA samples) were included. Quantification was performed using standard curves produced by the Opticon Monitor software v. 3.1 (Bio-Rad, Hemel Hempstead, United Kingdom). The absolute mRNA copy number was normalized to cDNA concentration per gram of tissue. Mean cytokine mRNA copies for control and *S. equi* subsp. *zooepidemicus*-infected dogs were compared by a *t* test (SPSS Statistics).

RESULTS

Lung tissue samples from all dogs included in this study were screened by PCR for known respiratory pathogens, and the results are summarized in Table 5. All dogs were negative for canine adenovirus (CAV) and canine distemper virus (CDV) by PCR.

Within groups with positive *S. equi* subsp. *zooepidemicus* isolation (groups 1 to 3), 66.7% (26/39) of dogs were diagnosed with pneumonia, whereas only 6.3% (1/16) of the control dogs

TABLE 3. Pneumonia classification based on histopathological scores[a]

Pneumonia category	Criteria for pneumonia category (histopathological scores)
No pneumonia	Neutrophil score of ≤1 and fibrin score of ≤1 and hemorrhage score of ≤1
Pneumonia	
Fibrinous	Fibrin score of ≥2 or fibrin score of ≥1 and necrosis or fibrin score of ≥1 and hemorrhage score of 1 and necrosis
Hemorrhagic	Hemorrhage score of ≥2 or hemorrhage score of ≥1 and necrosis
Fibrino-suppurative	Fibrin score of ≥2 and neutrophil score of ≥1
Fibrino-suppurative and necrotizing	Fibrin score of ≥2 and neutrophil score of ≥1 and necrosis
Fibrino-suppurative, necrotizing, and hemorrhagic	Fibrin score of ≥2 and neutrophil score of ≥1 and hemorrhage score of ≥2

[a] Histopathological scores are from Table 2. The red blood cell scores from Table 2 are the hemorrhage scores.

TABLE 4. Primer sequences for amplification of *szeF*, *szeN*, and *szeP* superantigen genes

Gene and primer[a]	Sequence (5′ to 3′)
szeF	
F1	ATC-CGA-ATG-TAT-ACT-GAC-GAA-AAG
F2	TTG-CTA-GCA-GAT-GAC-AGG-AAG-ATA
F3	TTT-TAT-CTT-GTG-GCT-TTC-GTT-A
F4	CTA-TTT-CTT-GGG-CTG-TTA-CTA-T
szeN	
N1	AAA-TGC-TCA-AAA-GTG-CGA-CAG-G
N2	ATA-GAA-GGT-AGA-GCC-CCA-AGA-TAA-GAT-A
N3	AAT-GTA-AGT-TTA-TCC-GAA-GAA
N4	AAT-ATC-CAG-TTG-AGA-AAT-CC
szeP	
P1	TGT-CGC-ACT-TTT-GAG-CAT-TTT-G
P2	CTG-AGC-GAT-TTT-AAC-ATA-GTA-GTC
P3	AAG-CGA-GTT-AAT-GGG-ATA-CGA-T
P4	TCA-CCC-TTT-ACA-AAT-TTA-CCT-T

[a] Two sets of primers were designed for each gene: one pair to amplify the full-length gene (primers 1 and 2 [e.g., primers F1 and F2 for the *szeF* gene]) and another pair to amplify a shorter internal sequence (primers 3 and 4 [e.g., primers F3 and F4 for the *szeF* gene]).

TABLE 5. Average histopathological lung scores for all dogs infected with *S. equi* subsp. *zooepidemicus* and for dogs coinfected with *S. equi* subsp. *zooepidemicus* and common respiratory viruses and bacteria

Dog infection category[a]	Avg histopathological score for:		*P* value
	Infected dogs (no. of dogs)	Uninfected dogs (no. of dogs)	
All dogs infected with *SZ*	14.5 (39)	4.9 (16)	0.0003
Dogs infected with *SZ* and another virus (CHV and/or CPIV and/or CRCoV)	11.7 (14)	13.6 (16)	0.5947
Canine parainfluenza virus (CPIV)	10.2 (5)	14.9 (29)	0.3357
Canine respiratory coronavirus (CRCoV)	12.5 (6)	13.2 (23)	0.8816
Canine herpesvirus (CHV)	16.5 (6)	14.1 (33)	0.5859
Dogs infected with *SZ* and bacteria			
Mycoplasma spp.	14.6 (17)	14.4 (21)	0.9610
Mycoplasma cynos	16.0 (7)	14.2 (31)	0.6598
B. bronchiseptica	9.0 (10)	16.4 (29)	0.0354

[a] *SZ*, *S. equi* subsp. *zooepidemicus*.

