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Characterization of a mucoid clone of *Streptococcus zooepidemicus* from an epizootic of equine respiratory disease in New Caledonia

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ABSTRACT

Streptococcus equi subspecies *zooepidemicus* (*Sz*) is a tonsillar and mucosal commensal of healthy horses with the potential to cause opportunistic infections of the distal respiratory tract stressed by virus infection, transportation, training or high temperature. The invasive clone varies from horse to horse with little evidence of lateral transmission in the group. Tonsillar isolates are non-mucoid although primary isolates from opportunist lower respiratory tract infections may initially be mucoid. In this study, a novel stably mucoid *Sz* (*Sz*NC) from a clonal epizootic of respiratory disease in horses in different parts of New Caledonia is described.

*Sz*NC (ST-307) was isolated in pure culture from transtracheal aspirates and as heavy growths from 80% of nasal swabs ($n = 31$). Only 4% of swabs from unaffected horses ($n = 25$) yielded colonies of *Sz*. A viral etiology was ruled out based on culture and early/late serum antibody screening. Evidence for clonality of *Sz*NC included a mucoid colony phenotype, SzP and SzM sequences, and multilocus sequence typing. *Sz*NC, with the exception of isolates at the end of the outbreak, was hyaluronidase positive. Its SzP protein was composed of an N2 terminal, and HV4 variable region motifs and 18 carboxy terminal PEPK repeats. Biotin labeling of surface proteins revealed DnaK and alanyl-tRNA synthetase (AlaS) on the surface of clonal isolates, but not on non-clonal non-mucoid *Sz* from horses in the epizootic or unrelated US isolates. Reactivity of these proteins and SzP with convalescent serum indicated expression during infection.

Introduction

Streptococcus equi subspecies *zooepidemicus* (*Sz*) of Lancefield group C is a normal commensal of the tonsils of healthy horses, but it is an important opportunist cause of rhinitis, bronchiolitis and pneumonia in foals and horses stressed by virus infection, high temperature, training or transport (Edwards, 1933; Hoffmann et al., 1993; Timoney, 2004; Wood et al., 2005). Multiple serovars of *Sz* containing the SzP protein are found in the lingual and palatine tonsils of healthy horses (Anzai et al., 2000), single clones of which dominate in the lungs of foals and donkeys with pneumonia. These clones vary from case to case in the same group of animals. Colonies of *Sz* from the tonsil are uniformly non-mucoid but are often briefly mucoid in primary cultures from lung or tracheal washes (Edwards, 1933; Hoffmann et al., 1993; Anzai et al., 1999). The amount of capsular hyaluronic acid is responsible for the mucoid colony phenotype and for resistance to phagocytosis (Anzai et al., 1999; Wibawan et al., 1999). There is evidence it may facilitate adherence and invasion (Wibawan et al., 1999).

Although most cases of equine respiratory disease associated with *Sz* reflect endogenous secondary infection by a single clone selected stochastically from a tonsillar site, epizootics of respiratory disease in horses and dogs with specific virulent clones that vary in multilocus sequence typing (MLST) have been reported recently (Pesavento et al., 2008; Björnsdóttir et al., 2012; Lindahl et al., 2013). Outbreaks of disease in other hosts such as the pig, monkey, guinea pig and dog are almost always clonal and seldom endogenous (Seastone, 1939; Feng and Hu, 1977; Salasia et al., 2004; Pesavento et al., 2008). Colony appearance can be influenced by hyaluronidase. For example, phage encoded hyaluronidases change the mucoid colony phenotype of some *S. equi* (*Se*) to matt at the end of the exponential phase of growth (Timoney et al., 1991). Unlike *Sz*, the gene for host encoded hyaluronidase in *Se* is inactivated by a frame shift mutation and so capsule production is abundant and constant (Holden et al., 2009). Conversely, most strains of *Sz* produce an active hyaluronidase and colonies usually appear non-mucoid with the exception of some newly isolated

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Table 1
Isolates of *Streptococcus zooepidemicus* (*Sz*) from affected horses in representative outbreaks of respiratory disease in different parts of New Caledonia.

Sz	Outbreak number	Date	Area	*Sz* colony phenotype	Hyaluronidase (mm)	
					Mucoid	Non-mucoid
NC28	3	27/10/97	Bourail (a)	Mucoid + non-mucoid	8	8
NC29	3	27/10/97	Bourail (a)	Mucoid + non-mucoid	13	10
NC30	3	27/10/97	Bourail (a)	Mucoid	9	NA
NC32	4	27/10/97	Bourail (b)	Mucoid + non-mucoid	14	7
NC37	1	31/10/97	Nouméa	Mucoid	13	NA
NC41	6	12/11/97	Bouffeneche	Mucoid + non-mucoid	10	10
NC42	7	12/10/97	Jego Boglea	Mucoid	8	NA
NC54	8	12/11/97	Mont-Meu	Mucoid + non-mucoid	10	10
NC78	11	21/11/97	Bourail (c)	Mucoid + non-mucoid	10	9
NC88	13	29/07/98	E Royallei	Mucoid	0	NA
NC89	13	29/07/98	Galea	Mucoid	0	NA
NC91	13	29/07/98	Armistice	Mucoid	0	NA

Bourail a, b, c were different stables; NA, not applicable.

strains from the lower respiratory tract (Sting et al., 1990; Anzai et al., 2000).

The aim of this study was to characterize a mucoid clone (NC) of *Sz* and associated immune responses from an epizootic of respiratory disease in horses in New Caledonia that persisted for 10 months and for which there was no evidence of a primary viral etiology. Surface exposed proteins, hyaluronidase activity and reactivity with convalescent sera were studied.

