***Streptococcus equi* with truncated M-proteins isolated from outwardly healthy horses**

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The M-protein genes of *Streptococcus equi* isolated from 17 outwardly healthy horses after 4 strangles outbreaks had ended, including a quarantined animal, were compared with those of *S. equi* isolates from 167 active cases of strangles across 4 countries. The healthy horses included 16 persistent *S. equi* carriers, at least one from each of the four outbreaks. These carriers, despite being outwardly healthy, had empyema of the guttural pouch(es), an enlargement of the equine Eustachian tube. A persistent carrier from two of these outbreaks, the quarantined animal and a healthy animal with normal guttural pouches, from which *S. equi* was isolated only once, were colonized by variant *S. equi* with truncated M-protein genes (24% of outwardly healthy animals with *S. equi*). The truncated M-protein genes had in-frame deletions in slightly different positions between the signal sequence and the central repeat region, equivalent to approximately 20% of the mature expressed protein. Immunoblotting with antibody to recombinant M-protein confirmed that the variants expressed a truncated form of the M-protein. In contrast to the outwardly healthy *S. equi* carriers, only 1/167 of *S. equi* isolates from strangles cases possessed a truncated M-protein gene (< 1%; Fisher's exact test, $P = 0.0002$). Compared with isolates from healthy horses with a truncated M-protein, much more of the N terminus of the truncated M-protein was retained in the variant *S. equi* from a strangles case. Variant *S. equi* from outwardly healthy animals were more susceptible to phagocytosis by neutrophils *in vitro* than typical isolates. This is the first report of detection of *S. equi* with a truncated M-protein. The distribution of the variants between strangles cases and carriers suggests that the 80% of the M-protein retained in the variants may contribute to colonization whilst the deleted portion of the gene may be needed for full virulence.

Keywords: truncated M-protein, phagocytosis, persistent colonization, guttural pouch colonization, *Streptococcus equi*

INTRODUCTION

Strangles in horses, caused by *Streptococcus equi*, is characterized by pyrexia, mucopurulent nasal discharge and abscesses of the lymph nodes of the head. As many as 10% of cases may die from disseminated abscessation or purpura haemorrhagica and it is one of the most frequently reported equine diseases worldwide. There

are currently no published records demonstrating effective vaccines or treatments for strangles.

Streptococcus pyogenes, an important human respiratory pathogen, produces a class of surface M-proteins with separate characteristic and conserved regions associated with cell wall anchorage (C-terminal), central tandem repeats and an N-terminal signal sequence (Kehoe *et al.*, 1996). Following the signal sequence, some M-proteins have a hypervariable region that can elicit production of opsonic antibody, believed to be important for protective immunity, and that generally does not cross-react with other M-types. One or more

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The EMBL accession numbers of the M-protein gene partial sequences of *S. equi* isolates A1–A3 and B–E are AJ249868–AJ249874, respectively.

separate M-proteins can be produced by individual strains, each with one or more host-protein-binding capabilities. In the absence of opsonic antibody, the opsonogenic M-proteins can increase resistance of cells to phagocytosis and this activity is dependent on the presence of fibrinogen. *S. equi* produces a surface M-protein (Timoney *et al.*, 1997) that also increases resistance of cells to phagocytosis *in vitro* in serum supplemented with equine fibrinogen (Chanter *et al.*, 1994) and it is highly opsonogenic *in vivo*, but not protective (Timoney & Eggers, 1985).

The DNA sequence of the *S. equi* M-protein gene (Timoney *et al.*, 1997) is more than 99% identical to the sequence of a recently detected *S. equi* gene for a fibrinogen-binding protein (Meehan *et al.*, 1998). Apart from regions involved in cell wall anchorage, cell wall spanning and the signal sequence, there is little sequence or structural similarity between the *S. equi* and *S. pyogenes* M-proteins. The amino acid residue repeat sequences within the central region of the *S. equi* protein are smaller and generally fewer in number than those present within the *S. pyogenes* proteins. Incongruity between the variation seen in the M-proteins of *S. pyogenes* and other markers of genetic variation suggests a good deal of horizontal gene transfer (Whatmore *et al.*, 1994). Further, the M-proteins of *S. pyogenes* can vary in size during infections and outbreaks due to intragenic recombination between identical central tandem repeats (Fischetti *et al.*, 1986), and insertion and point mutations can occur in the hypervariable N terminus (Harbaugh *et al.*, 1993). In contrast, the M-protein gene of *S. equi* does not appear to have transferred to the closely related *Streptococcus zooepidemicus* (Timoney *et al.*, 1997) and changes have hitherto not been detected in the M-protein, which has been regarded as invariant (Galan & Timoney, 1988).