(group 0) showed microscopic evidence of pneumonia (one case of hemorrhagic pneumonia). The types of pneumonia diagnosed among *S. equi* subsp. *zooepidemicus*-infected dogs were fibrino-suppurative, necrotizing, and hemorrhagic (65.4%; $n = 17$), fibrinous (15.4%; $n = 4$), hemorrhagic (15.4%; $n = 4$), and fibrino-suppurative (3.8%; $n = 1$). Interestingly, within group 3 (the group with the most severe clinical respiratory signs), 80.0% (12/15) of dogs had the most severe form of pneumonia, i.e., fibrino-suppurative, necrotizing, and hemorrhagic pneumonia (Fig. 1), whereas only 23.5% (4/17) and 14.3% (1/7) of dogs in groups 2 and 1, respectively, were assigned this diagnosis.

The total histopathological scores for apical and diaphragmatic lobes were not found to be significantly different ($P = 0.7$), thus scores were combined to give an overall histopathological lung score for each dog that was used for all subsequent analyses.

Statistically significant differences in total histopathological scores were recorded for *S. equi* subsp. *zooepidemicus*-infected dogs versus uninfected dogs ($P < 0.001$). Similar analyses were performed for coinfections with other common respiratory pathogens, although no significant differences in the histopathological score were identified (Table 5).

The mean histopathological scores for each study group were compared, and statistically significant differences were identified between groups 0 and 3 ($P < 0.001$) and groups 2 and 3 ($P < 0.001$). There was no statistically significant correlation between the length of time in the kennel and the histopathological score ($P = 0.3$).

PCR amplification and sequencing of superantigen genes. All isolates were positive for *sodA* gene and PCR amplification using internal primer pairs for the *szeF*, *szeN*, and *szeP* genes was performed on genomic DNA from 47 canine isolates of *S. equi* subsp. *zooepidemicus* (Fig. 2). Specifically, 36.2% (17/47) of isolates were positive for all three genes; the remaining 63.8% (30/47) of isolates were negative for all three genes. There was no significant association between the presence or absence of genes and the total histopathological score ($P = 0.4$) or the respiratory score ($P = 0.3$). For one isolate that was PCR positive for all three genes (isolate *Sz*BHS7), sequences were amplified using external primer pairs, cloned into a plasmid vector, and sequenced. The sequences were compared to that of isolate *Sz*BHS5; DNA and amino acid sequence identity were 100% for SzeF, SzeN, and SzeP (23a).

Canine cytokine and chemokine mRNA quantification. Quantification of mRNA copies of canine TNF-α, IL-6, and IL-8 in samples of lung tissue from 10 control dogs and 10 dogs with *S. equi* subsp. *zooepidemicus*-associated pneumonia showed that *S. equi* subsp. *zooepidemicus*-infected dogs had significantly higher levels of TNF-α, IL-6, and IL-8 mRNA than uninfected control dogs did ($P = 0.002$, $P < 0.001$, and $P < 0.001$, respectively). Specifically, the mean number of mRNA copies (per nanogram of cDNA) were 5.8 and 42.3 (TNF-α), 2.4 and 634.9 (IL-6), and 380.5 and 27929.2 (IL-8) for control and *S. equi* subsp. *zooepidemicus*-infected animals, respectively, corresponding to 7.3-, 264.5-, and 73.4-fold increases for TNF-α, IL-6, and IL-8, respectively (Fig. 3).

DISCUSSION

This study systematically characterized pneumonia due to *S. equi* subsp. *zooepidemicus* in dogs using a specifically designed and objective histopathological scoring scheme. The majority of *S. equi* subsp. *zooepidemicus*-infected dogs with pneumonia had severe fibrino-suppurative, necrotizing, and hemorrhagic pneumonia, which is strongly associated with severe clinical signs. A small number of dogs (group 1) had no clinical signs of respiratory disease despite isolation of *S. equi* subsp. *zooepidemicus* from a postmortem lung wash. It is possible that these dogs were either carriers of *S. equi* subsp. *zooepidemicus*, perhaps they had previously been infected and recovered, or bacterial and/or host factors contributed to the dogs not developing clinical disease.

