Genetic Diversity of *Streptococcus equi* subsp. *zooepidemicus* and Doxycycline Resistance in Kennelled Dogs

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The genetic diversity and antibiotic resistance profiles of 38 *Streptococcus equi* subsp. *zooepidemicus* isolates were determined from a kennelled canine population during two outbreaks of hemorrhagic pneumonia (1999 to 2002 and 2007 to 2010). Analysis of the *szp* gene hypervariable region and the 16S-23S rRNA intergenic spacer region and multilocus sequence typing (MLST) indicated a predominant *tetO*-positive, doxycycline-resistant ST-10 strain during 1999 to 2002 and a predominant *tetM*-positive doxycycline-resistant ST-62 strain during 2007 to 2010.

*S. equi* subsp. *zooepidemicus* is associated with severe respiratory disease in kennelled dogs (4, 11, 16). It is rarely found in household pet dogs with respiratory disease symptoms (5). Typing of equine isolates by analysis of the 16S-23S rRNA gene intergenic spacer (IGS) region (6) and the *szp* gene (17) and multilocus sequence typing (MLST) (19) indicate that *S. equi* subsp. *zooepidemicus* is a diverse species with over 272 different sequence types (STs) recorded in the *S. equi* subsp. *zooepidemicus* MLST database (http://pubmlst.org/szooepidemicus/) (10). Relatively little is known about the genetic diversity of canine isolates of *S. equi* subsp. *zooepidemicus*, although isolates from outbreaks of hemorrhagic pneumonia in the United Kingdom and United States were shown by MLST to be genetically related (19). Equine isolates of *S. equi* subsp. *zooepidemicus* can show resistance to a range of antibiotics, including trimethoprim-sulfonamide, enrofloxacin, and tetracycline (7, 9). Canine *Streptococcus canis* isolates can harbor resistance to tetracycline, enrofloxacin, clindamycin, and erythromycin (12, 15). This is the first study to present data regarding the antibiotic resistance and genetic diversity of canine *S. equi* subsp. *zooepidemicus* isolates from two persistent outbreaks of hemorrhagic pneumonia (1999 to 2002 and 2007 to 2010) in a kennelled rehoming center population.

A total of 42 canine isolates of *S. equi* subsp. *zooepidemicus* were analyzed in this study. Thirty-eight isolates were from a kennelled dog population; 2/38 were from apparently healthy dogs (lungs), 17/38 were from dogs with mild respiratory disease, defined as a cough with or without nasal discharge (14 lungs, 1 tonsil, 2 oropharynx), 18/38 were from dogs with hemorrhagic pneumonia (lungs), and 1/38 was from a dog with otitis externa (ear canal). Four additional canine isolates were obtained from pet dogs, including a dog with rhinitis (nasal swab), dermatitis (skin swab), heart failure (pleural effusion), and hemorrhagic pneumonia (lungs).

Additional isolation of *S. equi* subsp. *zooepidemicus* on Columbia CNA agar (Becton Dickinson, United Kingdom) and PCR identification (1) was performed on oropharyngeal swabs from dogs on the day of entry into the study (*n* = 33) and those kennelled for 7 days or longer (*n* = 45) during 2010. On entry into the study, 0/33 (95% confidence interval [95% CI], 0.000 to 0.1239) were culture positive for *S. equi* subsp. *zooepidemicus*, increasing to 9/45 (20%; 95% CI, 0.1067 to 0.3404) in dogs kennelled for more than 7 days (*P* = 0.009 by Fisher’s exact two-sided test), indicating that dogs were infected after entering the kennel where the infection was apparently enzootic.

