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Title: Characterization of *Streptococcus equi* subsp. *ruminatorum* isolated from spotted hyenas (*Crocuta crocuta*) and plains zebras (*Equus burchelli*), and identification of a M-like protein (SrM) encoding gene

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1 RT: *Streptococcus equi* subsp. *ruminatorum* from wildlife

2

3 Characterization of *Streptococcus equi* subsp. *ruminatorum* isolated from spotted hyenas
4 (*Crocuta crocuta*) and plains zebras (*Equus burchelli*), and identification of a M-like protein
5 (SrM) encoding gene

6

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20 **ABSTRACT.**

21 Thirteen strains of *Streptococcus equi* subsp. *ruminatorum* from free-ranging spotted hyenas
22 (*Crocuta crocuta*) and plains zebras (*Equus burchelli*) in Tanzania were characterized by
23 biochemical and molecular-biological methods. Although the colony appearance of the *S.e.*
24 *ruminatorum* wildlife strains differed from that of the *S.e. ruminatorum* type strain CECT
25 5772^T, all biochemical reactions of the wildlife strains were similar to those of the type strain.
26 In addition, all wildlife strains produced hyaluronidase and were capable of hydrolysing
27 arginine, three strains (23%) synthesized acetoin, but only eight strains (62%) produced acid
28 from ribose. Rep-PCR indicated that different clones of *S.e. ruminatorum* were distributed
29 among the hyena and zebra populations in the study area. Identical rep-PCR patterns in hyena
30 and zebra strains suggest that a direct transmission of *S.e. ruminatorum* between these species
31 may occur. The presence of a M-like protein (SrM) gene was demonstrated in all *S.e.*
32 *ruminatorum* strains including the type strain. Sequencing of the M-like protein gene revealed
33 a hypervariable region within the deduced amino acid sequence. Most of the strains clustered
34 with previously described strains based on the hypervariable region of the *S.e. zooepidemicus*
35 SzP protein. Sequencing also demonstrated that identical SrM protein sequences were shared
36 among *S.e. ruminatorum* strains from different host species.

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43 **Keywords:** *Streptococcus equi* subsp. *ruminatorum*; wildlife; characterization; 16S rRNA; M-
44 like protein; SrM; rep-PCR

45

46 1. Introduction

47 Between 2002 and 2003, spotted hyenas (*Crocuta crocuta*) in Tanzania, Africa, suffered
48 from a disease outbreak, during which affected animals displayed strangles-like clinical
49 symptoms with severe swelling of the mandibular lymph nodes, formation and drainage of
50 abscesses, and respiratory distress. Two hyenas most likely died of this condition, caused by
51 *Streptococcus equi* subsp. *ruminatorum* (*S.e. ruminatorum*) (Höner et al., 2006).

52 *Streptococcus e. ruminatorum*, a Gram-positive beta-haemolytic bacterium belonging to
53 Lancefield's group C, was first described in Spanish livestock with clinical and subclinical
54 mastitis (Fernández et al., 2004). Its closest phylogenetic relatives are *Streptococcus equi*
55 subsp. *equi* (*S.e. equi*), the causative agent of strangles in solipeds (Timoney, 2004), and *S.*
56 *equi* subsp. *zooepidemicus* (*S.e. zooepidemicus*) which causes disease in a variety of
57 mammals and is a frequently isolated opportunistic pathogen of horses. Compared to
58 *Streptococcus equi*-associated infections in domestic animals, very little has been reported
59 about their occurrence in free-ranging wildlife (Soedarmanto et al., 1996; Akineden et al.,
60 2005; Höner et al., 2006).

61 Pathogenicity factors have been characterized in some detail in *S.e. equi* and *S.e.*
62 *zooepidemicus* (reviewed in Harrington et al., 2002; Timoney, 2004). Cell wall-associated M-
63 like proteins, designated SeM and SzP in *S.e. equi* and *S.e. zooepidemicus*, respectively, have
64 been linked to virulence and are important in the pathogenesis of infection (Chanter et al.,
65 2000; Walker and Runyan, 2003).

66 In the study by Höner et al. (2006), *Streptococcus* strain determination was possible in four
67 out of nine animals on the basis of 16S rRNA sequence analysis and Lancefield typing. In the
68 present study, we characterized *S.e. ruminatorum* isolated from free-ranging spotted hyenas
69 and plains zebras (*Equus burchelli*) by conventional and molecular-biological methods to
70 compare them with the respective features of the *S.equi* type strains, and we investigated the
71 presence and variability of a M-like protein-encoding gene within this bacterial subspecies.

72 2. Materials and methods

73 2.1. Bacterial strains

74 Bacteriological investigations were carried out on different samples available from 18
75 spotted hyenas (five with clinical symptoms and one with enlarged manibular lymph nodes)
76 and two zebras. According to the severe strangles-like clinical symptoms in diseased hyenas
77 and with respect to the common habitat of streptococci, samples were collected from tonsils,
78 mucous membranes of the oropharynx and affected tissues, whenever possible. In addition,
79 swabs were taken both from the tonsil and a minor skin wound of a plains zebra that was
80 immobilized for veterinary investigations as well as from a zebra carcass. A total of 13 group
81 C streptococci (GCS; in most cases grown in pure cultures) were isolated from 4 spotted
82 hyenas and 2 zebras (Table 1). One of these hyenas was diseased, two were seemingly healthy
83 and from the fourth one only remnants of the carcass were found.

84 Because only 16S rRNA information was available on the five GCS strains that had already
85 been described previously (strains 88/03-1, 15/04-1, 15/04-2, 16/04 and 216/04-1; Höner et
86 al., 2006), we characterized all 13 isolated GCS strains in the present study. The bacteria were
87 cultivated on columbia sheep blood agar (CSA) plates (Oxoid, Wesel, Germany) under
88 aerobic conditions at 37°C, 5% CO₂ for 24–48 h. Type strains of *S.e. equi* DSM 20561^T, *S.e.*
89 *zooepidemicus* DSM 20727^T, and *S.e. ruminatorum* CECT 5772^T were used as references (the
90 latter was kindly provided by J. F. Fernández-Garayzábal).