Four strangles outbreaks investigated by endoscopy after cases had convalesced were each shown to include persistent carriers in which *S. equi* most frequently resided in the guttural pouches, often associated with clinically silent inflammation (Newton *et al.*, 1997, 2000). Some of these carriers were colonized by *S. equi* with a truncated M-protein gene. The objective of this study was to characterize the isolates and the nature of the M-protein gene truncation.

METHODS

Samples from carriers of *S. equi*. Nasopharyngeal samples were taken by passing an unguarded, wire-mounted gauze swab to the level of the common pharynx and soft palate via the ventral nasal meatus (Newton *et al.*, 1997). The swab was then removed from the nose and immersed in 4 ml isotonic PBS (1^{-1} : 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 , 0.2 g KH_2PO_4 , pH 7.2), containing 2% (v/v) foetal calf serum and 0.0005% (w/v) amphotericin B (Sigma-Aldrich) for transporting to the laboratory on ice. The fluid phase of nasopharyngeal samples was collected after squeezing the swabs between sterile forceps. The guttural pouches were sampled by endoscopy after sedation with intravenously administered detomidine (12 µg Domosedan kg^{-1} ; Norden Laboratories) and butor-

phenol (24 µg Torbugesic kg^{-1} ; Forte Dodge Animal Health). Nasopharyngeal swabs were always taken before endoscopy to avoid contamination of the nasopharynx and nasal cavities by guttural pouch material on the endoscope. Guttural pouch lavage samples were collected by the installation and aspiration of 50 ml sterile PBS (Oxoid) using a sterile polythene catheter passed through the biopsy channel of the endoscope.

***S. equi* isolates.** One hundred and sixty-seven isolates of *S. equi* from separate cases of strangles were compared with 17 isolates from 17 outwardly healthy carriers. The 167 isolates were composed of 136 from clinical samples submitted to the Animal Health Trust's diagnostic laboratory from 40 veterinary practices in 22 counties of mainland Britain between 1990 and 1998, 14 from Australia, 7 from Ireland and 10 from Canada. The 17 isolates from 17 healthy animals were largely detected by repeated endoscopic examination and sampling of the guttural pouches of cases and contacts ($n = 150$ horses) in 4 outbreaks (Newton *et al.*, 1997, 2000). Fifteen of these isolates were from persistent carriers that had been either cases or contacts during the outbreaks. The sixteenth isolate was cultured from an outwardly healthy horse, also with guttural pouch empyema, shortly after it arrived in quarantine. The seventeenth isolate was from the only *S. equi* sample from a healthy pony with normal guttural pouches that was subsequently *S. equi* negative. Isolates were stored on Cryobeads (ProLab) at -50°C after two or three passages on 5% (v/v) horse blood agar. Studies were conducted after 2–3 further subcultures of the frozen stock on 5% horse blood agar. All 184 isolates failed to ferment trehalose, sorbitol, ribose and lactose but fermented salicin, and all were Lancefield group C (Grant *et al.*, 1993) and 16S–23S RNA gene intergenic spacer type D1, typical of *S. equi* (Chanter *et al.*, 1997). All isolates were tested by PCR1 (see below) for the M-protein gene after two further passages of single colonies on 5% horse blood agar. There were considerable variations in colony morphology after 18 h incubation at 37°C . The colonies of many isolates were white, dry and 1 mm in diameter, whilst others were honey-coloured, glossy and 2 mm in diameter, fitting the typical description for *S. equi*. The colonies of all but one isolate had zones of haemolysis extending from 2 to 6 mm in diameter and this variation had no apparent relationship with other aspects of colony morphology. The different colony morphology of each of the isolates was stable with further passages.

PCR for M-protein gene fragments. Three to five colonies were resuspended in 500 µl isotonic PBS. Resuspended bacteria or samples of the nasopharynx and guttural pouch were centrifuged at 10000 g for 5 min. The supernatant was discarded and the deposit was resuspended in $1 \times$ GeneAmp buffer II (Perkin Elmer; supplied as $10 \times$ concentration), 0.5% (v/v) Tween 20 and 100 µg proteinase K ml^{-1} (Sigma-Aldrich). A 25 µl aliquot of buffer was generally used, but if a large deposit was produced, buffer was added to at least twice the volume of the pellet. The samples were incubated at 55°C for 30 min and then boiled for 5 min. After centrifugation at 10000 g for 5 min, the supernatant fraction was recovered for immediate PCR. Aliquots of the original samples were stored at -20°C .

