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The Antiphagocytic Activity of SeM of *Streptococcus equi* Requires Capsule

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Resistance to phagocytosis is a crucial virulence property of Streptococcus equi (Streptococcus equi subsp. equi; Se), the cause of equine strangles. The contribution and interdependence of capsule and SeM to killing in equine blood and neutrophils were investigated in naturally occurring strains of Se. Strains CF32, SF463 were capsule and SeM positive, strains Lex90, Lex93 were capsule negative and SeM positive and strains Se19, Se1-8 were capsule positive and SeM deficient. Phagocytosis and killing of Se19, Se1-8, Lex90 and Lex93 in equine blood and by neutrophils suspended in serum were significantly ($P \leq 0.02$) greater compared to CF32 and SF463. The results indicate capsule and SeM are both required for resistance to phagocytosis and killing and that the anti-phagocytic property of SeM is greatly reduced in the absence of capsule.

Key words: *capsule, phagocytosis, SeM, Streptococcus equi*

J. Equine Sci.
Vol. 25, No. 2
pp. 53–56, 2014

Streptococcus equi (*Streptococcus equi* subsp. *equi*; Se), a Lancefield group C streptococcus causes strangles, a highly contagious disease of the upper respiratory tract of horses characterized by tonsillitis and metastatic abscessation of draining lymph nodes [9]. Se produces 4 virulence factors that affect uptake or killing of Se by neutrophils. These are SeM, factor H binding Se18.9, IdeE and a constitutively expressed hyaluronic acid capsule [1–3, 15, 16]. A commercial modified live attenuated non-encapsulated vaccine is of low virulence for mice and horses [6]. The capsule confers a characteristic wet mucoid colony morphology, whereas non-encapsulated variants form small dry colonies on solid media.

SeM, a 58 kDa cell wall anchored fibrillar protein, binds fibrinogen and limits deposition of C3b on the bacterial surface by an unknown mechanism [2]. Specific antibodies are opsonizing and mouse protective [4, 5, 11, 12]. Hyaluronic acid (HA), a polymer of glucuronic acid and N-acetylglucosamine repeating units is anti-phagocytic by mechanisms not well understood. It may interfere with phagocytic ingestion by steric interference or by charge repulsion [17].

Phagocytosis of Se has been correlated with the presence of capsule and with SeM [1, 3]. Hyaluronidase treatment of Se abolishes resistance to *in vitro* phagocytosis in a dose dependent manner [1]. However, hyaluronidase treatment does not abolish resistance to phagocytosis in the presence of fibrinogen to the extent it does in its absence [3]. This suggests the hyaluronic acid (HA) capsule is required for full functionality of SeM.

The aim of this study was to investigate the contribution of capsule and SeM to resistance to phagocytosis in three pairs of Se exhibiting the following phenotypes, HA^+ SeM^+; HA^+ SeM^- and HA^- SeM^+.

Se strains CF32, SF463, Lex 90, Lex 93 and Se19 had been isolated from abscesses or nasal swabs of horses as described in Table 1. All failed to ferment lactose, sorbitol and trehalose. Strains cultured overnight at 37°C on Columbia-colistin nalidixic acid (CNA) blood agar showed either a mucoid or non-mucoid colony phenotype (Fig. 1). Se1-8 is a SeM negative mutant of CF32 produced using Tn*916* insertional mutagenesis [14].

Proteins released by boiling acidified (pH – 2.5) bacterial pellets were separated on 12% SDS-PAGE gels and electrophoretically transferred to nitrocellulose membrane. The blots were sequentially incubated in SeM specific antiserum, peroxidase-conjugated protein G (1:4000) and developed in 4-chloro-1-naphthol.

Received: March 7, 2014
Accepted: April 8, 2014
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Table 1. Strains of *Streptococcus equi*

Strain	Source	Description
CF32	Mandibular abscess, NY, 1981	SeM^+, mucoid colony, SeM allele-2
SF463	Nasal swab, KY, 1993	SeM^+, mucoid colony, SeM allele-30
Lex90	Nasal swab, KY, 1990	SeM^+, non-mucoid colony, SeM allele-2
Lex93	Nasal swab, KY, 1993	SeM^+, non-mucoid colony, SeM allele-2
Se1-8	Tn*916* insertional library of CF32	SeM^-, intact SeM sequence, mucoid colony
Se19	Nasal swab, Ireland, 1985	SeM^-, intact SeM sequence, mucoid colony, SeM allele-1