91

92 2.2. Biochemical properties

93 Biochemical features were determined by use of the commercial API[®] 20 Strep system
94 (V.6.0, bioMérieux, Nürtingen, Germany) according to the manufacturer's instructions. The
95 CAMP (Christie-Atkins-Munch-Petersen) reaction was conducted as described by Hahn
96 (1980) with *Staphylococcus (S.) aureus* DSM 12463^T. All biochemical reactions were also
97 performed with the above mentioned *S. equi* type strains.

98 2.3. *Hyaluronidase production*

99 Hyaluronidase production was tested according to Winkle (1955) using mucoid *S.e. equi* as
100 substrate for enzyme activity. The hyaluronidase-producing *S. aureus* strain DSM 12463^T was
101 used as a positive control.

102

103 2.4. *PCR conditions*

104 *16S rRNA gene analysis:* A lawn of bacteria was resuspended in 10 ml Todd Hewitt
105 broth medium (THB;, Oxoid) and incubated 24 h at 37°C. Bacterial DNA was extracted with
106 the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's
107 recommendations for gram-positive bacteria, yielding 200 µl of DNA solution (mean
108 concentration: 67 ng/µl) which was used for subsequent analyses. Amplification with
109 universal 16S rRNA primers TPU1 and RTU8 (Wyss et al., 1996) was carried out on a *T*-
110 Gradient thermocycler (Biometra, Göttingen, Germany) in 50 µl reaction volumes containing
111 10 µl 5x GoTaq Reaction buffer (1.5 mM MgCl₂ f.c.; Promega, Mannheim, Germany), 200
112 µM dNTPs, 0.5 µM of each primer, 1.25 units of GoTaq DNA polymerase (Promega), and 2
113 µl of template DNA. Reaction mixtures were subjected to the following cycling protocol:
114 initial denaturation (94°C: 3 min), 30 cycles (94°C: 60 s; 55°C: 60 s; 72°C: 60 s) and final
115 extension (72°C: 30 min) to equalize fragment length for subsequent sequencing. PCR
116 products were purified using the QIAquick PCR Purification Kit (Qiagen), and directly
117 sequenced applying the fluorescent BigDye Terminator Cycle Sequencing Kit 3.1 (ABI,
118 Darmstadt, Germany) followed by fragment separation and analysis on an ABI model
119 A3130x1 Genetic Analyzer using the software SEQUENCING ANALYSIS v.5.2 (ABI). The newly
120 obtained sequences (740 bp) were aligned with corresponding *S. equi* ssp. sequences retrieved
121 from the GenBank database (<http://www.ncbi.nlm.nih.gov/>) using CLUSTALX v.1.8
122 (Thompson et al., 1997). The phylogenetic tree for 16S rRNA genes was constructed applying
123 the Neighbor-Joining (NJ) algorithm (Saitou and Nei, 1987) implemented in the software

124 package MEGA3 (Kumar et al., 2004). Parameters required for tree construction, such as
125 model of nucleotide substitution and site mutation rate heterogeneity, were estimated using a
126 combination of PAUP* (v.4.b10, Swofford, 2002) and MODELTEST (v.3.7, Posada & Crandall,
127 1998; Posada, 2003).

128 *Repetitive sequence-based PCR (rep-PCR)*: Genomic fingerprinting by rep-PCR was
129 performed using the Enterobacterial Repetitive Intergenic Consensus primer ERIC1R and
130 ERIC2 as described by Al-Ghamdi et al. (1991). For the reactions, 4 µl of the extracted DNA
131 were used as template.

132 *M-like protein encoding gene PCR*: Our approach to search for a M-like protein
133 encoding gene in *S.e. ruminatorum* was based on the existence of such proteins in the other
134 two *S. equi* subspecies. PCR reaction mixtures (50 µl) contained 1.25 µM each of primer
135 *StrepM1* (5'-AAACAAAAGGGGAATAAAATG-3') and *StrepM2* (5'-
136 TTAGTTTTCTTTGCGTCTTGT-3') (Pabst, 1999), 10 µl 5x GoTaq Reaction buffer
137 (Promega), 200 µM dNTPs, 1.25 units of GoTaq DNA polymerase (Promega), and 5 µl of
138 template DNA. Amplification was carried out on a *T*-Gradient thermocycler (Biometra) as
139 follows: 60s at 94°C, 30 cycles (30 s at 94°C; 30 s at 50°C; 30 s at 72°C) and 7 min at 72°C.
140 Bidirectional sequencing was carried out as described above for 16s rRNA. Determination of
141 open reading frames (ORF) in all newly obtained *Streptococcus* M-like protein sequences and
142 subsequent amino acid sequence alignments were performed using the software package
143 BIOEDIT v.7.0.5.3 (Hall, 1999). Phylogenetic trees for M-like protein genes were constructed
144 as described above for 16S rRNA genes except that the nucleotide sequence alignment was
145 manually corrected according to the ORF. Identification of the M-like protein hypervariable
146 (HV) region followed the approach by Walker and Timoney (1998). The number of
147 synonymous differences per synonymous site (K_s) as well as the number of non-synonymous
148 differences per non-synonymous site (K_{ns}) were calculated using DNASP (ver. 4.10, Rozas et
149 al., 2003) with the exclusion of all gap containing positions. After removal of undefined

150 terminal nucleotides, the remaining 807 bp overlapping nucleotide sequence of the M-like
151 proteins included in this study translated into 269 amino acids (aa). The basal nucleotide
152 alignment was then manually corrected according to the aa alignment in order to guarantee
153 the proper nucleotide position assignment for the calculations of synonymous and non-
154 synonymous substitutions. Both K-values were calculated both for all 269 aa and for the 68 aa
155 of the HV region alone. A ratio of $K_{ns}/K_s < 1$ indicates purifying selection, while a ratio of
156 $K_{ns}/K_s > 1$ indicates positive selection.