The position of the primers for PCR1 were selected to amplify most of the M-protein gene (Fig. 1) from DNA isolated from cultures. To reduce the possibility of amplification of a related sequence, the reverse primer was chosen from sequences just upstream of the putative anchor sequence to avoid potential homology with the genes of other cell-wall-anchored proteins. Details of the oligonucleotide sequences of the primers are

Fig. 1. Diagrammatic representation of the M-protein gene of *S. equi* showing the relative locations of the signal sequence (▨), the deleted region of the gene from variant *S. equi* from outwardly healthy carriers (▤▤), the central repeat region (■), cell wall anchor region (●●●●) and the primers for PCR1, PCR2 and sequencing.

Table 1. Oligonucleotide primer sequences used in PCR amplification from *S. equi* isolates and for sequencing based on the *S. equi* M-protein gene sequence

PCR primers		
	Sequence (forward and reverse; 5'–3')	Amplification product size in bp (between nucleotide base numbers of the M-protein gene)
PCR1	ATGTTTTTGAGAAATAACAAGC; TTGACCTGCTTTAGCAGTTG	1547 (1–1547)
PCR2	TAGCCATAAGTGGAGATGCC; CTCTAGATTTCGAAGTTGGG	344 (176–519)
Sequencing primers		
	Sequence (5'–3')	Primer position on the M-protein gene
Primer 1	ATGTTTTTGAGAAATAACAAGC	1–22
Primer 2	TCTACCAAGCGGTCTAGACTAC	931–880

given in Table 1. Sample DNA (5 µl) was mixed with 2.5 µl 10 × GeneAmp PCR buffer (Perkin Elmer), 0.5 µl 10 mM dNTP mix, 1 µl of each primer at 25 pmol µl⁻¹, 0.1 µl (0.5 U) AmpliTaq (Perkin Elmer), 14.9 µl water and 30 µl light mineral oil for PCR (Sigma-Aldrich). The mixture was heated at 95 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 2 min and 72 °C for 3 min, and then a period of 7 min at 72 °C.

The position of the primers for PCR2 (Fig. 1, Table 1) were selected to assess if the DNA deleted from the M-protein gene was present elsewhere within the DNA isolated from the variant *S. equi*. Sample DNA (5 µl) was mixed with 5 µl buffer J (PCR Optimizer kit; Invitrogen), 0.5 µl 10 mM dNTP mix, 1 µl of each primer at 25 pmol µl⁻¹, 0.1 µl (0.5 U) AmpliTaq (Perkin Elmer), 17.4 µl water and 30 µl light mineral oil for PCR (Sigma-Aldrich). The mixture was heated at 95 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 2 min and 72 °C for 2 min, and then a period of 5 min at 72 °C.

DNA sequencing. DNA from the product of PCR1 was sequenced using the Big Dye Terminator Cycle Sequencing kit (Perkin Elmer) after purification with QIAquick columns (Qiagen). The DNA concentrations were estimated from absorbance at 260 nm and diluted in water to 30–90 ng DNA µl⁻¹. Diluted DNA (1 µl) was added to 8 µl Big Dye Terminator mix, 2 µl sequencing primer 1 or 2 (Fig. 1; Table 1) at 1.6 pmol µl⁻¹ and 9 µl water. The mixture was amplified

by 25 cycles of 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min. The DNA was precipitated from the sequencing reaction by mixing with 2 µl 3 M sodium acetate pH 4.6 and 50 µl 95 % (v/v) ethanol for 15 min. After centrifugation at 10000 g for 20 min, the supernatant fraction was discarded, the pellet rinsed in 250 µl 70 % ethanol, recentrifuged and air-dried at 37 °C for 10 min. The samples were then sequenced on an ABI Prism 377 apparatus (Perkin Elmer).

Immunoblotting. Aliquots (1 ml) of cultures of *S. equi* grown overnight at 37 °C in Todd-Hewitt broth were centrifuged at 10000 g for 10 s. The pellets were resuspended in 100 µl Laemmli sample buffer and heated at 100 °C for 5 min before centrifugation at 10000 g for 5 min. The polypeptides in 35 µl of the supernatant fractions were separated by electrophoresis through 12 % (w/v) acrylamide and molecular masses were estimated by comparison with those of pre-stained molecular mass standards (Sigma-Aldrich). After electrophoresis, the separated polypeptides were transferred onto nitrocellulose (Towbin *et al.*, 1979) by electroblotting at 150 V for 4 h. The nitrocellulose sheet was air-dried before incubating for 1 h at 20 °C in 1 % (w/v) dried skimmed milk dissolved in 0.1 % (v/v) Tween 20 in PBS (PBST) before washing in three changes of PBST and shaking for 1 h at 20 °C in mouse antiserum to recombinant M-protein (Meehan *et al.*, 1998), diluted 1/2000 in PBST. The blot was washed in five changes of PBST before incubation with shaking for 1 h at 20 °C in goat antiserum to