While a number of other viral and bacterial pathogens were present in the dog population studied, none of these pathogens were associated with pneumonia as assessed by using the histopathological score. Moreover, histopathological lung lesions typical for infection with canine herpesvirus (CHV), canine parainfluenza virus (CPIV), canine adenovirus (CAV), and canine distemper virus (CDV) were absent. Several of these other agents, in particular canine respiratory coronavirus (CRCoV), are associated with upper respiratory tract infections, and thus, their involvement in pneumonia would not be expected. Interestingly, dogs coinfected with *B. bronchiseptica* had a significantly lower histopathological score ($P = 0.04$) than dogs not coinfected with *B. bronchiseptica*. This should be interpreted with caution because of the borderline significance of the association; however, this is supportive of *B. bronchiseptica* having a tropism for the canine upper respiratory tract. In addition, the presence of two different bacteria competing within the same organ system could reduce the overall lung load of *S. equi* subsp. *zooepidemicus*. An interesting potential implication of this result is that the widespread use of kennel

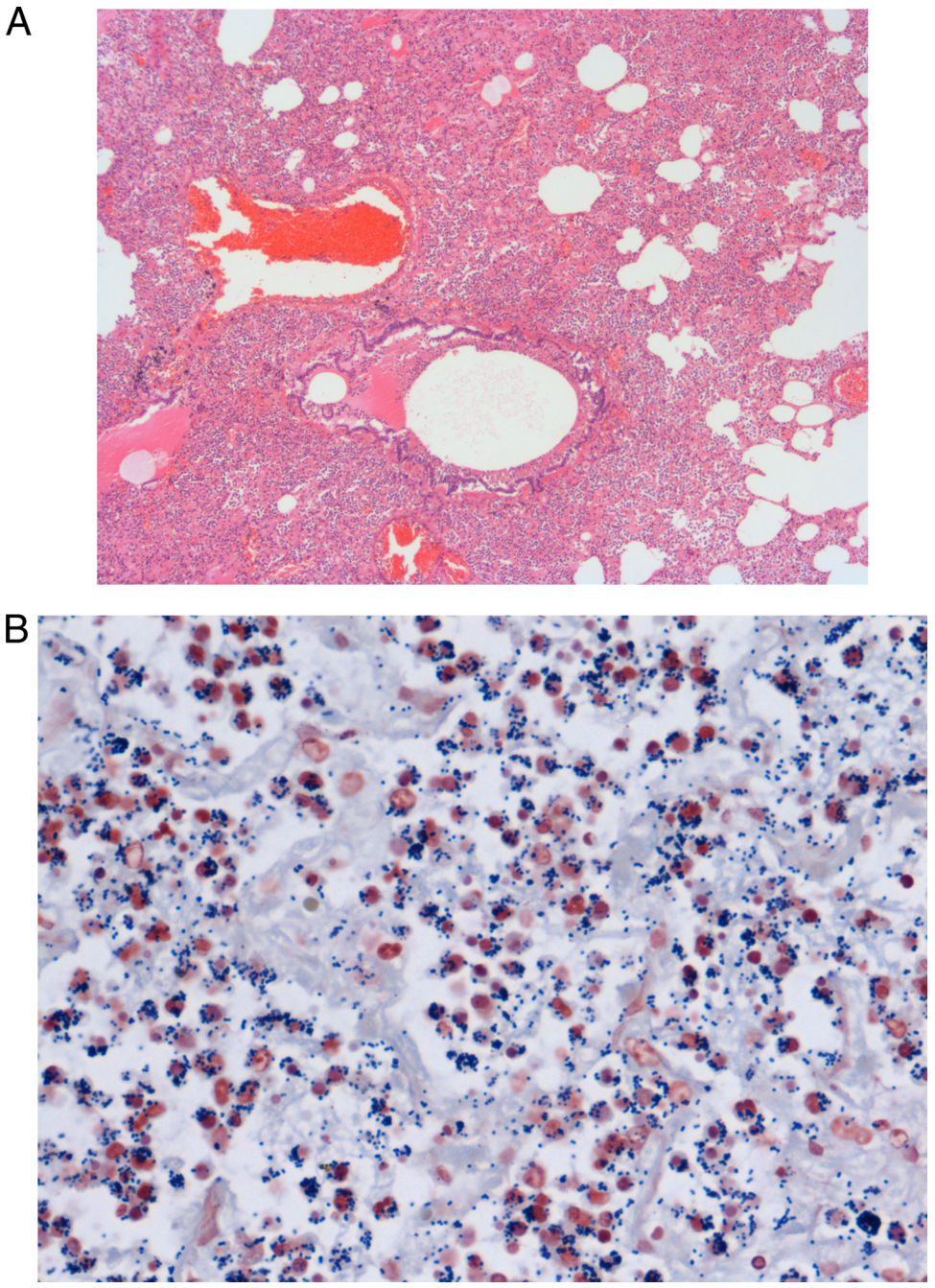