157

158 *2.5. Whole-cell protein profile*

159 One loop of bacteria was inoculated into 3 ml THB and incubated 24 h at 37°C. Cultures were
160 centrifuged (relative centrifugal force = 580, 10 min), supernatants discarded, the bacteria
161 resuspended in distilled water, and centrifuged again. Pellets were chilled at -80°C for 5 min.
162 Extraction of protein was performed using the Bacterial-PE LB™ Kit (KMF, Lohmar,
163 Gemany) according to the manufacturer's instructions. Bacterial protein was boiled (95°C, 10
164 min) in the presence of 100 µl sample buffer containing 48 mM Tris-HCl (pH 6.8), 2% SDS,
165 5% beta-mercaptoethanol, 25% glycerol, and 0.01% bromophenol blue. Twenty µl of the
166 solubilized proteins were subsequently examined by SDS-12% polyacrylamide gel
167 electrophoresis (PAGE) with a Criterion Cell™ apparatus (BioRad, Munich, Germany) and
168 stained with the SilverSNAP® Stain Kit II (Perbio, Rockford, IL, USA).

169

170

171 **3. Results**

172 *Bacterial culture and identification of strains*

173 Colonies of 11 isolates were small (0.5-1 mm) and glossy, with a white centre and a
174 vitreous margin. Two isolates (15/04-2, 132/06-4) appeared as pinpoint-like colonies (≤ 0.5
175 mm). All isolates were surrounded by a zone of complete beta-haemolysis and grew in

176 nutrient broth, producing sediment and clear supernatant with the exception of isolate 131/06
177 which produced an even turbidity.

178 All isolates belonged to Lancefield's serological group C and were identified as *S.e.*
179 *ruminatorum* based on 16S rRNA gene sequence analysis (Fig. 1). The new sequences were
180 deposited into the GenBank database (acc.nos.: EU078549-556).

181 Using API[®] 20 Strep the biochemical reactions of all wildlife strains were similar to those
182 of the *S.e. ruminatorum* type strain CECT 5772^T (Table 2). However, in contrast to the type
183 strain, all wildlife strains were uniformly non-mucoid and secreted arginine dihydrolase; three
184 strains (23%) from one hyena produced acetoin, but only eight strains (62%), isolated from
185 one hyena and the two zebras, produced acid from ribose. All isolates were positive in the
186 CAMP reaction.

187 The colonies of a mucoid *S.e. equi* strain grown in proximity to all of the *S.e. ruminatorum*
188 wildlife strains appeared small and non-mucoid, suggesting that all of these isolates produced
189 hyaluronidase.

190

191 *Rep-PCR*

192 Comparison of rep-PCR generated fingerprints revealed different fragment patterns among
193 the beta-haemolytic streptococci strains compared (Fig. 2). Four different banding patterns
194 were detected among the wildlife strains. Isolates from the same individual had identical
195 profiles with the exception of the two strains 15/04-1 and 15/04-2 from one hyena (table 1).
196 The patterns of strains 15/04-2 (hyena), 16/04 (hyena), 216/04-1 and -2 (both zebra), and
197 131/06 (hyena) appeared similar. The type strains had patterns that clearly distinguished them
198 from the wildlife strains: *S.e. ruminatorum* CECT 5772^T was highly similar to *S.e.*
199 *zooepidemicus* DSM 20727^T, whereas *S.e. equi* DSM 20561^T revealed a rep-PCR generated
200 fingerprint pattern different from all other strains applied on the gel.

201

202 *M-like protein gene sequences*

203 PCR amplification of M-like protein genes generated a single amplicon of 1149 bp length
204 for both all isolates and the type strains of *S.e. equi* (DSM 20561^T) and *S.e. zooepidemicus*
205 (DSM 20727^T). The respective *S.e. ruminatorum* (CECT 5772^T) PCR-fragment was shorter
206 and had a size of approximately 1050 bp (data not shown). The multi-sequence alignment of
207 the newly obtained *S.e. ruminatorum* sequences and GenBank-retrieved *S. equi* sequences
208 revealed an overlapping stretch of 807 bp (including indels) which was subsequently used to
209 construct the NJ-tree (Fig. 3). The latter showed that M-like protein gene sequence
210 distribution did not follow the 16S rRNA gene derived subspecies classification (Fig. 1). The
211 deduced aa sequence (ranging from aa 13-282) is assumed to have an alpha-helical secondary
212 protein structure (Timoney et al., 1995). The M-like protein gene sequences of three *S.e.*
213 *ruminatorum* isolates (88/03-1 to -3), all retrieved from one hyena (table 1), clustered with the
214 homologous type strain sequence of *S.e. ruminatorum* CECT 5772^T, whereas the other newly
215 sequenced M-like protein genes were either sister sequences of the one from *S.e.*
216 *zooepidemicus* DSM 20727^T (sequences 15/04-1, 15/04-2, 131/06, 216/04-1 and -2) or
217 clustered with the homologous sequences from other *S.e. zooepidemicus* strains (sequences
218 16/04, 132/06-1 to -4). The NJ-tree also revealed a sister relationship of sequences from
219 streptococci isolated from either zebra (216/04-1, -2) or hyena (15/04-1, 15/04-2, 131/06). All
220 newly obtained sequences were deposited into the GenBank database (acc. nos.: EU069396-
221 411).

222 The greatest heterogeneity of the M-like protein across all 13 GCS isolates was found in
223 the region from amino acid residues 106 to 166, a region whose heterogeneity has already
224 been described for the SzP protein (Walker and Timoney, 1998). Four isolates (132/06-1 to -
225 4) from one zebra clustered with hypervariability (HV) type 1, three isolates (88/03-1 to -3)
226 from one hyena comprised HV type 3 and one isolate (16/04) from another hyena was
227 classified as HV type 4 (Fig. 4). The HV regions of the remaining five isolates (15/04-1 and -

228 2, 131/06, 216/04-1 and -2) from two additional hyenas and another zebra were most similar
229 to the HV2 group but showed a unique amino acid motif, DAKAAWDKVLFD, which was
230 neither present in HV type 2 nor in HV3. Thus, these isolates were assigned to a new HV
231 type, the intermediate type HV2v. The introduction of HV2v followed the designation of
232 HV4v by Walker and Runyan (2003).

