Characterization and protective immunogenicity of SzM of Streptococcus zooepidemicus NC78 from a clonal outbreak of equine respiratory disease

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Streptococcus zooepidemicus (Sz) of Lancefield group C is a highly variable tonsillar and mucosal commensal usually associated with opportunistic infections of the respiratory tract of vertebrate hosts. More virulent clones have caused epizootics of severe respiratory disease in dogs and horses. Virulence factors of these strains are poorly understood. The anti-phagocytic SeM is a major virulence factor and protective antigen of S. equi (Se), a clonal biovar of an ancestral Sz. Although the genome of SzH70, an equine isolate, encodes a partial homolog (szm) of sem, expression of the gene has not been documented. We have identified and characterized SzM from an encapsulated Sz from an epizootic of equine respiratory disease in New Caledonia. SzMNC78 has a predicted predominantly alpha-helical fibrillar structure with a LPSTG cell surface anchor motif and resistance to hot acid. A putative binding site for plasminogen is present in its B-repeat region the sequence of which shares homology with repeats of the plasminogen binding proteins of human group C and G streptococci. Equine plasminogen is activated in a dose response manner by rSzMNC78. Only 23.20 and 25.46% DNA homology is shared with SeM of prototype strains SeCF32 and Se4047 and with SzM proteins of other Sz for which homology ranges from 19.60 to 54.70%. As expected, SzMNC78 reacted with convalescent sera from horses with respiratory disease associated with strains of Sz. SzMNC78 resembles SeM in binding equine fibrinogen and eliciting strong protective antibody responses in mice. Sera of vaccinated mice opsonized SzNC78 and SzW60, the SzM of which shared partial amino acid homology with SzMNC78. We conclude that SzM is a protective antigen of SzNC78 and was strongly reactive with serum antibody of horses during recovery from Sz associated respiratory disease.

Keywords: Streptococcus zooepidemicus, SzM, fibrinogen binding, protective antigen.
INTRODUCTION

*Streptococcus zooepidemicus* (*Streptococcus equi* subsp. *zooepidemicus*; Sz) of Lancefield group C is a normal tonsillar and mucosal commensal of the upper respiratory tract of Equidae. Although a variety of serovars are present in tonsils of healthy horses, respiratory disease is associated with a single clone which is usually present in large numbers in bronchial and nasopharyngeal secretions (1). Unlike its clonal derivative *S. equi* (Se), comparisons of genome sequences of Sz in databases confirm genetic variability and extensive rearrangement/recombination as suggested by early studies (2, 3). Sz opportunistically produces respiratory disease in situations of virus infection, heat stress, and prolonged transportation (4). Select clones can be devastating pathogens in intensively housed dogs and guinea pigs and in humans following consumption of contaminated milk and cheese (5, 6, 7).

Few virulence factors of Sz have been recognized. SzP, an anti-phagocytic, hypervariable and protective M-like protein is a mosaic of 2 variable N-termini, at least 5 variable central regions and a variable number of PEPK C-terminal repeats (8). Vaccination with recombinant SzP of SzW60 protected mice against homologous intraperitoneal challenge (9). An intranasal live attenuated *Salmonella enterica* serovar Typhimurium MGN707 expressing SzP from an MB9 serovar of Sz was effective in reducing persistence of SzMB9 *in utero* (10). However, there is evidence that other protective antigens exist. A SzP deletion mutant of SzATCC35246 protected mice against intramuscular challenge (11).

The 58 kDa anti-phagocytic SeM is a major virulence factor and protective antigen in Se, a host specific clonal biovar of an ancestral Sz that causes equine strangles. SeM binds fibrinogen which reduces deposition of C3b on the bacterial surface and phagocytosis by neutrophils (12). SeM elicits strong serum IgG and mucosal IgA responses following infection.
(13) and vaccines rich in SeM reduce disease severity and morbidity (14). Although the N-terminal sequence of SeM varies, different isolates are uniformly susceptible to the opsonic-bactericidal effect of a single opsonic serum suggesting some opsonogenic epitopes are invariant (15, 16, 17). Whole genome annotation of SzH70 has revealed a partial sem homolog designated szm (18). Expression of SzM by Sz and stimulation of antibody response and protective efficacy have not been documented.