		Residue no.
P	MFLRNNKPKFSIRKLSAGAASVLVATSVLGGTTVKAN SEVSRTATPRLSRDLKNRLSDIAISGDASSAQK	70
A1	----- LG GGTTVKANSEVSRTATPRLSRDLKNRLSDIAIS R DASSAQK	
A2	----- LG GGTTVKANSEVSRTATPRLSRDLK I *****	
A3	----- LG GGTTVKANS*****	
B	----- LG GGTTVKANSEVSRTATPRLSRDLKNRLSDIAIS*****	
C	----- LG GGTTVKA*****	
D	----- LG GGTTVKANS*****	
E	----- LG GGTTVKANSEVS Y *****	
P	VRNLLKGASVGDQLQALLRGLDSARAAYGRDDYYNLLMHLSSMLNDKPDGDRRQLSLASLLVDEIEKRIAD	140
A1	VRNLLKGASVGDQLQALLRGLD P ARAAYGRDDYYNLLMHLSSMLNDKPDGDRRQLSLASLLVDEIEKRIAD	
A2-E	*****	
P	GDRYAKLLEAKLAAIKSQQEMLRERDSQLRNLEKEKEQELTKAKDERQALTESFNKT-----	210
A1	GD S YAKLLEAKLAAIKSQQEMLRERDSQLRNLEKEKEQEL Q KAKDERQALTESFNKT-----	
A2	***** S K G KEQEL Q KAKDERQALTESFNKT-----	
A3	***** L Q KAKDERQALTESFNKT-----	
B	*****KDERQALTESFNKT-----	
C	*****KDERQALTESFNKT-----	
D	*****KDERQALTESFNKT-----	
E	*****KDERQALTESFNKT-----	

Fig. 2. Amino acid sequence deduced from the DNA sequence across the region of deleted DNA within the M-protein gene from the variant *S. equi* isolates. Asterisks indicate the deleted sequence; the initial letters in bold typeface represent the amino acids of the signal sequence; letters in bold, italic typeface and underlined represent amino acids which differ from those in the previously published M-protein deduced amino acid sequence (P). Sequences are from the following isolates: A1, from a case of strangles that became a carrier; A2, taken 4 months after A1, after the pony had recovered and become established as a carrier; A3, taken 6 months after A1; B, from a case of strangles in a second outbreak; C, from a carrier in a third outbreak; D, from a healthy pony in contact with the carrier of isolate C; E, from a carrier detected whilst in quarantine as a new introduction to the premises where C was isolated.

mouse immunoglobulin conjugated to horseradish peroxidase (Dako) diluted 1/500 in PBST. After five washes in PBST, the blots were developed using the enhanced ECL Chemiluminescence system (Amersham Pharmacia Biotech) and autoradiography.

Phagocytosis. Previously described methods for testing the susceptibility of *S. equi* to phagocytosis (Chanter *et al.*, 1994) were used, with the exception that a standard serum from an animal known to have had no contact with strangles (stored at -70°C) was used in place of specific-pathogen-free foal serum. Neutrophils were purified from a pony (also known to have had no contact with strangles) on different days for serial replicates of the test. As specified in the results, some cultures were grown with $30\ \mu\text{g}$ hyaluronidase (Sigma-Aldrich) ml^{-1} to remove the hyaluronate capsule. Some of the phagocytosis tests were conducted with $3\ \text{mg}$ equine fibrinogen (Sigma-Aldrich) ml^{-1} added since this has previously been shown to increase resistance to phagocytosis (Chanter *et al.*, 1994). Since fibrinogen also binds to the full M-protein (Meehan *et al.*, 1998), it was possible that deficiencies in resistance to phagocytosis by the truncated M-protein variants of *S. equi* would be more apparent under these conditions. Observations were made of colony morphology of pairs of isolates from the same outbreak, as described above, to see if there were variations in capsulation or haemolytic activity that might explain differences in resistance to phagocytosis. Portions of the bacterial cultures were also spread onto glass slides, dried,

stained by the Gram method and examined microscopically for differences in bacterial cell chain length that might affect resistance to phagocytosis. Forty chains were counted from fresh cultures on three separate days for each strain of *S. equi*.

