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
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 (140 μM NaCl, 0.2 μM KCl, 1.15 μM NaHPO₄, 0.2 μM KH₂PO₄, 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 butorphanol (24 μg Torbugesic kg⁻¹; Fort 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× GeneAmp buffer II (Perkin Elmer; supplied as 10× 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 prepared, 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

<table>
<thead>
<tr>
<th>PCR primers</th>
<th>Sequence (forward and reverse; 5′–3′)</th>
<th>Amplification product size in bp (between nucleotide base numbers of the M-protein gene)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR1</td>
<td>ATGTTTTTGGAGAAATAACAAAGC; TGGACCTGCTTTAGCAAGTTG</td>
<td>1547 (1–1547)</td>
</tr>
<tr>
<td>PCR2</td>
<td>TAGCCATAGTGAGATGGCC; CTCTAGATTTCGAAGTTGG</td>
<td>344 (176–519)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Sequencing primers</th>
<th>Sequence (5′–3′)</th>
<th>Primer position on the M-protein gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>ATGTTTTTGGAGAAATAACAAAGC</td>
<td>1–22</td>
</tr>
<tr>
<td>Primer 2</td>
<td>TCTACCAAGCGGTCTAGACTAC</td>
<td>931–880</td>
</tr>
</tbody>
</table>

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 1 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 1 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 7 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 10 000 g for 20 min, the supernatant fraction was discarded, the pellet rinsed in 250 µl 70 % ethanol, re-centrifuged 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 10 000 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 10 000 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 % (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

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**Streptococcus equi** with truncated M-proteins
**Residue no.**

<table>
<thead>
<tr>
<th></th>
<th>P</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<tr>
<td><strong>P</strong></td>
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<td><strong>A3</strong></td>
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<td><strong>C</strong></td>
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**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.

**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 µg hyaluronidase (Sigma-Aldrich) ml⁻¹ to remove the hyaluronate capsule. Some of the phagocytosis tests were conducted with 3 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 capsules 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*ₚ, and a *P* value calculated by GraphPad Prism Software; a positive *r*ₚ 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.
E was cultured from the only sample positive for premises where the carrier of isolate C was kept. Isolate healthy pony shortly after arrival in quarantine at the outbreaks. Isolate D was cultured from an outwardly carriers of isolates A3 and C were from separate had unilateral or bilateral guttural pouch empyema. The carriers of isolates A3, C and D had contact with the carrier of isolate C. Isolate E 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, 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 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.

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.

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.

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

<table>
<thead>
<tr>
<th>Culture and test conditions*</th>
<th><strong>S. equi</strong> with full-length M-protein from a case of strangles (Fig. 3a, lane 2)†</th>
<th><strong>A3</strong> (truncated M-protein)‡</th>
<th>Survival of A3‡</th>
<th><strong>S. equi</strong> with full-length M-protein from a case of strangles (Fig. 3a, lane 5)†</th>
<th><strong>C</strong> (truncated M-protein)†</th>
<th>Survival of C‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>+H+F</td>
<td>2.10</td>
<td>1.29</td>
<td>61.4</td>
<td>35.3</td>
<td>21</td>
<td>59.5</td>
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<tr>
<td>+H+F</td>
<td>9.09</td>
<td>2.64</td>
<td>29.0</td>
<td>31.9</td>
<td>13</td>
<td>40.8</td>
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<tr>
<td>+H+F</td>
<td>18.16</td>
<td>1.25</td>
<td>6.9</td>
<td>46.6</td>
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<td>76.3</td>
</tr>
<tr>
<td>+H–F</td>
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<td>56.0</td>
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<td>4.3</td>
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</table>

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

Table 2 shows the 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.

The nucleotide sequence of the *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
Spearman test of effective pairing

variant

than the other variants, had slightly higher molecular

sizes of their M-protein genes (Fig. 3b).

sizes reacting with the antiserum, proportional to the

A1–A3 contained polypeptides of incrementally smaller

length M-protein, was significantly more resistant to

phagocytosis (Tables 2 and 3). Isolate A1, with the full-

length M-protein gene, 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 sta-

tistically 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 sam-

pling 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 re-

quired 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,

mission and infection of contact animals with poten-

tially 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

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 sta-

tistically significant differences in mean bacterial cell


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.

<table>
<thead>
<tr>
<th>Phagocytosis conditions*</th>
<th>A1‡</th>
<th>A2‡</th>
<th>Survival of A2‡</th>
<th>A3‡</th>
<th>Survival of A3‡</th>
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<tr>
<td>+ F</td>
<td>53</td>
<td>18</td>
<td>340</td>
<td>27</td>
<td>509</td>
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<tr>
<td>+ F</td>
<td>10·5</td>
<td>0·79</td>
<td>7·5</td>
<td>0·43</td>
<td>4·1</td>
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<tr>
<td>+ F</td>
<td>21·9</td>
<td>0·94</td>
<td>4·3</td>
<td>0·106</td>
<td>0·5</td>
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<tr>
<td>+ F</td>
<td>24</td>
<td>1·4</td>
<td>3·8</td>
<td>1·9</td>
<td>7·9</td>
</tr>
<tr>
<td>+ F</td>
<td>35</td>
<td>2·2</td>
<td>6·3</td>
<td>2·1</td>
<td>6·0</td>
</tr>
<tr>
<td>− F</td>
<td>16·5</td>
<td>21</td>
<td>127·3</td>
<td>197</td>
<td>1194</td>
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<tr>
<td>− F</td>
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<td>9·1</td>
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<td>− F</td>
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<td>− F</td>
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<td>0·02</td>
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<th>Test</th>
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<td>Isolate 2 vs 3</td>
<td>0·63</td>
<td>0·89</td>
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</table>

* 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 $T_o$ remaining at $T_x$ h.

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

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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 case 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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REFERENCES


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