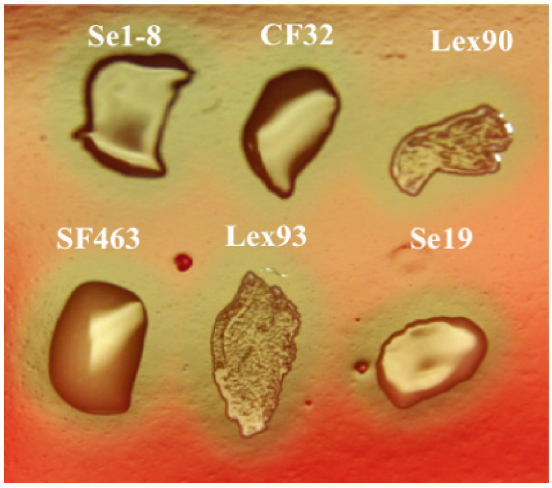

Fig. 1. Mucoid and non-mucoid colonies of *Streptococcus equi*.

The bactericidal assay was performed with heparinized whole blood and with neutrophils from Welsh ponies raised in isolation on the University of Kentucky Farm with no exposure to Se or its antigens. The assay consisted of 1 m*l* aliquots of blood mixed with 200 μl of each Se strain cultured overnight at 37°C in Todd-Hewitt broth with 0.2% yeast extract and diluted in phosphate buffered saline (PBS) to give a final concentration of 5.0×10^3 CFU/m*l*. After mixing, 600 μl were placed on ice ($\mathbf{t}_0$) and the remainder rotated for 60 min at 37°C in polypropylene tubes and then placed on ice ($\mathbf{t}_{60}$). Pour plates in triplicate were made by mixing 200 μl each $\mathbf{t}_0$ and $\mathbf{t}_{60}$ blood-bacteria suspension with 15 m*l* CNA agar and 1 m*l* heparinized equine blood. Plates were incubated overnight at 37°C and hemolytic colonies counted. % survival was calculated for each strain using CFU at $\mathbf{t}_0$ and $\mathbf{t}_{60}$. The assay was repeated 9 times for each strain and the counts pooled for each pair of strains of similar phenotype.

The assay was also done using equine neutrophils isolated from heparinized blood of Welsh ponies and suspended in Hank's balanced salt solution (HBSS) as described by Sedgwick *et al* [8]. 200 μl of neutrophil suspension (1.0×10^6 cells/m*l*) were mixed with 1 m*l* fresh normal pony serum and with 200 μl of overnight culture of each Se strain diluted in PBS to contain 5.0×10^3 CFU/m*l*. The neutrophil-bacteria suspensions were then divided into 2 aliquots of 600 μl. One aliquot ($\mathbf{t}_0$) was placed in ice; the second was rotated in a polypropylene tube for 60 min and then placed on ice ($\mathbf{t}_{60}$). Pour plates in triplicates were prepared as described above for the bactericidal assay in blood, incubated overnight at 37°C and hemolytic colonies counted at $\mathbf{t}_0$ and $\mathbf{t}_{60}$. The assay was repeated 9 times for each strain and the counts pooled for each pair of phenotypically similar strains.

The Wilcoxon signed rank test was used to test for significance of difference in % survival between each pair of strains.

SeM-specific antiserum reacted strongly with 41 and 46 kDa SeM fragments of all strains except Se19 and Se1-8 (Fig. 2). Survival of CF32/SF463 in blood or in neutrophils suspended in serum was very significantly ($P<0.001$) greater than that of Lex90/Lex93 or Se19/1-8 (Table 2). The non-encapsulated SeM^+ Lex90 and 93 showed moderately greater survival in blood ($P=0.00096$) and neutrophils ($P=0.0002$) than the SeM^- encapsulated Se19 and Se1-8. Survival of Se19 and Se1-8 in blood was greater than in neutrophils ($P=0.019$).

Absence of detectable SeM from Se19 and Se1-8 was confirmed by immunoblot analysis with SeM-specific antiserum (Fig. 2). Conversely, CF32, SF463, Lex90 and Lex93 were positive for the 41 and 46 kDa fragments of SeM. Sequence analysis of *sem* in both Se19 and Se1-8 revealed an intact gene in each strain. Lack of expression involved down-regulation at either the transcriptional or translational levels. Resolution of these possibilities is the subject of current investigation.

Absence of capsule rendered both SeM-positive strains susceptible to phagocytosis/killing, an indication that the anti-phagocytic activity of SeM requires capsule. Therefore, the conclusion of previous investigators that capsule is an important virulence factor, is fully valid only when capsule and SeM are present together [1, 3, 10]. Loss of SeM is associated with much reduced pathogenicity for the mouse and horse consistent with its crucial role in virulence of Se [7, 14].