Statistical methods. Fisher's exact test was used to test the null hypothesis that there was no difference in the proportion of isolates that were variant (producing a truncated M-protein) between *S. equi* recovered from clinical cases ($n=167$) and outwardly healthy animals ($n=17$).

The Wilcoxon test was used to test the null hypothesis that there was no difference between variant and typical *S. equi* isolates in their resistance to phagocytosis by purified neutrophils in serum under different test conditions. Analyses were conducted between sequential isolates (A1, A2 and A3) from a single outwardly healthy carrier, as well as variant and typical isolates from separate horses in the same outbreaks. The effectiveness of the pairing of laboratory tests on variant and typical isolates in the Wilcoxon test was assessed using the Spearman non-parametric correlation coefficient, r_s , and a P value calculated by GraphPad Prism Software; a positive r_s and a low P value indicate effective pairing.

The unpaired t -test [after checking for equal variances (F -test)] was used to test the null hypothesis that there was no difference in mean chain length between variant and typical *S. equi* isolates from different horses, between and within outbreaks. The analyses were conducted using pooled data

(total of 120 chains per isolate) comprising observations made on 40 chains on three separate days.

All statistical tests were conducted using GraphPad Prism Software with a significance level set at 5%.

RESULTS

PCR detection of variant *S. equi* with a truncated M-protein gene

A PCR amplification based on primer positions encompassing most of the M-protein gene (PCR1, Fig. 1) gave a 1.5 kb product as expected of the full-length gene when applied to *S. equi* isolated from empyema of the guttural pouches of 13/17 outwardly healthy horses (76%). A product of 1.2 kb was obtained from the isolates from the remaining four healthy horses (isolates A3, C, D and E). The carriers of isolates A3, C and D had unilateral or bilateral guttural pouch empyema. The carriers of isolates A3 and C were from separate outbreaks. Isolate D was cultured from an outwardly healthy pony shortly after arrival in quarantine at the premises where the carrier of isolate C was kept. Isolate E was cultured from the only sample positive for *S. equi*, from a healthy pony with normal guttural pouches that had contact with the carrier of isolate C. Isolate E possessed features typical of *S. equi* (16S–23S RNA gene intergenic spacer, sugar fermentation pattern and Lancefield Group C antigen), apart from being consistently non-haemolytic on repeated passage.

Two earlier isolates were available from the carrier of isolate A3. PCR of A2, taken 2 months earlier, gave a product larger than obtained from A3 but smaller than expected of the full-length M-protein gene. PCR of A1, taken 6 months before A3 when the pony had typical signs of strangles, gave a product consistent with the presence of the full-length M-protein gene.

The PCR test gave a 1.5 kb product as expected of the full gene when applied to 166/167 *S. equi* isolates from 167 strangles cases in mainland Britain, Australia, Canada and Ireland (99% of active cases). Isolate B, from the remaining strangles case, gave a PCR product of approximately 1.2 kb. Isolation of variant *S. equi* was statistically significantly less likely from strangles cases than from outwardly healthy yet *S. equi* positive horses (Fisher's exact test, $P=0.0002$).

Sequential nasopharyngeal or guttural pouch samples taken over several months were available from only two of the carriers (A3 and C). PCR amplification of these samples gave homogeneous products of sizes corresponding to the genes detected in the isolates. This suggested that only one type of *S. equi*, whether variant or typical, was present in the samples. Variant *S. equi* with an M-protein gene of comparable size to that of A3 were isolated from an empyemic guttural pouch on numerous occasions over a 2.5 month treatment period before infection and inflammation were eventually eliminated. The carrier of isolate C harboured variant *S. equi* for at least 2 years.

Fig. 3. Polypeptides in extracts of whole cells of *S. equi* separated by SDS-PAGE that reacted with mouse antiserum to recombinant fibrinogen-binding protein, synonymous with the M-protein. (a) Typical and variant isolates. Lanes: 1, laboratory reference strain *S. equi* 4047 with the complete M-protein gene; 2, *S. equi* with the complete M-protein gene from a pony with typical strangles; 3, A3, from a carrier in the same outbreak as the isolate in lane 2; 4, B, from a case of strangles in a second outbreak; 5, *S. equi* with the complete M-protein gene from a typical case of strangles from a third outbreak; 6, C, from a carrier in a third outbreak; 7, E, from the carrier in quarantine; 8, D, from a healthy pony in contact with the carrier of isolate C. (b) Sequential isolates from a pony that became a guttural pouch carrier. Lanes: 1, A1, from a nasopharyngeal swab taken before carrier status was identified by endoscopy of the guttural pouches; 2, A2, taken from the guttural pouch 4 months after A1; 3, A3, taken from the guttural pouch 6 months after A1.