The aims of these studies were to clone and express SzM from SzNC78 from a clonal epizootic of equine respiratory disease, compare its amino acid sequence to that of SeM, determine its fibrinogen binding ability, opsonogenicity, reactivity with convalescent sera and evaluate its protective efficacy in mouse immunization and challenge experiments.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Sz isolates from different cases and outbreaks of equine respiratory disease are listed in Table 1. Isolates from a case of peritonitis in a pony and one isolate from an outbreak of canine hemorrhagic pneumonia are also included. SzNC78 was a representative isolate from an epizootic of equine respiratory disease in New Caledonia in 1997-98. The epizootic persisted for 10 months and involved weanling and adult horses on at least 13 riding premises in different parts of New Caledonia. Clinical signs included coughing and purulent nasal discharge. A specific clone of mucoid Sz (ST-307) was isolated in pure culture from transtracheal aspirates from some affected animals and as heavy growths from the majority of nasal swabs (n = 56). Only 4% of swabs from unaffected horses were positive for Sz. Virus culture combined with early/late serum antibody screening for Influenza, EHV1, Adenovirus and Rhinovirus failed to incriminate a viral etiology. szp of mucoid strains of Sz
isolated from stables in the epizootic expressed a protein with N1 terminal and HV4 hypervariable domains (GenBank accession numbers # HM 565772; HM 565773; HM 565774). This isolate was subsequently cultured overnight at 37°C in Todd Hewitt Broth (THB) + 0.2% yeast extract.

pET-15b, *Escherichia coli* strains Novablue and BL21 were obtained from Novagen (Madison, WI). pBluescript phagemid, lambda ZAP II pre-digested vector, ExAssist helper phage, *E. coli* strains XL1-Blue MRF+ and SOLR were from Stratagene (La Jolla, CA). All *E. coli* strains were grown at 37°C in LB medium and supplemented with ampicillin (100 µg/ml) whenever required.

**Convalescent and hyper-immune sera.** Convalescent equine sera were from the Gluck Equine Research Center collection. All samples screened at 1:200 dilution were from weanling and adult horses with clinical evidence (nasal discharge, cough, fever, lung consolidation) of respiratory disease and detection of large numbers of Sz in cultures of nasal swabs and nasopharyngeal lavages. Polyclonal antiserum was raised in yearling goats by subcutaneous administration of 150 µg of purified rSzM with QuilA (10 mg/ml) as adjuvant. Booster injections contained 100 µg of recombinant protein and were administered 14 and 28 days after the primary immunization. Serum was obtained 2 weeks after final booster. Antibody titers in sera collected on Days 0, 28 and 42 were determined by ELISA using rSzM as antigen.

**Genomic DNA library.** A genomic DNA library of SzNC78 was constructed as described previously (19).

**Library screening.** The library was screened with a pool of 5 convalescent sera (1:200) from horses from the epizootic of respiratory disease. Bound antibody was detected with horseradish peroxidase (HRP)-labeled protein G (Zymed, San Francisco, CA) diluted 1:1,000 followed by 4-
chloro-1-naphthol. Positive plaques on agar plugs were allowed to elute overnight at 4°C in 500 µl of SM buffer. Reactive plaques were rescreened until clonal. Plasmids containing inserts of Sz DNA from selected reactive phages by using ExAssist helper phage and E. coli SOLR according to the manufacturer’s protocol. Following SDS-PAGE, proteins in lysates of each reactive phage were transferred to nitrocellulose, blotted using the convalescent equine serum pool and molecular weights of proteins represented by reactive bands calculated.

**DNA sequencing and analysis.** Plasmid DNA was isolated using a Zippy™ plasmid miniprep kit (Zymo Research, Irvine, CA) and sequenced in a commercial sequencing facility (Eurofin MWG operon, Alabama) using standard T3 and T7 primers. The complete nucleotide sequences from inserts were then compared with SzH70 (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_zooepidemicus). Putative coding sequences were predicted using Sequence manipulation suite, PSORT and SignalP, and protein secondary structure and transmembrane domains were predicted using SABLE (http://www.expasy.ch/tools/).

**Sequencing of SzM from different Sz isolates.** The open reading frames of szm’s from SzNC78, RT, NH55426, NH38, NH182 and W60 were amplified by PCR using chromosomal DNA as template and szm primers (SzMF – 5’ ATA AAG AAG TTC CTG TCA TTA 3’ and SzMR – 5’ CAA CAG ACA GGA GAC TGT TGC 3’). The PCR protocol consisted of 30 cycles of 94°C for 30 sec, annealing at 55°C for 30 sec, and 72°C for 2 min. Amplicons were purified using GeneJET PCR purification kit (Fermentas) and sequences (Eurofins MWG Operon) were obtained using primers from the initial amplification. Sequences were analyzed as described above.