233 The HV region of *S.e. zooepidemicus* DSM 20727^T, classified as HV2, was identical to
234 that of the *S.e. zooepidemicus* prototype strain SzPW60 (acc. no. U04620), while *S.e.*
235 *ruminatorum* CECT 5772^T clustered with *S.e. ruminatorum* 88/03-isolates and the *S.e.*
236 *zooepidemicus* sequences retrieved from GenBank (Fig. 4). *Streptococcus e. equi* DSM
237 20561^T, *S.e. zooepidemicus* (GenBank acc. nos. AF519473 and AF519476) as well as
238 another *S.e. equi* sequence (acc. no. U73163) were placed in the HV type 4 group. The
239 determination of synonymous versus non-synonymous substitutions across the 807 bp
240 fragment of 32 unique M-like proteins (retrieved from Fig. 3) resulted in a K_{ns}/K_s -ratio of
241 0.454. When calculated for the hypervariable region alone, that ratio rose to 3.016 (table 3).

242

243 *Whole-cell protein profiles*

244 The protein fingerprints of all 13 wildlife *S.e. ruminatorum* strains investigated revealed
245 almost identical banding patterns. Type strain *S.e. ruminatorum* CECT 5772^T displayed a
246 protein profile similar to that of the wildlife *S.e. ruminatorum* strains, whereas the protein
247 patterns of type strains *S.e. equi* DSM 20561^T and *S.e. zooepidemicus* DSM 20727^T differed
248 distinctly from those of all other strains analysed (figure not shown).

249

250

251 **4. Discussion**

252 All 13 beta-haemolytic streptococci investigated in the present study were isolated
253 from Tanzanian spotted hyenas and plains zebras. Based on serological features and 16S

254 rRNA gene sequence analysis these strains belonged to Lancefield's serological group C and
255 were identified as *S.e. ruminatorum*. In general, the wildlife strains showed all standard
256 biochemical reactions of the *S.e. ruminatorum* type strain. There were, however, also some
257 differences. In contrast to the *S.e. ruminatorum* CECT 5772^T type strain, colonies of the
258 wildlife strains were uniformly non-mucoid and grew with different growth characteristics on
259 solid and in liquid medium. All wildlife strains were capable of hydrolysing arginine, three
260 isolates (23%) synthesized acetoin, but only eight strains (62%) produced acid from ribose.
261 Moreover, in contrast to *S.e. ruminatorum* CECT 5772^T all wildlife strains produced
262 hyaluronidase as demonstrated by the decapsulation test using a mucoid *S.e. equi*.
263 *Streptococcus e. ruminatorum* strains with colony appearance and biochemical reaction
264 outcomes deviating from the CECT 5572^T type strain were also recently described by
265 Baverud et al. (2007).

266 Encapsulation protects streptococci against phagocytosis and *in vivo* pathogenicity is
267 probably related to the amount of capsule present (Anzai et al., 1999). Furthermore, the
268 hyaluronic acid capsule is required for the functionality of surface exposed proteins
269 (Timoney, 2004). Strains of *S.e. equi* isolated from cases of strangles are usually encapsulated
270 (Timoney, 2004). Non-encapsulated mutant strains were described as less virulent for horses
271 and mice (Timoney and Galán, 1985; Anzai et al., 1999). Although capsule production in *S.e.*
272 *zooepidemicus* is highly variable and quickly lost during culture, isolates recovered from the
273 tonsils of healthy animals are almost always non-encapsulated (Anzai et al., 2000; Timoney,
274 2004). During residence of *S.e. zooepidemicus* on mucosal sites of the host hyaluronic acid
275 production has been suggested to be spontaneously modulated (Anzai et al., 2000). The
276 Spanish *S.e. ruminatorum* isolates were uniformly mucoid irrespective of being cultured from
277 animals with clinical or subclinical mastitis (Fernández et al., 2004). In the present study,
278 capsule expression appeared unrelated to specific disease symptoms as all *S.e. ruminatorum*
279 strains were uniformly non-mucoid.

280 Although originally used to determine the genetic variation of *S.e. equi* (Al-Ghamdi et
281 al., 2000) rep-PCR results of the present study indicate that this method can also be used to
282 differentiate *S.e. ruminatorum* isolates, even though only a limited number of wildlife strains
283 was investigated. Rep-PCR generated fingerprints revealed 4 different patterns among the 13
284 *S.e. ruminatorum* isolates, indicating that different bacterial clones were distributed within the
285 hyena and zebra populations in the study area. Most interestingly, rep-PCR patterns were
286 similar among three hyena strains (15/04-2, 16/04, 131/06) and two zebra strains (216/04-1,
287 216/04-2). This lead to the assumption that direct transmission of streptococci may have
288 occurred between the two mammal species. One hyena was found to carry two strains (15/04-
289 1, 15/04-2) having different morphological features as well as differences in rep-PCR
290 characteristics, in 16S rRNA and in SrM gene sequences, but being identical in both their
291 biochemical features tested and their protein profiles. The occurrence of these two different
292 *S.e. ruminatorum* strains within one host might be due to super-infection or the result of
293 mutagenesis of a residential clone. In contrast to the rep-PCR results, the analyses of the
294 whole cell protein profiles yielded similar protein banding patterns in all *S.e. ruminatorum*
295 isolates. Thus, the complementary application of both methods for strain characterization is
296 recommended.

297 All 13 wildlife isolates and the *S. ruminatorum* type strain revealed a M-like protein
298 encoding gene which we designate SrM protein. Its nucleotide and amino acid sequences
299 varied among isolates, similar to the variability already described for SzP proteins in *S.e.*
300 *zooepidemicus* (e.g. Timoney et al., 1991; Walker and Timoney, 1998; Walker and Runyan,
301 2003) and to the deduced amino acid variability reported for the *S.e. equi* SeM protein (Anzai
302 et al., 2005). The NJ-tree revealed a sister relationship of sequences from streptococci isolated
303 from either zebra (216/04-1, -2) or hyena (15/04-1, 15/04-2, 131/06) indicating either a M-
304 like protein gene sequence distribution independent of host species or a somewhat more
305 recent streptococci transmission between these host species. The lack of an apparent