DNA sequence analysis of the truncated M-protein genes

DNA sequencing of the PCR products from the variant *S. equi* (Fig. 1) showed that the M-protein genes contained deletions of 363–441 bp between the end of the signal sequence and the start of the central repeat region (Figs 1 and 2). The deletions were in-frame and retained the signal sequence. The deletion sites in the M-

Table 2. Resistance of *S. equi* to phagocytosis by isolated neutrophils in serum

Isolates with a truncated M-protein gene from guttural pouch carriers were compared with paired isolates with the full-length M-protein gene from the same outbreaks of strangles. The comparisons were made in matched pairs to allow for day to day variations in culture conditions and neutrophil preparation by the Wilcoxon rank signed test.

Culture and test conditions*	Outbreak 1 isolates			Outbreak 3 isolates		
	<i>S. equi</i> with full-length M-protein from a case of strangles (Fig. 3a, lane 2)†	A3 (truncated M-protein)†	Survival of A3‡	<i>S. equi</i> with full-length M-protein from a case of strangles (Fig. 3a, lane 5)†	C (truncated M-protein)†	Survival of C‡
+H+F	2.10	1.29	61.4	35.3	21	59.5
+H+F	9.09	2.64	29.0	31.9	13	40.8
+H+F	18.16	1.25	6.9	3.7	0.06	1.6
+H-F	0.25	0.14	56.0	46.9	36	76.8
+H-F	2.50	1.16	46.4	14.8	5.3	35.8
+H-F	6.29	0.56	8.9	0.45	0.1	22.2
-H+F	19.17	2.99	15.6			
-H+F	7.94	3.98	50.1			
-H+F	5.52	0.47	8.5			
-H-F	13.04	0.96	7.4			
-H-F	2.20	0.60	27.3			
-H-F	5.57	0.24	4.3			
	$P=0.0005$ (pairing was effective; Spearman $r_s=0.5315$, $P=0.0377$)			$P=0.0313$ (pairing was effective; Spearman $r_s=0.9429$, $P=0.0083$)		

* Bacteria were grown either with hyaluronidase (+H) or without (-H). The phagocytosis assay was conducted either with fibrinogen (+F) or without (-F).

† Values are percentages of c.f.u. at T_0 remaining at T_2 h.

‡ Values represent survival of isolates with truncated M-proteins as a percentage of survival of *S. equi* with full-length M-protein.

protein genes from separate variants on different premises were slightly different, suggesting that they had emerged independently. The translated DNA sequence suggested that between 0 and 6 amino acid residues of the N terminus of the mature surface-expressed M-protein were retained in the variant *S. equi* from the healthy horses (A3, C, E, D). In contrast, the translated M-protein gene from the only variant that came from a strangles case (B) retained 26 amino acid residues of the N terminus. The region of the M-protein gene of isolate A1 encompassing, and just downstream of, the region of the deletions in isolates A2 and A3, had 8 bp variations from the published sequence (Timoney *et al.*, 1997), translating into four amino acid changes. The sequence for isolate A2 had a further 3 bp changes, two bases downstream of the deletion site. The M-protein gene sequence from A1 varied from the sequence published by Timoney *et al.* (1997) and resulted in three amino acid residue changes (G63R, R143S and T181Q) that were also present in the sequence published by Meehan *et al.* (1998). Examination of the full-length M-protein gene over the putative deletion sites did not reveal any inverted repeat DNA sequences. A search of the DNA databases did not reveal any homology between the DNA sequence encompassing the deletions or flanking regions and any insertion elements or transposons.

A PCR test was devised to amplify the region of DNA deleted from the M-protein gene of the variant *S. equi* (PCR2 in Fig. 1). The test gave the expected product from *S. equi* with the full-length M-protein gene but no product from the variant *S. equi*. This indicated that the deleted DNA was not present outside the M-protein gene elsewhere in the genome of the variant *S. equi*, and, therefore, the full-length gene could not be restored by intragenic DNA recombination.