Materials and methods

Bacterial strains and media

Mucoid and non-mucoid *Sz* originally cultured from nasal swabs and transtracheal aspirates were from affected weanling and adult horses in a widespread epizootic of contagious respiratory tract disease in New Caledonia (Table 1). Strains were cultured in Todd Hewitt Broth with 0.2% yeast extract using 1% inocula or plated on CNA-horse blood agar plates at 37 °C overnight. Equine *Sz* strains of US origin included for comparison of protein profiles were *Sz*631 (peritonitis); UK30, W60 (lymphadenitis); RT, E69 (pneumonia); NH182; 7e (rhinitis).

Polymerase chain reaction (PCR) amplification and sequencing of szp

Selection of isolates for SzP typing was based on the appearance of mucoid and non-mucoid colonies of each on CNA-horse blood agar. The SzP protein is an important protective antigen of *Sz* and the basis of Moore and Bryans typing system (Walker and Timoney, 1998). Primers IGSzPF (5′ CTT GCT AAA GTA ATG GTT GAC 3′) and IGSzPR (5′ GTT TGT GAG CAA GGC TTA GTC 3′) were designed 144 base pairs (bp) upstream and 138 bp down-stream of *szp*, respectively. These primers amplified a 1212 bp fragment that included SzP. The PCR protocol consisted of 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min. PCR products were purified using GeneJET PCR purification kit (Fermentas) and sequences (Eurofins MWG Operon) obtained using primers from the initial amplification.

MLST typing

MLST typing was performed as described elsewhere (Webb et al., 2008). Sequence type (ST) was assigned based on existing allelic profiles.[1]

Cell-associated hyaluronic acid

Hyaluronic acid was assayed as described by Wessels et al. (1991) with minor modifications. Overnight cultures in Todd Hewitt Broth (10 mL) at 37 °C were centrifuged at 16,000 *g* for 20 min. The pellet was gently washed with phosphate buffered saline (pH 7.2), re-suspended in 2 mL of distilled water, and then transferred into a capped glass tube and shaken vigorously for 1 h with 5 mL chloroform. After centrifugation at 2500 *g* for 10 min, the aqueous phase was assayed by the carbazole method (Bitter and Muir, 1962). Hyaluronic acid from *Sz* (Sigma) was used as a control.

[1] See: http://pubmlst.org/szooepidemicus/.

Hyaluronidase

Hyaluronidase from *Sz* cultures grown at 37 °C was assayed by a modification of the rapid plate method (Smith and Willett, 1968). Aliquots (10 μL) of overnight cultures in Todd Hewitt Broth incubated at 37 °C were placed in wells (triplicate) of Todd Hewitt Broth agar supplemented with 0.3 mg hyaluronic acid/mL from *Sz* (Sigma) and 10 mg bovine serum albumin per mL. The plate was incubated at 37 °C for 16 h and zones of clearing following addition of 2 M acetic acid measured in mm.

Sera

Sera from *Sz* infected horses in New Caledonia collected 6 months after recovery were stored at −80 °C. A goat was immunized intramuscularly with 1.5 mL of a suspension of mucoid *Sz*NC78 prepared from 10 mL of culture (1.5×10^8 CFU/mL) heat-killed at 56 °C for 90 min, 15 μL of merthiolate (1:1000) and 60 μL Quil A (10 mg/mL). Two booster doses were given at 15 day intervals. Other goats were separately immunized IM with 1.8 mL of solutions of 150 μg of rSzPNC78 or rSzMNC78, 18 μL of merthiolate (1:1000) and 72 μL Quil A (10 mg/mL). *Sz*NC78 was a typical mucoid isolate from outbreak 11 (Table 1). Two booster doses of 100 μg of rSzP or rSzM were given at 15 day intervals. Sera from both goats were harvested 14 days later, aliquoted and stored at −20 °C. These sera were diluted 1:200 for immunoblots.

Biotin labeling and preparation of surface proteins

Bacteria were grown as described above and harvested by centrifugation at 16,000 *g* for 20 min. The pellet was washed three times by re-suspension in 0.1 M sterile phosphate buffered saline (pH 7.2) and tagged with 0.5 mg/mL biotin for 30 min with gentle shaking (Pierce). Bacteria were then harvested and washed three times prior to extraction with mutanolysin.

Labeled bacteria were suspended in 1 mL of 50 mM phosphate buffer (pH 7.2) containing 0.5 M sucrose, 0.2 mM phenylmethylsulfonyl fluoride and lysozyme (3 mg/mL). After addition of mutanolysin (150 U/mL, Sigma–Aldrich), the mixture was incubated for 90 min at 37 °C with gentle shaking and then centrifuged at 3000 *g* for 10 min. Protein was estimated using BCA (Pierce) and the samples stored at −20 °C.

Gel electrophoresis and immunoblotting

The proteins in streptococcal mutanolysin extracts were separated on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels and electrophoretically transferred to nitrocellulose as described elsewhere (Timoney et al., 1995). Blots were developed with equine convalescent or goat hyperimmune sera (1:200) followed by protein G conjugated to horseradish peroxidase (1:1000) (Zymed) or with extravidin conjugated to horseradish peroxidase (1:1000) (Sigma). Reactive bands were visualized with 4-chloro-1-naphthol.

Identification of proteins

Protein bands were analyzed in the proteomics core facility of the Department of Molecular and Cellular Biochemistry, University of Kentucky by tandem mass spectrometry. Briefly, gel slices were vacuum dried and alkylated by addition of 50 mM NH_4HCO_3 containing 50 mM iodoacetamide and incubated for 30 min in the dark at room temperature. Proteins were digested in-gel using proteomics grade trypsin at a concentration 20 ng/μL on ice for 1 h. Extracted peptides were then analyzed by an Applied Biosystems 4800 MALDI TOF/TOF Proteomics Analyzer. Peak