306 correlation between *S.e. ruminatorum* SrM protein sequences and host mammal species had
307 also been demonstrated for the M-like SzP-protein of *S.e. zooepidemicus* (Nicholson et al.,
308 2000) which was found to be shared among strains isolated from different mammalian hosts
309 such as human, horse and guinea pig. Another interesting find was the apparent clonal
310 relationship between an isolate from a hyena (15/04-2) and two isolates from one zebra
311 (216/04-1 and -2), again indicating potential horizontal transmission between these species.
312 As expected, the greatest variability within the SrM protein was found in the HV region,
313 which in all of the isolates clustered with previously described SzP-protein HV types
314 (Timoney et al., 1995; Walker and Runyan, 2003). This high variability of the hypervariable
315 region is clearly positively selected for, as demonstrated by the calculation of synonymous
316 versus non-synonymous substitutions ($K_{ns}/K_s = 3.016$), while the additional inclusion of 201
317 aa flanking the HV region lead to a ratio of $K_{ns}/K_s = 0.454$, indicating the purifying selection
318 acting on the amino acids surrounding the HV region. All of the isolates and the three type
319 strains of *S.e. equi*, *S.e. zooepidemicus* and *S.e. ruminatorum* were assigned to either HV type
320 1, type 2, 3 or 4 but not HV4v or HV5. The HV types of three isolates from two hyenas
321 (131/06, 15/04-1, 15/04-2) and two isolates of one zebra (216/04-1, 216/04-2) fell between
322 HV type 2 and type 3. Because they did not match either type exactly but appeared to be close
323 to the HV2 cluster, the HV regions of these isolates were newly designated HV2v. Two
324 isolates (15/04-1, 15/04-2), that were clearly distinguishable by rep-PCR analysis, had highly
325 similar SrM protein gene sequences with an identical HV region. This corroborates findings
326 observed in *S.e. zooepidemicus* which revealed that similarity of M protein serotypes does not
327 necessarily indicate close relatedness (Walker and Runyan, 2003). Because there was only
328 one diseased animal that died in the study presented here (hyena M149), any assumption of an
329 association of M-like protein types with particular disease conditions would be purely
330 speculative.

331 Comprehensive long-term studies on wildlife health status in general or monitoring
332 programs for the detection of disease reservoirs are scarce as they require markedly large
333 manpower to collect data and samples, and trained personnel. The spotted hyena population in
334 the Ngorongoro Crater has been monitored for more than 10 years, but has only suffered from
335 a single bacterial disease outbreak so far (Höner et al., 2006). This is unexpected because
336 predators and scavengers such as spotted hyenas are likely to acquire infectious diseases when
337 feeding on large carcasses of diseased animals. The outbreak was attributed to *S.e.*
338 *ruminatorum*, which so far had only been associated with mastitis in small ruminants
339 (Fernández et al., 2004) but was also found in affected and seemingly healthy hyenas. This
340 emphasizes the need to study the epidemiology of this infection and to acquire data regarding
341 possibly underlying diseases in spotted hyenas. In search for a possible reservoir in local
342 livestock, bacterial investigations of udder tissue and swabs from different mucosal sites were
343 performed in local sheep and goats. *Streptococcus e. ruminatorum* was not detected in any of
344 the samples from sheep (n=7) and goats (n=15) investigated so far (data not shown).

345 In summary, our data revealed that *S.e. ruminatorum* isolated from free-ranging
346 spotted hyenas and plains zebras showed all standard biochemical reactions of the CECT
347 5772^T type strain. However, in contrast to the latter, all wildlife isolates produced
348 hyaluronidase and were uniformly non-mucoid.

349 To our knowledge, this is the first demonstration of the presence of a M-like protein
350 gene in *S.e. ruminatorum* (SrM). It was found in all *S.e. ruminatorum* strains investigated.
351 The importance of M-like proteins as virulence factors in *S.e. equi* and *S.e. zooepidemicus*
352 (reviewed by Harrington et al., 2002) as well as in *S.e. ruminatorum* is reflected by the strong
353 positive selection the HV region is under, thereby increasing the fitness of the streptococci in
354 their interaction with the host organisms. In-depth studies on *S.e. ruminatorum* are still
355 lacking, e.g. a more extensive investigation of wildlife *S.e. ruminatorum* isolates is needed in

356 order to understand their epidemiologic features and to clarify their role in the disease
357 outbreak in spotted hyenas in Tanzania.

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359

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367

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- 483

484 **Table 1.** *Streptococcus equi* subsp. *ruminatorum* isolated from spotted hyenas and plains zebras

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Isolate Nr.	Source	Sample type
88/03-1, 88/03-2, 88/03-3	hyena M149, m, ad, clinical signs, died	soft tissue, lung, mandibular lymph node
15/04-1, 15/04-2	hyena E118, m, y, no clinical signs, killed	tonsil
16/04	hyena A084, m, ad, no clinical signs, killed	tonsil
216/04-1, 216/04-2	zebra EQ003, m, ad, healthy, immobilized	pus, skin wound
131/06	hyena X051, f, ad, died	tonsil
132/06-1, 132/06-2, 132/06-3, 132/06-4	zebra EQ004, f, ad, died	tonsil, tonsil swab, throat swab

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487 m – male, f – female, ad – adult, y – yearling

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492 **Table 2.** Biochemical characteristics of *Streptococcus equi* subsp. *ruminatorum* strains and their closest phylogenetic relatives

Isolate no.	88/03 -1	88/03 -2	88/03 -3	15/04 -1	15/04 -2	16/04	216/04 -1	216/04 -2	131/06	132/06 -1	132/06 -2	132/06 -3	132/06 -4	<i>S.e.</i> <i>rumina-</i> <i>torum</i> type strain CECT 5772	<i>S.e.</i> <i>equi</i> type strain DSM 20561	<i>S.e. zoo-</i> <i>epidemicus</i> type strain DSM 20727
Source	hyena M149	hyena M149	hyena M149	hyena E118	hyena E118	hyena A084	zebra EQ003	zebra EQ003	hyena X051	zebra EQ004	zebra EQ004	zebra EQ004	zebra EQ004			
Test																
Acetoin production	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Hippurate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyrrrolinodyl arylamidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
alpha-Galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
beta-Glucuronidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
beta-Galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
alkaline Phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Leucinaminopeptidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	-	-	-	+	+	-	+	+	-	+	+	+	+	+	-	+
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycogen	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
beta-Hemolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CAMP test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hyaluronidase	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Capsule expression	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+

493 ¹ *S.e. ruminatorum* revealed a positive reaction in the study of Fernández et al. (2004); ² In Fernández et al. (2004) the outcome differed depending
494 on the *S.e. ruminatorum* isolate investigated. Deviations from the *S.e. ruminatorum* type strain reactions are shaded in grey.