**Recombinant SzMNC78.** The open reading frame of szm without signal sequence was amplified by PCR using chromosomal DNA of SzNC78 as template and szm specific primers.
(SzMNC78F - 5’ TTG CTC GAG GAG GAT TTT AAT GGC GCT AAT TCT 3’ and SzMNC78R - 5’ CAT GGA TCC TTA ACC TGC TTT AGG TGC TG 3’). The amplicon thus generated was digested using BamHI and XhoI and ligated into pre-digested pET-15b with poly-histidine residues. The ligate was then transformed into *E. coli* Novablue to increase plasmid copy number. Positive clones were identified by colony PCR and recombinant plasmids transformed into *E. coli* BL21. High level expression of poly-histidine tagged recombinant SzM (rSzM) was achieved by growing overnight in Overnight Express™ instant TB medium (Novagen, Madison, WI). Recombinant protein was extracted using Talon Superflow metal affinity resin (Clontech Laboratories, Inc.) in buffer containing 8M urea according to the manufacturer’s recommendations. rSzM was dialyzed against 20 mM Tris containing 50 mM NaCl (pH 7.5) and purity was checked by SDS-PAGE.

**Gel electrophoresis and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in an X-Cell SureLock Mini-Cell (Invitrogen, Carlsbad, CA) for 2 h at 100 V in Tris-glycine running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.8). Samples adjusted to equalize protein concentrations were mixed with equal volumes of 2X gel loading buffer (100 mM Tris-Cl [pH 6.8], 10% SDS, 50% glycerol, 500 mM dithiothreitol, and 0.1% bromophenol blue) and boiled for 2 - 3 min before loading. Gels were rinsed twice in distilled water and stained with 0.3% Coomassie brilliant blue R-250 (Sigma). Separated proteins were also transferred electrophoretically to nitrocellulose (0.2 µM; Schleicher & Schuell, Keene, NH) and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (50 mM Tris, 150 mM NaCl, 0.05% Tween 20 [pH 7.5]). The membranes were then incubated with goat antiserum against rSzMNC78 (1:200) followed by Protein G conjugated to
horseradish peroxidase (1:1000) (Zymed, San Francisco, CA). Bound conjugate was detected by using 4-chloro-1-naphthol.

**Hot-acid extracts.** The procedure of Lancefield and Perlmann (20) was used to prepare hot-acid extracts of SzNC78.

**ELISA.** 96 well flat bottom polystyrene ELISA plates (Costar, Corning, NY) were coated with rSzM (1 µg/well) in 100 µl of coating buffer (0.1M carbonate bicarbonate, pH 9.2) overnight at 4ºC. Optimum concentrations of antigen and antibody were determined by checker board titration. After washing in PBS-T (PBS, 0.05% Tween 20), plates were blocked for 1 h at 37ºC with 5% non-fat dried milk in PBS-T. Convalescent sera diluted 1:200 were then added and incubated for 2 h at 37ºC. Bound IgG was detected by incubation with HRP-Protein G diluted 1:8000 (Zymed, San Francisco, CA) for 1 h at 37ºC. Plates were then developed with 15 mg O-phenylenediamine (OPD, Sigma, St. Louis, MO) in 15 ml of 0.1 M citrate buffer and 20 µl H2O2, terminated by the addition of 2M H2SO4, and OD’s read at 490 nm. Wells without coating antigen and serum served as negative controls for background reactivity.

**Fibrinogen binding.** Wells of 96 well polystyrene ELISA plate were coated overnight with 100 µl rSzM solution, ranging in concentration from 0.005 to 10.0 µg per well. After washing and blocking with 5% (w/v) non fat milk, equine fibrinogen (3.0 µg/well) was added to separate wells and incubated for 2 h at 37ºC. After washing, 100 µl of rabbit antiserum (1:100) specific for equine fibrinogen were added and incubated at 37ºC for 2 h. Controls consisted of wells from which fibrinogen or rabbit antiserum was omitted. Binding of fibrinogen specific rabbit antibody was detected by incubation with goat-anti rabbit IgG – HRP. Plates were then developed as described under ELISA. The dose response assay was repeated 3 times in duplicate.
**Plasminogen activation.** Wells of 96 well polystyrene ELISA plate were coated overnight with 100 µl rSzM solution, ranging in concentration from 10.0 to 0.625 µg per well. After washing and blocking with 5% (w/v) non fat milk, equine plasminogen (5.0 µg/well) (Molecular Innovations, Inc., Michigan, USA) was added to separate wells and incubated for 2 h at 37°C. After washing, 100 µl of recombinant streptokinase solution (6.0 µg/well) along with equine fibrinogen (5.0 µg/well) were added to each well and incubated at 37°C. After 10 min of incubation, 50 µl of plasmin substrate D-VLK-pNA (25 µg/mL) (Molecular Innovations, Inc., Michigan, USA) was added and incubated at 37°C for 1 h and OD₄₀₅nm was recorded. Controls consisted of wells from which plasminogen or rSzM or streptokinase was omitted. The assay was repeated twice in duplicate.