Expression of a truncated M-protein from variant *S. equi* detected by antibody to recombinant M-protein

Several polypeptides in extracts of the variant *S. equi* separated by SDS-PAGE and transferred to nitrocellulose reacted with antiserum to the M-protein (Fig. 3a). No reaction was detected using pre-immune serum. Extracts of cultures of typical *S. equi* also contained several polypeptides reacting with the antiserum but their molecular masses were larger than for the variant *S. equi*. The slowest migrating and strongest reacting polypeptide in the typical *S. equi* was estimated to be approximately 17 kDa larger than the slowest and strongest reacting polypeptide in the variant *S. equi*. The calculated differences between the molecular masses of the truncated and full-length M-protein gene products

Table 3. Resistance of *S. equi* isolates A1 (with the full-length M-protein gene), A2 and A3 (with progressively larger truncations of the M-protein gene) from the same carrier pony to phagocytosis by purified neutrophils in serum

The comparisons were made in matched pairs to allow for day to day variations in culture conditions and neutrophil preparation by the Wilcoxon rank signed test.

Phagocytosis conditions*	A1†	A2†	Survival of A2‡	A3†	Survival of A3‡
+F	53	18	34.0	27	50.9
+F	10.5	0.79	7.5	0.43	4.1
+F	21.9	0.94	4.3	0.106	0.5
+F	24	1.4	5.8	1.9	7.9
+F	35	2.2	6.3	2.1	6.0
–F	16.5	21	127.3	19.7	119.4
–F	5.5	0.5	9.1	0.38	6.9
–F	1.27	1	78.7	0.135	10.6
–F	2.5	1.3	52.0	1.8	72.0
–F	0.07	0.02	28.6	0.03	42.9
Test	Wilcoxon rank signed test		Spearman test of effective pairing		
	<i>P</i> value		Spearman <i>r_s</i>	<i>P</i> value	
Isolate 1 vs 3	< 0.01		0.78	< 0.01	
Isolate 1 vs 2	< 0.01		0.75	0.01	
Isolate 2 vs 3	0.63		0.89	< 0.01	

* The phagocytosis assay was conducted either with fibrinogen (+F) or without (–F). All bacteria were grown with hyaluronidase.

† Values are percentages of c.f.u. at T_0 remaining at T_2 h.

‡ Values represent survival of isolates A2 or A3 as a percentage of survival of isolate A1.

were from 13.1 to 16.6 kDa. Polypeptides reacting with the antiserum in isolate B, with a larger M-protein gene than the other variants, had slightly higher molecular masses than the reactive polypeptides in the other variant *S. equi* (A3, C, D and E). The three serial isolates A1–A3 contained polypeptides of incrementally smaller sizes reacting with the antiserum, proportional to the sizes of their M-protein genes (Fig. 3b).

Resistance to phagocytosis by equine neutrophils of variant and typical *S. equi*

Typical *S. equi* were more resistant to phagocytosis by purified equine neutrophils than variant *S. equi* isolated from the same outbreaks. This was the case whether or not hyaluronidase was used to remove the capsule layer, or fibrinogen was incorporated to increase resistance to phagocytosis (Tables 2 and 3). Isolate A1, with the full-length M-protein, was significantly more resistant to phagocytosis than either of the variant isolates A2 or A3; no difference could be found between isolates A2 and A3 (Table 3). There were no variations in colony or cellular morphology indicative of capsulation or haemolytic activity that could explain the differences in resistance to phagocytosis. Also, there were no statistically significant differences in mean bacterial cell

chain length or chain length distribution between pairs of variant and typical *S. equi* from the same outbreak.

DISCUSSION

Intensive nasopharyngeal swab and endoscopic sampling of 150 horses from four strangles outbreaks over a 5-year period has provided the first indication of the existence of *S. equi* with a truncated surface M-protein. Hitherto, *S. equi* was regarded as virtually uniform, particularly in relation to M-protein (Galan & Timoney, 1988). Although relatively intensive sampling was required to detect the variants they were found after examining only four outbreaks and they were from at least three discernibly separate sources. Two of the variant *S. equi* carrying animals were from separate outbreaks and a third was detected with guttural pouch empyema soon after arriving into quarantine from a third source. The ability of the variants to persist, particularly in carriers (Fisher's exact test, $P=0.0002$), would provide the opportunity for repeated transmission and infection of contact animals with potentially significant epidemiological and immunological consequences.