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509 **Table 3.** Nucleotide substitutions (synonymous/ non-synonymous) of 32 unique SrM proteins
 510 of *Streptococcus equi* ssp. strains

mean	SynDif*	SynPos*	K _s *	NSynDif*	NSynPos*	K _{ns} *	K _{ns} / K _s
across 269 AS**	34.97	173.57	0.242	56.34	558.43	0.110	0.454
HV (69 AS***)	57.76	600.31	0.104	33.99	141.69	0.315	3.016

511

512 SynDif: number of synonymous differences; SynPos: number of synonymous positions within
 513 sequences; K_s: number of synonymous differences per synonymous positions; NSynDif:
 514 number of non-synonymous differences; NSynPos: number of non-synonymous positions
 515 within sequences; K_{ns}: number of non-synonymous differences per non-synonymous
 516 positions; *: values rounded; **: Due to nucleotide alignment gaps at 25 amino acid positions
 517 only 244 codons were included in the calculations; *** Due to nucleotide alignment gaps at
 518 10 amino acid positions only 59 codons were included in the calculations; Mean: mean value
 519 calculated from 496 pairwise sequence comparisons.

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531 **Figure 1.** Neighbour-joining tree based on 16S rRNA gene sequences showing the
 532 phylogenetic relationships of *S. equi* subsp. *ruminatorum* isolates. Sequences of
 533 *Streptococcus equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi* were used as outgroups.
 534 Statistical support for nodes was obtained by bootstrapping (1000 replicates), only values
 535 $\geq 50\%$ are shown. Scale indicates number of substitutions per site. New sequences are given in
 536 bold face.

537 Se_e = *S.e. equi*; Se_r = *S.e. ruminatorum*; Se_z = *S.e. zooepidemicus*

538

539 **Figure 2.** Rep-PCR generated DNA fingerprinting of Lancefield's group C streptococci.

540 MW 100-bp DNA marker; **lane 1** isolate 88/03-1; **lane 2** isolate 88/03-2; **lane 3** isolate
 541 88/03-3; **lane 4** isolate 15/04-1; **lane 5** isolate 15/04-2; **lane 6** isolate 16/04; **lane 7** isolate
 542 216/04-1; **lane 8** isolate 216/04-2; **lane 9** isolate 131/06; **lane 10** isolate 132/06-1; **lane 11**
 543 isolate 132/06-2; **lane 12** isolate 132/06-3; **lane 13** isolate 132/06-4; **lane 14** *S.e.*
 544 *ruminatorum* CECT 5772^T, lane 15; **lane 15** *S.e. equi* DSM 20561^T, **lane 16** *S.e.*
 545 *zooepidemicus* DSM 20727^T; numbers at the bottom refer to patterns, lower case characters
 546 indicate variation within pattern 3.

547

548 **Figure 3.** Neighbor-joining tree based on M-like protein (SrM) gene sequences showing the
 549 phylogenetic relationships among *S. equi* subsp. *ruminatorum* isolates and the two other
 550 subspecies *S. equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi*. The tree was obtained by
 551 applying pairwise gap deletion and a substitution rate heterogeneity among sites of $\alpha = 1.529$.
 552 Statistical support for nodes was obtained by bootstrapping (1000 replicates), only values
 553 $\geq 50\%$ are shown. Scale indicates number of substitutions per site.

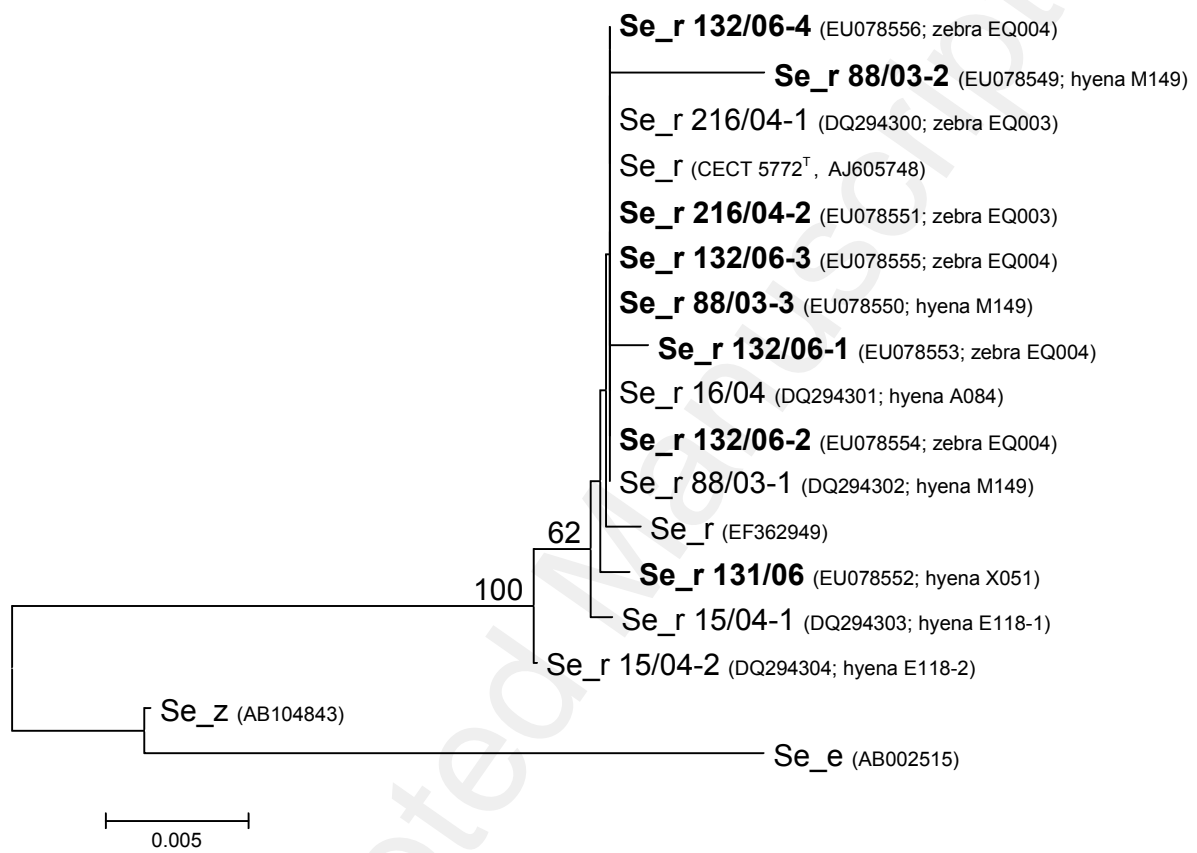
554 Se_e = *S.e. equi*; Se_r = *S.e. ruminatorum*; Se_z = *S.e. zooepidemicus*. Host species of
 555 isolates are given on the right. New sequences are given in bold face.