**Mouse immunization and challenge.** Two sets of 10 (5 male and 5 female) outbred 8 week old ICR (CD-1): HSD mice (weight: 12 – 14 g) were immunized by sub-cutaneous inoculation of 40 µg of rSzM in 0.15 ml PBS with QuilA (25 µg/mice). Two booster doses were given at a 2 week interval by sub-cutaneous inoculation of 40 and 20 µg of protein in 0.15 ml of PBS. A set of 5 male and 5 female mice immunized with sterile PBS plus QuilA served as controls.

Vaccinated and control groups of mice were challenged 2 weeks after the final vaccine booster. The challenge dose was 3 x 10⁴ CFU of log phase cultures of SzNC78 inoculated intraperitoneally into female mice and 3 x 10³ CFU into male mice. In preliminary trials, these doses caused 100% mortality between 2 and 6 days of inoculation. A lower dose was used in male mice because a shorter time to death was noted when 3 x 10⁴ CFU were inoculated suggesting greater susceptibility of male mice. Mice were observed at approximately 8 h intervals after inoculation for 8 days for signs of illness (depression, rough coat, lack of activity). Sick mice were immediately euthanized and heart blood collected for culture on CNA blood agar.
and assay of SzM specific antibody in serum. Heart blood was also cultured from mice that died in the interval between observations. β-hemolytic colonies were identified as Sz by fermentation of lactose and sorbitol.

Surviving mice were euthanized at 8 days and heart blood collected for culture and assay of serum antibody. Cumulative morbidity curves were prepared for vaccinated and control mice. A Chi square test was used to test for significance in differences in morbidity between groups of control and vaccinated mice. The immunization/challenge protocol requiring prompt euthanasia for humane reasons was required by the University of Kentucky Animal Care and Welfare Committee.

**Opsonophagocytic assay.** Overnight cultures of SzNC78 in THB were diluted $10^{-4}$ in sterile PBS. One hundred µl of this dilution were then combined with 50 µl pooled serum from 3 SzM immunized mice and incubated for 30 min at 37ºC. Opsonized bacteria were then added to 1.0 ml of fresh heparinized horse blood and mixed well. An aliquot of 575 µl was immediately removed and then placed on ice ($T_0$). The remaining suspension was rotated at 37ºC for 90 min and placed on ice ($T_{90}$). Pour plates in triplicate consisted of 15 ml THB agar at 56ºC, 500 µl heparinized horse blood and 150 µl test sample. Colonies were counted after overnight incubation at 37ºC and % reduction in counts from $T_0$ to $T_{90}$ calculated. The donor horse was selected based on a low level of SzP specific serum antibody.

**Accession numbers.** The nucleotide sequence of *szm*’s of NC78, RT, NH38, NH55426, NH182, W60 and UK30 have been deposited in GenBank under accession numbers JX014303, KC146014, KC146015, KC146016, KC146017, KC146018 and KC146019 respectively.

**Statistical analysis.** P values for significance of differences in morbidity were calculated using Chi-square test ($\chi^2$).
RESULTS