FIG. 1. Canine lung sections. (A) Typical fibrino-suppurative, necrotizing, and hemorrhagic pneumonia due to *S. equi* subsp. *zooepidemicus*, characterized by diffuse filling and obliteration of alveolar spaces by numerous neutrophils, fibrin, and proteinaceous fluid. Bronchioles contain hemorrhaged blood, proteinaceous fluid, and sloughed necrotic cells. This section was stained with hematoxylin and eosin. Magnification, ×20. (B) Neutrophils frequently contain numerous intracellular Gram-positive cocci. This section was Gram stained. Magnification, ×40.

cough vaccines, containing *B. bronchiseptica*, may have created a "niche" for *S. equi* subsp. *zooepidemicus* infection in dogs. Of the mycoplasmas, only *Mycoplasma cynos* has been associated with lower respiratory tract infections (28). However, in this study, there was no association between the presence of this bacterium and the histopathological score.

Three superantigen genes, *szeF*, *szeN*, and *szeP*, the products of which have 59%, 34%, and 49% amino acid sequence identity to SpeH, SpeL, and SpeM of *S. pyogenes*, respectively, were previously identified in the genome of isolate *Sz*BHS5 (23a). In order to try to explain differences in both the clinical signs and lung pathology of dogs known to be infected with *S. equi* subsp. *zooepidemicus*, isolates obtained from postmortem lung washes were screened for the presence of these genes by PCR. Thirty-six percent of isolates in this study were positive, and the identity of the amplified products of one strain was confirmed