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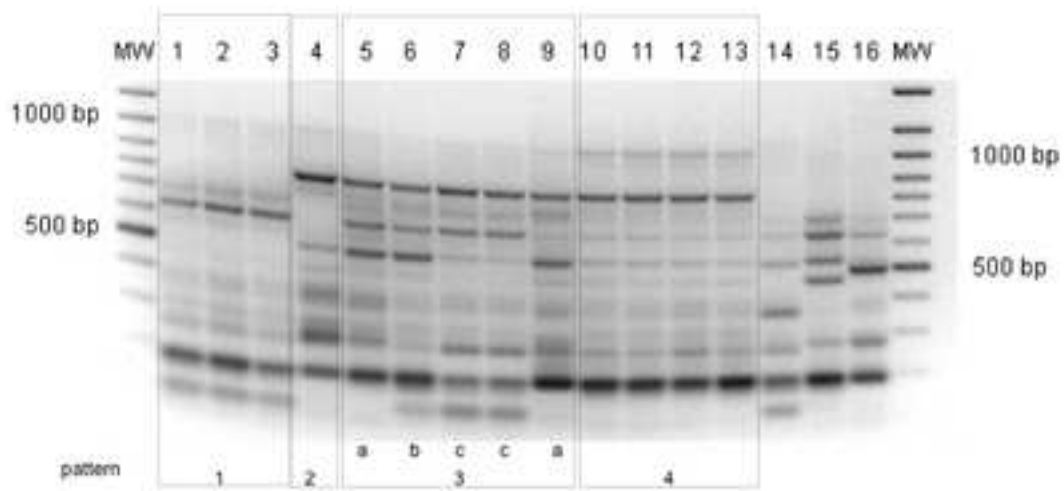
557 **Figure 4.** Amino acid sequence alignment of the hypervariable region (HV) of M-like
558 proteins of *Streptococcus equi* subspecies.

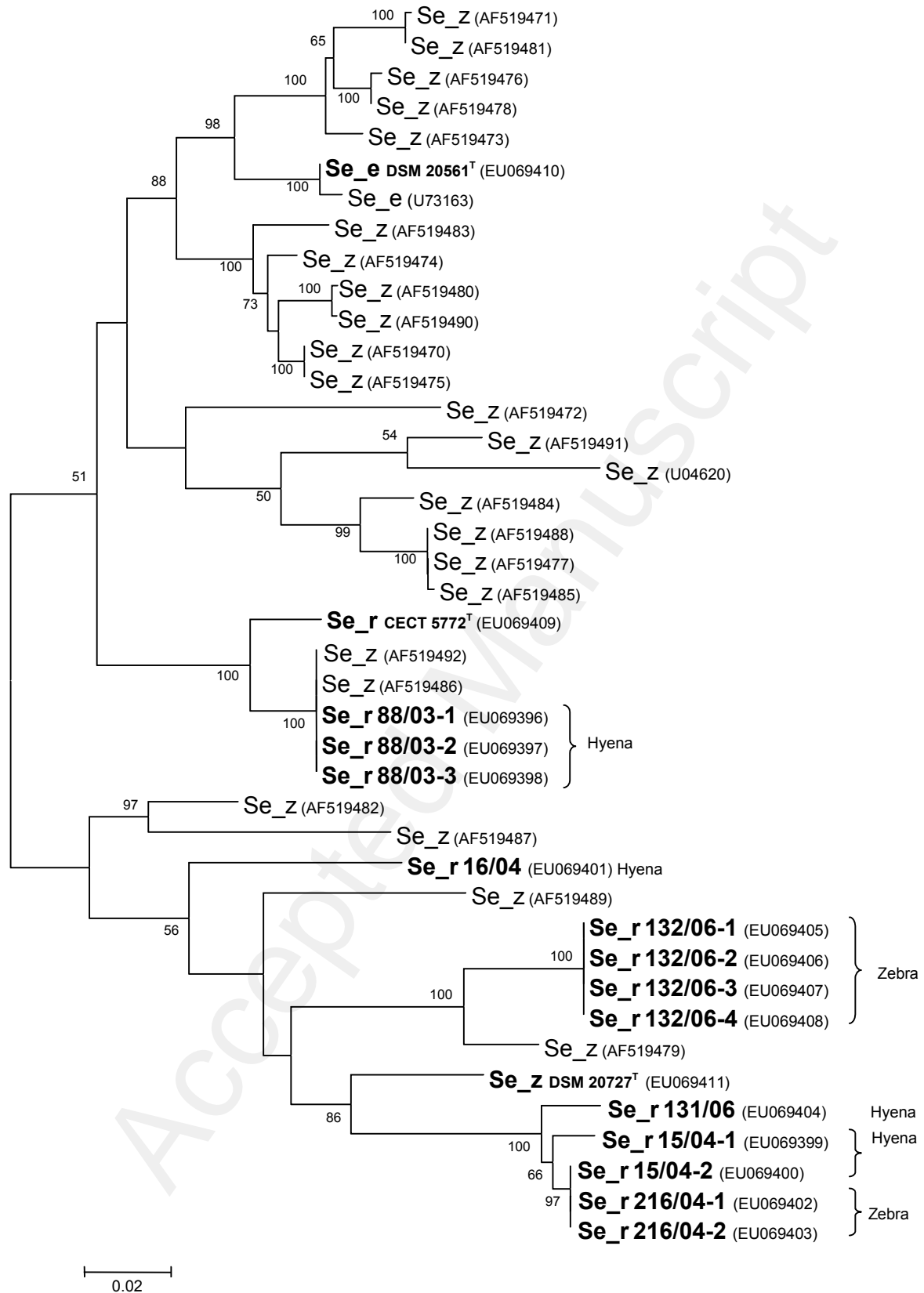
559 Sequences retrieved from GenBank are given with their accession numbers. Different
560 GenBank entries but with identical sequences are only represented once. Conserved sequence
561 motifs flanking the variable region are depicted in dotted boxes. Shaded areas identify residue
562 differences within HV types. Newly obtained SrM sequences are given in bold face. The
563 cluster HV type 2v was newly assigned. *Amino acid positions are given in reference to the
564 emmSzW60 protein (acc.no. U04620).