MLST analysis was performed on 36 isolates as previously described (19) except that sequencing primer *nrdE3* was replaced by *nrdE5* (5′-AGC-ATA-GGTT-GCA-TGA-TGA-T-3′), *proS2* was replaced by *proSS* (5′-GAG-TTC-TTA-GAG-CCA-GCC-AG-3′), and additional reverse primer *yzqL5* (5′-ATG-ACC-AAG-AG C-AAAT-ACC-3′) was used. Allele numbers and sequence types were assigned using the *S. equi* subsp. *zooepidemicus* MLST database at http://pubmlst.org/szooepidemicus/ (10) and analyzed using eBURST (http://eBURST.mlst.net) (8). MLST data for 6 isolates were taken from the literature (19). All 42 isolates were subjected to *szp* gene hypervariable (HV) region PCR amplification (18) and sequencing using *szp5* (5′-CTA-GCA-GCA-GAA-GGA-GCA-ATC-AC-3′) and *szp6* (5′-TTG-ACC-GAG-TCA-ATC-TTC-TTG-GTA-GCA-3′) as well as 16S-23S rRNA intergenic spacer region PCR analysis (6). Eleven MLST sequence types were identified, encompassing six different HV types and three IGS types (Table 1). The four isolates from household pet dogs were MLST types ST-6 (C2HV2), ST-26 (A1HV4), ST-118 (A1HV3), and ST-200 (A1HV2) and were distinct from those from kennelled dogs (Fig. 1). Within the kennelled population during the outbreak in 1999 to 2002, six different MLST sequence types and four IGS/HV types were identified (Table 1). During this period, two MLST types predominated; ST-123 was isolated from early pneumonic cases in 1999 and was replaced by ST-10 as the predominant type in 2000. ST-10 then persisted until 2002. During the second outbreak (2007 to 2010), a single MLST/HV/IGS type was isolated, ST-62/A1HV1. This MLST type was not isolated prior to 2007, and this clonal infection persisted and was enzootic in the kennelled population over a period of at least 3 years.
years. All typing methods gave consistent results and overall divided the isolates into the same groups (Table 1) with MLST providing the greatest discrimination. Studies indicate far greater genetic diversity of *S. equi* subsp. *zooepidemicus* isolates from horses than from the dogs in this study (13), reflecting the commensal nature of infection in horses. In dogs, this species is not a common commensal. It was not isolated from dogs on entry to the kennel and is rarely isolated from household pet dogs (5). The absence of *S. equi* subsp. *zooepidemicus* in dogs on entry to the kennel supports enzootic infection maintained within the population with the introduction of new strains being less common. Of the 11 STs identified, 7 (including the predominant ST-10 and ST-62) had previously been identified in other animals (horses, cattle, and dogs) or humans. ST-6, ST-22, ST-47, and ST-123 are so far restricted to canines.

MICs for doxycycline (Sigma, United Kingdom) were determined with 0.5 to 128 mg/liter using agar dilution (2) and the following breakpoints: sensitive, ≤4 mg/liter; intermediate, 8 mg/
liter; and resistant, ≥16 mg/liter (3). The presence of the tetracycline resistance genes tetK, tetL, tetM, and tetO was examined by PCR (14). In total, 22/42 isolates were resistant to doxycycline. All 17 ST-10 strains and two single-locus variants (SLVs) (ST-22 and ST-47) had the tetO gene and a corresponding MIC of 64 mg/liter for doxycycline. Five isolates of ST-62 from 2007 to 2008 were sensitive to doxycycline (MIC of 0.5 mg/liter), whereas three ST-62 isolates from 2009 to 2010 with a MIC of 64 mg/liter had acquired the tetM gene. All other isolates analyzed were sensitive to doxycycline, and tetracycline resistance genes were absent in these strains. Doxycycline was used as a treatment against respiratory disease in this kennelled population, and it is possible that this led to selection of the tetO-positive doxycycline-resistant ST-10 strain. As doxycycline-sensitive isolates of ST-10 or its SLVs were not identified, it is possible that the resistance gene was acquired by this strain before introduction into this population. The outbreak in 2007 to 2010 was attributed to ST-62. Acquisition of the tetM gene occurred in this ST between 2008 and 2009. Sequence analysis of the szp gene revealed 100% DNA identity from 2007 to 2010 (data not shown), supporting the hypothesis that acquisition of tetM in ST-62 occurred in the kennel.

The emergence of doxycycline-resistant strains within this population on at least two occasions was likely driven by selective pressure from empirical antimicrobial use, and regular sensitivity profiling may be beneficial in future outbreaks. Control measures within kennelled populations that minimize contact and ensure stringent hygiene measures to prevent indirect spread of S. equi subsp. zooepidemicus via fomites may prove more successful than screening of new arrivals.

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