Identification and analysis of szm. Seventy immunoreactive plaques were identified during screening of the lambda library of SzNC78 with convalescent equine serum from the New Caledonia epizootic. Plasmids rescued from these phages were sequenced and compared with the annotated genomic sequences of SzH70; SzATCC35246 and SzMGCS10565. Sequencing analysis coupled with immunoblot pattern confirmed 33 plaques expressing different proteins. Thirty three open reading frames (ORF) were identified in inserts, some of which overlapped. A subset of these ORFs was predicted to have surface exposure based on LPxTG or LXYC or a structure typical of a transmembrane domain. Sequence from one phagemid revealed an open reading frame encoding a protein, designated SzM (GenBank accession number - JX014303), of 591 amino acids with a predicted molecular mass of 66 kDa that shared 23.20 and 25.46% homology with SeM of SeCF32 and Se4047 respectively. SzMNC78 shared 19.66, 22.30, 23.30, 24.05, 24.17, 43.52 and 54.66% homology with SzMs of SzH70, SzNH55426, SzUK30, SzNH38, SzRT, SzW60 and SzNH182. The N and C-termini of SzM carried a 42-amino acid signal sequence (VKA↓ED) and a specific sortase recognition sequence LPSTG respectively. SzMNC78 is hot-acid resistant (Fig. 1c) with a secondary structure predicted to contain an extensive region of alpha-helix extending from residue 48 to 511. The secondary-structure prediction shows loops in the vicinity of residues 1 to 48 and 511 to 590 with a small β-strand at its C-terminus (data not shown). Comparisons of the amino acid sequences of SzMNC78 with SeM and SzM’s of different strains of Se and Sz revealed 2 Clades (Fig. 2). Clade 1 composed of Sz strains RT, NH55426, NH38, UK30, H70, ATCC35246 and S. equi CF32 and 4047 and Clade 2 composed of Sz strains NC78, NH182 and W60. SzMNC78 showing two tandem repeats (A: 308 – 332 a.a and 336 – 360 a.a and B: 374 – 404 a.a and 416 – 446 a.a) and 9 to 10 proline-rich...
repeats in the C-terminus. Proline-rich repeats appear to be conserved and unique to Clade 2 strains of Sz and were absent from SeM and from all Clade 1 Sz strains. Comparison of repeat sequences (Table 2) in SeM with SzM’s of the 9 strains of Sz reveals some interesting features. Clade 1 Sz strains all had B repeats with sequence similar to the B repeats of Se. However, 3 different A repeats (A1, A2, A3) are present in Clade 1 strains. The A3 (A3a, A3b, A3c) repeats in both Se and four Sz strains probably represent degenerate versions of each other (Table 2).

The B repeat of SzMNC78 is homologous to the C2, C3 and C4 repeats of the plasminogen binding proteins (MLC36 and MLG72) of human group C and G streptococci and also with C repeats of the Arp4 protein of S. pyogenes (Fig. 3).

Expression of rSzM of SzNC78. Purity of the recombinant SzM was confirmed by SDS-PAGE and staining with Coomassie brilliant blue. An immunoblot was also performed with the convalescent equine serum pool to confirm that rSzM showed the same reactivity as native SzM (Fig. 1a and 1b). Antisera specific for rSzM raised in yearling goats, had antibody titers greater than 1:106400.

Expression of SzM by other equine isolates of Sz. SzM expression was detected in mutanolysin extracts of Sz isolates (NC32 and NC88) from different stables during the New Caledonian epizootic by reactivity with rSzM specific goat antisera (Fig. 4). No signal was detected in extracts of other Sz strains except SzNH182 and Sz7e indicating absence of the protein or low level cross reactivity. Reactivity of the SzM antiserum with a hot-acid extract of SzNC78 confirmed resistance of SzM to acid and heat (Fig. 1c).

SzM-specific antibody levels in convalescent sera. SzM specific antibody levels in convalescent sera of horses with Sz respiratory disease are shown on Fig. 5. Sera from many Sz infected equines including cases from the New Caledonia epizootic had elevated antibody levels.
to rSzMNC78, indicating expression of this protein during lung infection. Sera from healthy horses showed very low reactivity in the ELISA indicating low or absence of non-specific binding of equine IgG by SzM. This was confirmed by substituting HRP-conjugated mouse anti-horse IgG for Protein G, which binds to the same domain on SzM as IgG (21).

**Fibrinogen binding.** SzM showed strong binding to equine fibrinogen immobilized on wells of ELISA plates (Fig. 6). Dose dependent binding of equine fibrinogen to immobilized SzM revealed 320 ng of rSzM as the saturation concentration for 3 µg of fibrinogen.

**Plasminogen activation.** Equine plasminogen showed strong affinity for rSzMNC78 immobilized on wells of ELISA plate (Fig. 7). Plasmin released decreased with decreasing concentration of rSzMNC78, suggesting SzM contributes to protease activity of Sz in tissue.

**Efficiency of SzM as a protective antigen in mice.** Preliminary dose titration revealed that $10^4$ CFU of SzNC78 administered intraperitoneally caused illness (ruffled coat, coughing, and depression) followed by death in few hours in 100% of normal mice. Prompt euthanasia was therefore preformed once signs of illness were observed in the vaccination/challenge study.

Cultures of heart blood (10 µl) from sick mice consistently yielded heavy growth of Sz. No immunized mice became sick and cultures of heart bloods of these mice were negative for Sz following euthanasia at 8 days. A highly significant difference ($p \leq 0.01$) in susceptibility (illness/bacteremia) was observed in immunized mice compared to controls (Fig. 8).