The emergence of carriers of variant *S. equi* lacking the N terminus of the M-protein and the relative lack of

such variants from active strangles cases may be an indication of the roles of different domains of M-protein in persistence and disease. One hypothesis regarding the mechanism of the emergence of the variants is that colonization for many months stimulates sufficient immunity to force an escape mutation. M-protein is a potent opsonogenic determinant and a large proportion of antibody responses are directed to the N terminus (Timoney *et al.*, 1999). The occurrence of M-protein size variations in the same strain of *S. pyogenes* in a single patient and individual outbreaks (Fischetti *et al.*, 1986) and the appearance of point and insertion mutations in single M-types (Harbaugh *et al.*, 1993) could be due to the selective pressure of host immunity (Fischetti, 1991). However, an effective assessment of the role of immunity in selecting an escape mutation in *S. equi* would require serial serum samples from animals that become carriers from before infection until after the emergence of variant *S. equi*. This would require large numbers of samples to be taken from many animals because it is not possible at the moment to predict which horses will become carriers and in which of these variants of *S. equi* will emerge. The mechanism of M-protein gene truncation is as unclear as the driving force behind the emergence of the variants. The deletion sites and the flanking DNA did not contain nucleotide sequences indicative of inverted repeats or potential insertion sequence or transposon activity. One feature of the process is the creation of nucleotide base variations in M-protein gene sequence in the regions immediately flanking the deletions, some of which result in changes in amino acid residues. Nonetheless, the identification of three serial isolates from a single carrier with different-sized M-protein genes suggests that the full extent of M-protein gene deletion in at least some variant *S. equi* might occur incrementally. Preliminary application of PFGE to RFLPs, as part of an ongoing, major molecular typing study, has identified eight types and isolates A1, A2 and A3 all fall into one of these (unpublished observations).

M-protein is strongly immunogenic and opsonogenic; it is exposed on the bacterial cell surface and increases resistance to phagocytosis by neutrophils in the presence of fibrinogen *in vitro* (Chanter *et al.*, 1994), and it binds to fibrinogen (Meehan *et al.*, 1998). The region of M-protein responsible for fibrinogen binding by recombinant M-protein is the part missing from the truncated M-protein of variant *S. equi* (Meehan *et al.*, 1999). The greater occurrence of variant *S. equi* among carriers compared with strangles cases suggests that truncation of the M-protein could be linked to a reduction in virulence. On the other hand, a truncated M-protein may contribute significantly to persistence of *S. equi* in empyemic guttural pouches since the variants retained the ability to express approximately 80% of the M-protein, as indicated by the detection of truncated M-protein using antisera to recombinant M-protein, under selective pressure to lose 20% of its N terminus.

The reaction of antiserum to recombinant M-protein with several polypeptides in each isolate may be indicative of post-translational processing. The reactive

polypeptides in each of the isolates with the full-length M-protein had very similar, if not identical, sizes. The sizes of the different reactive polypeptides in the variants were also of similar molecular masses (Fig. 3). Even those of isolate B, with a larger M-protein gene, gave a similar pattern of polypeptides to the other variants although they were consistently of slightly higher molecular mass. This pattern suggests specific post-translational cleavage sites within the M-proteins. Several polypeptides of different molecular masses with identical N-terminal amino acid sequences have been detected in single M-protein extracts, suggesting specific C-terminal truncation sites (Timoney & Mukhtar, 1993) and this may explain the presence of the polypeptides described here. *S. equi* produces at least two easily detectable proteases (Collin *et al.*, 1999) and these could be involved in the cleavage of M-protein.

The variant *S. equi* from carriers had reduced resistance to phagocytosis, perhaps partly caused by the deletion in their M-protein genes, yet paradoxically these isolates persisted in their hosts in the presence of purulent inflammation. Full resistance to phagocytosis, however, is likely to be the sum of the activities of a number of *S. equi* products, including the capsule. Consequently, the reduced resistance to phagocytosis of the variant *S. equi* may only be partial and insufficient to compromise the ability to persist in the guttural pouches.

The extent of the deletion at the N terminus of the mature surface-exposed M-protein might particularly influence virulence. One variant was from a strangles case and this had 20 more amino acids of the N terminus than were found in the M-protein of variants from outwardly healthy carriers. However, the truncation of M-protein may only partly explain the apparent lack of virulence of variant *S. equi* from healthy horses. One variant lacked haemolytic activity and it is possible that the other variants have additional alterations to one or more putative virulence determinants. Nonetheless, the naturally occurring variations in M-protein in *S. equi* from carriers indicate potentially revealing genetic locations for the generation of in-frame allelic replacement mutants for use in experimental infections to characterize the importance of different domains of M-protein for virulence. The naturally emerging variant *S. equi* may be important for elevating herd immunity and could be used for live vaccination provided the M-protein truncation causes attenuation without compromising the ability to colonize and immunize. Better understanding of the potential immunological and epidemiological significance of these variants will come from detailed investigations of more strangles outbreaks by endoscopy and M-protein PCR, and by experimental tests of virulence and immunity from variant challenge.

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