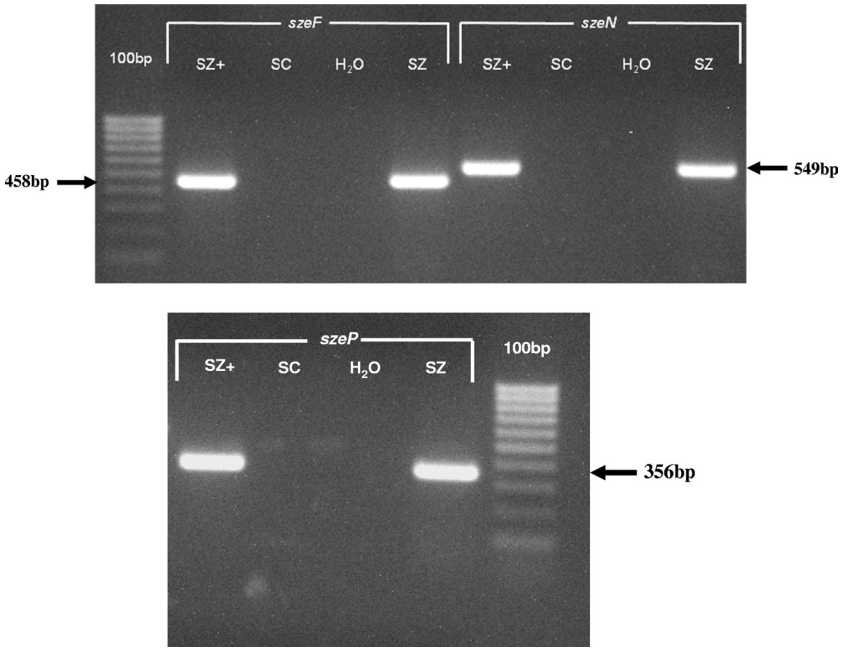

FIG. 2. PCR amplification of *S. equi* subsp. *zooepidemicus* DNA using internal primers to the superantigen genes *szeF*, *szeN*, and *szeP*. Lanes: SZ+, *S. equi* subsp. *zooepidemicus* isolate *Sz*BHS5; SC, *S. canis* control; H_2O, water control with no DNA added; SZ, *S. equi* subsp. *zooepidemicus* isolate from one dog (isolate *Sz*BHS7).

by sequencing. The remaining 64% of isolates were negative for all three genes, and there was no association between the presence or absence of these genes and clinical respiratory or histopathological scores. This suggests that either these proteins do not have superantigen activity *in vivo* or that other, as yet unidentified, virulence factors may be associated with severe *S. equi* subsp. *zooepidemicus*-associated disease in dogs.

The mRNA levels of TNF-α, IL-8, and IL-6 were significantly increased in the lungs of dogs with severe pneumonia induced by *S. equi* subsp. *zooepidemicus* than in uninfected dogs without lung pathology, suggesting that raised levels of these proinflammatory cytokines contribute to the pathogenesis of the infection in canine lungs. Studies in humans have shown a direct correlation between serum levels of proinflammatory cytokines, including TNF-α and IL-6 and the severity of streptococcal toxic shock syndrome (STSS) (22). It has recently been shown that plasma levels of IL-6 in dogs are predictive for the severity of sepsis and systemic inflammatory response syndrome (SIRS) (26). The role of these cytokines in severe pneumonia is also documented for other bacteria and in other species, particularly in pigs with *Mycoplasma hyopneumoniae* (1, 27). Host inflammatory factors are involved in lung lesion development in porcine *Actinobacillus pleuropneumoniae* infection, where TNF-α together with IL-1 stimulates the synthesis and rapid release of IL-8 by pulmonary macrophages following acute insult (14). IL-8 is strongly chemotactic for neutrophils and given the presence of numerous neutrophils observed microscopically, would be expected to be significantly elevated. The presence of large numbers of neutrophils adds to the pulmonary injury via the release of inflammatory mediators, e.g., leukotrienes and proteases, a process termed acute respiratory distress syndrome (ARDS) (29).

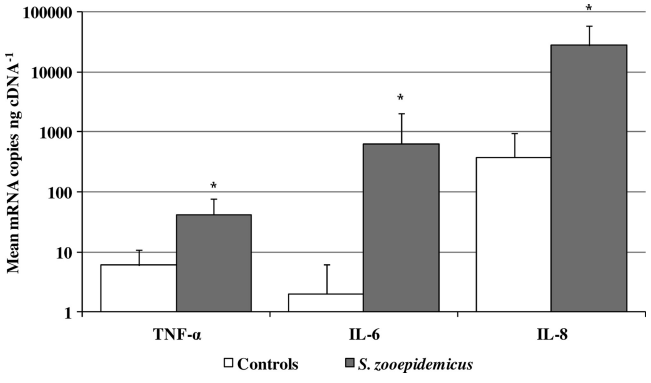

FIG. 3. Mean number of canine cytokine and chemokine mRNA copies in samples of lung tissue from control and *S. equi* subsp. *zooepidemicus*-infected dogs. Values that are statistically significantly different ($P < 0.005$) in the control lung and infected lung are indicated by an asterisk. The values are means plus 1 standard deviation (error bars).

S. equi subsp. *zooepidemicus* is frequently associated with acute and often fatal clinical disease in dogs. This study has shown that when pneumonia does occur, it is usually histopathologically severe and extensive. On the basis of detailed histopathological examinations, the *szeF*, *szeN*, and *szeP* genes are not essential factors in pathogenesis of the disease; however, the significant increases in proinflammatory cytokine mRNA within the lungs suggest that this bacteria can evoke potentially detrimental immune dysregulation in dogs. Histopathology alone may not be sensitive enough to establish the involvement of superantigens in the disease, and further investigations will be required to explore the many unanswered questions regarding the high pathogenicity of this organism in dogs.

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