**Opsonophagocytic activity of mouse antiserum to rSzM in horse blood.** Sera from mice immunized with purified rSzM with high levels (>1:106400) of antibody to rSzMNC78 in ELISA were opsonic for both SzNC78 and SzW60 (Table 3). Numbers of bacteria were decreased 9 to 21 fold following opsonization with immune serum, but increased following opsonization with normal mouse serum.
DISCUSSION

Early Ouchterlony studies of Se and Sz demonstrating a hot acid resistant M-like antigen together with evidence that extracts of the closely related Sz did not react with antiserum to SeM of Se suggested this antiphagocytic protein was uniquely expressed by Se (19, 22). However, later genomic studies revealed a homolog of SeM (SzM) in SzH70 (18). Our study for the first time documents expression of SzM by a strain of Sz (NC78) from a clonal outbreak of equine respiratory disease and describes its molecular features and functional characteristics. SzMNC78 resembles SeM in its mainly secondary alpha helical structure, a near identical signal sequence, fibrinogen binding, opsonogenicity and mouse protective properties.

Comparisons of the aminoacid sequences of SzMNC78 with SeM and SzM’s of different strains of Sz and Se revealed 2 Clades composed of strains RT, NH55426, NH38, H70, ATCC35246, UK30 and S. equi CF32 and 4047 (Clade 1) and Clade 2 composed of strains NC78, NH182 and W60. SzM sequences in Clades 1 and 2 shared 43 – 55 and 20 – 24 % homology respectively of their C-terminal halves with SeM suggesting Clade 1 strains may be more closely related to the putative ancestor of the almost clonal Se. Comparison of repeat sequences (Table 2) in SeM with SzM’s of the 9 strains of Sz reveals some interesting similarities and differences. Nine to ten proline-rich repeats in the C-terminus of SzMNC78, W60 and NH182 were absent from SeM and from all Sz strains of Clade 1. SzMNC78 also contained a set of tandem repeats (A: 308 – 332 a.a and 336 – 360 a.a and B: 374 – 404 a.a and 416 – 446 a.a). Clade 1 Sz strains all had B repeats with sequence similar to the B repeats of Se. However, three different A3 repeats (A3a, A3b, and A3c) in Clade 1 strains probably represent degenerate versions of each other. Taken together the repeat sequence data illustrate well the effects of recombination in the Sz genome as previously noted for SzP (8). An interesting feature
of the B repeat of SzMNC78 is its homology to the C2, C3 and C4 repeats of the plasminogen binding proteins of human group C and G streptococci (23). These plasminogen binding proteins (MLC36 and MLG72) were M-like in their resistance to hot acid but share no homology to SzMNC78 other than that of the C repeats. Although the Lancefield groups C and G streptococci usually isolated from clinical specimens from humans represent a distinctly separate genetic set than that from animals, their common ancestry and ability to receive DNA horizontally suggest great potential for sequence rearrangement and acquisitions that explain emergence of more virulent clones.

Sz has been implicated in a wide range of opportunistic infections of the respiratory and reproductive tracts in many vertebrate hosts. There is also evidence of emergence of specific clones associated with outbreaks of severe respiratory disease in shelter dogs, horses, pigs and monkeys (7, 24, 25). In the early 1900’s, pneumonia associated with Sz caused great loss in civil and military horse populations with mortality as high as 15% and prolonged convalescence (26, 27). Hypothetically, the association of disease outbreaks with large numbers of horses and dogs in close confinement suggests a scenario wherein a clone of Sz with enhanced virulence is selected and rapidly propagated and transmitted in the group. Effective control of in house outbreaks in earlier times was often achieved by segregating (picketing out) of affected horses (27) - an indication the epizootiology was that of a transmissible and not an opportunist infection model. Enhanced virulence for example could be explained by emergence and selection of a clone in which recombination and addition of sequences to szm resulted in greater resistance to phagocytosis. The addition of a plasminogen binding sequence would be expected to add to pathogenic potential by enhancing capture of a potential protease for activation by streptokinase (3). Generation of plasmin is known to enhance virulence of \textit{S. pyogenes} in the mouse (28). Also
survival of *S. canis* in phagocytosis analyses has been shown to be enhanced by plasminogen recruitment to the bacterial surface by M-protein and enolase (29).

Mouse antiserum to rSzMNC78 reduced proliferation of both SzNC78 and SzW60 in horse blood (Table 3) suggesting opsonins may be specific for an epitope encoded by secondary structure since the SzMNC78 and W60 amino acid sequences share only 43.52% similarity and have few predicted linear B cell epitopes in common. Consistent with this interpretation was the failure of goat antiserum specific for SzMNC78 to react with SzMW60 on an immunoblot (Fig. 4). The greater decrease (21.1x v 8.8x) in CFU after 90 min observed for the unencapsulated SzW60 opsonized with SzMNC78 specific antibody is explained by the greater resistance to phagocytosis of the encapsulated NC78.