Efficient phagocytosis/killing of the non-encapsulated

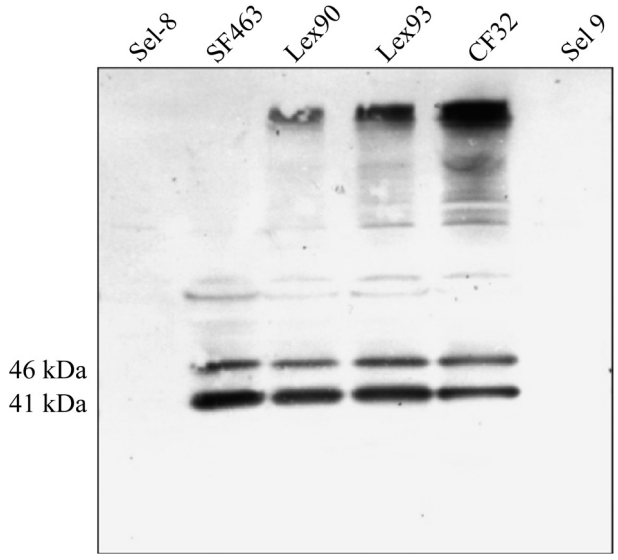

Fig. 2. Reactivities of hot acid extracts of encapsulated (Se1-8, SF463, CF32, Se19) and non-encapsulated (Lex90, Lex93) strains of *Streptococcus equi* with SeM specific rabbit antiserum. The immunoblot was prepared following SDS-PAGE and transfer of separated proteins to nitrocellulose.

Lex90 and 93 expressing normal amounts of SeM is consistent with previous studies [1]. SeM is the major fibrinogen binding protein of Se. Bound fibrinogen greatly enhances resistance of Se to killing by equine neutrophils possibly by binding complement control factor H [2]. However, the ability of SeM to reduce deposition of the opsonic forms of C3 on the surface of encapsulated organisms is not affected by fibrinogen binding [2]. It is tempting to speculate that optimal binding of fibrinogen as well as inhibition of C3 deposition requires the 3-dimensional conformation of SeM available only in the presence of capsule. In the absence of the hydrophilic capsule, Lex90 and 93 aggregate and sediment in THB, an effect of hydrophobic surface proteins including SeM. The greater survival of Se19 and Se1-8 in blood compared to neutrophils in serum may be explained either by reduction of phagocytosis due to binding of fibrinogen to SzPSe [13] or by loss of viability of neutrophils during isolation from blood.

It must be stressed that strains in this study with the exception of Se1-8 were naturally occurring clinical isolates. It is therefore possible that undetected differences other than SeM and capsule in each strain might have affected susceptibility to phagocytosis and killing. However, this is very unlikely given that the Se population is almost clonal and isolation of mutants with these altered phenotypes is extremely rare. Supporting this argument was the similarity of mean survival rates of strains within each pair i.e. CF32 and SF463, Lex90 and Lex93 and Se1-8 and Se19. It is likely these mutants were generated *de novo* in the horses from which they were isolated since their lack of virulence would not favor successful infection of a new host and onward transmission. Finally, Se1-8 was derived by Tn*916* mutagenesis by which a single copy of the transposon was inserted in CF32 with no discernible effect on its proteome other than loss of SeM expression (Artiushin S. and Timoney J.F, Unpublished data).

Acknowledgments

The study was supported by income from the Endowment of the Keeneland Association Endowed Chair in Infectious Diseases.

Table 2. Effect of capsule and SeM protein on survival of *Streptococcus equi* in equine blood and in neutrophils suspended in equine serum

Strain	Phenotype	Median % Survival (Range)	
		Blood (n=18)	Neutrophil Suspension (n=18)
CF32 SF463	HA^+, SeM^+	14.0 (7.0–20.0)	14.0 (6.0–35.0)
Lex90 Lex93	HA^-, SeM^+	6.0 (3.0–9.0) P=0.00096[a]	4.0 (1.0–8.0) P=0.0002[a]
Se 1-8 Se19	HA^+, SeM^-	4.0 (1.0–8.0) P=0.09[b]	2.0 (1.0–7.0) P=0.019[b]

Percentages were calculated from triplicate data from 9 separate experiments for each Se strain. a, P values calculated using Wilcoxon's signed rank test of significance of difference in survival between HA^+ SeM^+ and HA^- SeM^+ strains. P values for the comparison of HA^+ SeM^+ and HA^+ SeM^- were also 0.00096 (blood) and 0.0002 (neutrophils). b, P values calculated as above for differences in % survival between HA^- SeM^+ and HA^+ SeM^- strains. Survival of Se1-8 and Se19 was significantly greater (P=0.019) in blood than in neutrophils.

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