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HV type	strain	amino acid position
		102
		169
HV type 1	Se_z (AF519479)	LEQKMAELESKIQEKQKELKEVADKQLSAYKAINGRNGDAGLDQLDEQADEIKQAKVGLAEELQKTKKE
	Se_r 132/06-1	LEQKMNDLESKIQEKQKELKEVADKQLSAYKAINGRNGDAGLDQLDEQADEIKQVKVGLAEELQKVKKE
	Se_r 132/06-2	LEQKMNDLESKIQEKQKELKEVADKQLSAYKAINGRNGDAGLDQLDEQADEIKQVKVGLAEELQKVKKE
	Se_r 132/06-3	LEQKMNDLESKIQEKQKELKEVADKQLSAYKAINGRNGDAGLDQLDEQADEIKQVKVGLAEELQKVKKE
	Se_r 132/06-4	LEQKMNDLESKIQEKQKELKEVADKQLSAYKAINGRNGDAGLDQLDEQADEIKQVKVGLAEELQKVKKE
HV type 2	Se_z DSM 20727	LEQKMNDLESKIQEKQKAFEQVVLEQKKEQANPVDRNGDAEEDRLDELSGEIRREKAALEVELOKTKKE
	Se_z (U04620)*	LEQKMNDLESKIQEKQKAFEQVVLEQKKEQANPVDRNGDAEEDRLDELSGEIRREKAALEVELOKTKKE
	Se_z (AF519484)	LEQKIPELEKKIQEKEKEFEQVVLEQKMEQANPVNRGGDAEEDRLDELSGEIRRAKAAIKAELOKTKKE
	Se_z (AF519488)	LEQKMAELEKKIQEKEKEFEQVVLEQKMEQANPVNRGGDAEEDRLDELSGEIRRAKAAIKAELOKTKKE
	Se_z (AF519485)	LEQKMAELEKKIQEKEKEFEQVVLEQKMEQANPVNRGGDAEEDRLDELSGEIRRAKAAIKAELOKTKKE
HV type 2v	Se_r 131/06	LEQKISELEKKIQDAKAAWDKVLFDQKMEQAN-VFRRGDAEEDRLDELSGEIRREKAALEVELOKVKKE
	Se_r 15/04-1	LEQKISELEKKIQDAKAAWDKVLFDQKMEQAN-VFRRGDAEEDRLDELSGEIRREKAALEVELOKVKKE
	Se_r 15/04-2	LEQKISELEKKIQDAKAAWDKVLFDQKMEQAN-VFRRGDAEEDRLDELSGEIRREKAALEVELOKVKKE
	Se_r 216/04-1	LEQKISELEKKIQDAKAAWDKVLFDQKMEQAN-VFRRGDAEEDRLDELSGEIRREKAALEVELOKVKKE
	Se_r 216/04-2	LEQKISELEKKIQDAKAAWDKVLFDQKMEQAN-VFRRGDAEEDRLDELSGEIRREKAALEVELOKVKKE
HV type 3	Se_z (AF519492)	LEQKMAELESKIQETQ-----KKLDYENMPSSGGLHSDEDDTVARQLMSEKEAIKAELOKTKKE
	Se_r 88/03-1	LEQKMAELESKIQETQ-----KKLDYENMPSSGGLHSDEDDTVARQLMSEKEAIKAELOKTKKE
	Se_r 88/03-2	LEQKMAELESKIQETQ-----KKLDYENMPSSGGLHSDEDDTVARQLMSEKEAIKAELOKTKKE
	Se_r 88/03-3	LEQKMAELESKIQETQ-----KKLDYENMPSSGGLHSDEDDTVARQLMSEKEAIKAELOKTKKE
	Se_r CECT 5772	LEQKMAELESKIQETQ-----KKLDYENMPSSGGLHSDDKNDTVARQLMSEKEAIKAELOKTKKE
HV type 4	Se_z (AF519473)	LEQKIPELEKKIQEVQ-----EKLNYENRLSPERVGSDEDDTTEARRLIAEKEAIKAELOKTKKE
	Se_e (U73163)	LEQKIPELEKKIQEAQ-----EKLNYENRPSPKRVGSDEDDTVARQLMSEKEAIKAELOKTKKE
	Se_e DSM 20561	LEQKIPELEKKIQEAQ-----EKLNYENRPSPKRVGSDEDDTVARQLMSEKEAIKAELOKTKKE
	Se_r 16/04	LEQKIPELEKKIQETQ-----EKLKREDIPSPERVRSDEDDTVARQLISEKEAIKAELOKTKKE
HV type 4v	Se_z (AF519470)	LEQKIPELEKKIQETQ-----EKLNRENTPSPGRIVSDEDDTVARQLMSEKEAIKAELOKTKKE
	Se_z (AF519474)	LEQKIPELEKKIQETQ-----EKLNRENTPSPGRIISDEDDTVARQLMSEKEAIKAELOKTKKE
	Se_z (AF519482)	LEQKIPELEKKIQETQ-----EKLNRENTPSPGRIVSDEDDTTEARRLIAEKAALAELOKTKKE
	Se_z (AF519487)	LETKISDLEKKIQEAQ-----EKLNRENTLSPGRIVSDEDDTTEARRLIAEKEAIKAELOKTKKE
HV type 5	Se_z (AF519472)	LEQKMAELESKIQEKQKELEDIIRKQGPAAHAAIGGRNGDADAEQLDELAGEVSRKAAIKVELQKVKKE
	Se_z (AF519489)	LEQKMAELESKIQEKQKELEDIIRKQGPAAHAAIGGRNGDADAEQLDELAGEVSRKAAIKVELQKVKKE