Antibody induced by immunization of mice with rSzMNC78 was also strongly protective against homologous challenge. Cultures of heart blood from all mice showing signs (coat ruffling, depression) of infection showed heavy growths of Sz. Thus the read-out parameter was the approximate incubation period. For mice in the control group this ranged from 2 – 5 days which may reflect the outbred genetic status of ICR (CD-1): HSD strain.

The panel of equine sera tested for reactivity with rSzMNC78 was from cases of rhinitis/bronchiolitis and pneumonia on Kentucky farms from which Sz was cultured in large numbers from clinical samples. Information as to the presence/extent of pneumonia in all cases was not available. It is likely however, that clinically evident respiratory disease requiring sampling for laboratory testing involved the lung in many instances (30, 31). Since it is probable that sequences of SzM varied from Sz to Sz, the moderate to low reactivity of many sera in ELISA may be explained by lack of homology of SzM of the infecting strain to SzMNC78, the screening antigen used in ELISA. It is also likely that some sera were collected at an early stage
of the acquired immune response. It is noteworthy that the very low ELISA OD value obtained
with sera from healthy horses with no history of respiratory disease indicates SzMNC78, unlike
SeM, does not have an IgG-binding domain (32). ELISA OD values ranging between 1.0 to 1.8
for 11 sera (20%) confirm robust SzM specific antibody responses to infection of the respiratory
tract by some Sz strains. Nonetheless more detailed study of convalescent responses using SzM
of an infecting Sz will be required for more complete evaluation of this antigen as a serological
tool and as a correlate of protection engendered by a clonal epizootic.

Future study should also address the level of expression of SzM by different strains of Sz
and whether different SzM’s vary in degree of antiphagocytic/plasminogen binding function and
thus affect virulence of clones associated with severe respiratory disease.

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Figure legends

Fig. 1. Immunoblot showing rSzM and hot acid extracts of S. zooepidemicus NC78. Panel (a) shows Coomassie stained rSzM resolved by SDS-PAGE (12% gel), and panels (b) and (c) show reactivity of rSzM and a hot acid extract with rSzM specific goat hyperimmune serum respectively.

Fig. 2. Phylogenetic analysis of SzM and SeM in isolates of S. zooepidemicus and S. equi. The phylogenies were generated by neighborhood joining with 400 bootstrap replicates, rooted at midpoint.

Fig. 3. Homology between the B repeat of SzMNC78 and C repeats of the plasminogen binding proteins (MLC36 and MLG72) of human group C and G streptococci.

Fig. 4. Expression of SzM by different isolates of S. zooepidemicus. Mutanolysin extracts of 18 h cultures of each isolate were separated by SDS-PAGE and blotted with goat antiserum specific for rSzMNC78.

Fig. 5. SzMNC78 specific antibody levels (ELISA) in equine convalescent sera from cases of respiratory disease associated with S. zooepidemicus. Each value is the mean of 3 replications. The difference between OD values for the 2 groups was significant (p ≤ 0.01; 2-tailed t test).

Fig. 6. Dose dependent binding of equine fibrinogen to rSzMNC78 of S. zooepidemicus NC78.

Fig. 7. Dose dependent plasmin activity following addition of equine plasminogen and streptokinase to rSzMNC78. Plasmin activity was measured using hydrolysis of D-VLK-pNA.

Fig. 8. Cumulative morbidity curves for groups of 10 normal and 10 mice vaccinated with recombinant SzM protein of S. zooepidemicus NC78 and subsequently challenged intraperitoneally with 3 x 10³ CFU (males) and 3 x 10⁴ CFU (females) of S. zooepidemicus NC78.
Fig. 5

Box plot showing OD400nm values for convalescent and healthy horse sera. The convalescent group (n = 52) has a higher range of OD400nm values compared to the healthy group (n = 6).
Fig. 6
Fig. 7

OD$_{405nm}$ vs. rSzM (µg)

- 10 µg
- 5 µg
- 2.5 µg
- 1.25 µg
- 0.625 µg
Table 1. Isolates of *Streptococcus zooepidemicus*.

<table>
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<th>Isolate ID</th>
<th>Year</th>
<th>Place</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC78</td>
<td>1997 - 98</td>
<td>New Caledonia</td>
<td>Mucoid Sz from nasal swab</td>
</tr>
<tr>
<td>NC32</td>
<td>1997 - 98</td>
<td>New Caledonia</td>
<td>Mucoid Sz from nasal swab</td>
</tr>
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<td>NC88</td>
<td>1997 - 98</td>
<td>New Caledonia</td>
<td>Mucoid Sz from nasal swab</td>
</tr>
<tr>
<td>W60</td>
<td>1976</td>
<td>New York</td>
<td>Non mucoid Sz from mandibular lymph node abscess</td>
</tr>
<tr>
<td>RT</td>
<td>2009</td>
<td>Indiana</td>
<td>Non mucoid Sz from nasal discharge</td>
</tr>
<tr>
<td>NH55426</td>
<td>2011</td>
<td>Maryland</td>
<td>Non mucoid Sz from nasal swab</td>
</tr>
<tr>
<td>NH38</td>
<td>2011</td>
<td>Maryland</td>
<td>Non mucoid Sz from nasal swab</td>
</tr>
<tr>
<td>NH182</td>
<td>2011</td>
<td>Maryland</td>
<td>Non mucoid Sz from nasal swab</td>
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<td>631</td>
<td>1979</td>
<td>New York</td>
<td>Non mucoid Sz from case of peritonitis</td>
</tr>
<tr>
<td>UK30</td>
<td>2009 - 10</td>
<td>Kentucky</td>
<td>Non mucoid Sz from mandibular lymph node abscess of foal</td>
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<td>7e</td>
<td>1993</td>
<td>Kentucky</td>
<td>Mucoid Sz from pneumonic donkey</td>
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<tr>
<td>007</td>
<td>2006</td>
<td>Kansas</td>
<td>Non mucoid Sz from canine pneumonia</td>
</tr>
<tr>
<td>E69</td>
<td>2008</td>
<td>Washington</td>
<td>Non mucoid Sz from nasal swab</td>
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Table 2. Tandem repeats in SeM and SzM of S. equi and S. zooepidemicus

<table>
<thead>
<tr>
<th>Repeat</th>
<th>Tandem repeat sequences</th>
<th>Clade 1</th>
<th>Clade 2</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>S. equi</td>
<td>S. zooepidemicus</td>
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<td></td>
<td>CF32 4047</td>
<td>RT</td>
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<tr>
<td>A1</td>
<td>KDLDRINRRLLGNAKLDDKLSKEN</td>
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<td>-</td>
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<tr>
<td></td>
<td>KDLDRINRRLLGNAKGDKLASSK</td>
<td>* : ** : * : **</td>
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</tr>
<tr>
<td>A2</td>
<td>KEKEKARMTKELADKLSDKDRAIQITKELADKLS</td>
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<td>* : * : * : ** : **</td>
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<tr>
<td>A3</td>
<td>a) ASEKDKDKRAIQITTELANKL</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>A-ENSRDKAPAVSTELANKL</td>
<td>* : * : * : * : **</td>
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<tr>
<td></td>
<td>b) AEASRDKAPAVSKDLADKL</td>
<td>+</td>
<td>+</td>
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<td>AEASRDKAPAVSKDLADKL</td>
<td>* : ** : ** : ** : **</td>
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<td></td>
<td>C) ASEKDNRAIQITTELANKL</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>A-ENSRDKAPAVSKDLADKL</td>
<td>* : * : * : * : **</td>
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</tr>
<tr>
<td>B1</td>
<td>QKVAEANRGLKRDLEASRKEA gegenüber VEAELAD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>QKISEANRGLHRDEASRKEA gegenüber VEAELAD</td>
<td>* : ** : ** : ** : ** : ** : ** : **</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>AELQKQXDKALAB</td>
<td>+</td>
<td>+</td>
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<td>AELQKQXDAKVAR</td>
<td>** : ** : ** : ** : ** : ** : ** : **</td>
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</tbody>
</table>
Table 3. Opsonophagocytosis of *S. zooepidemicus* NC78 and W60 pretreated with mouse antiserum specific for rSzMNC78 or with normal mouse serum and incubated for 90 min in horse blood.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Serum</th>
<th>CFU</th>
<th>Change in CFU</th>
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<tbody>
<tr>
<td></td>
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<td>T0</td>
<td>T90</td>
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<tr>
<td>SzW60</td>
<td>Normal</td>
<td>248, 288, 300</td>
<td>492, 560, 624</td>
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<tr>
<td></td>
<td>Anti SzM</td>
<td>272, 292, 328</td>
<td>13, 17, 12</td>
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<tr>
<td>SzNC78</td>
<td>Normal</td>
<td>220, 216, 228</td>
<td>352, 272, 344</td>
</tr>
<tr>
<td></td>
<td>Anti SzM</td>
<td>252, 224, 260</td>
<td>30, 25, 30</td>
</tr>
</tbody>
</table>