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1 Rapid Communication

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3 Genetic characterisation of uninucleated cyst-producing *Entamoeba* spp. from ruminants ★

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20 ★ Note: Nucleotide sequence data reported in this paper are available in GenBank<sup>TM</sup> under  
21 the Accession Nos. **FN666248-FN666253**.

22

23 **Abstract**

24 Six *ssrRNA* gene sequences were obtained by PCR amplification of DNA from  
25 uninucleated *Entamoeba* cysts isolated from fresh faeces of sheep, cows, a roe deer and a  
26 reindeer. Phylogenetic analysis using sequences of non-, uni-, quadri- and octonucleate cyst-  
27 producing *Entamoeba* spp. for comparison showed that all six isolates formed a separate  
28 clade nested within the clade of quadrinucleate cyst producers. The data indicate that  
29 *Entamoeba bovis* can be isolated from ruminant hosts other than cattle, and we suggest that  
30 organisms clustering with the sheep and cattle isolates analysed in the present study be named  
31 *E. bovis*.

32

33 *Keywords:* *Entamoeba*, Parasite, Ruminants, Genetic diversity, PCR

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38 Organisms of the genus *Entamoeba* can be isolated from a variety of vertebrates and  
39 invertebrates and comprise parasitic species of varying pathobiological significance. Species  
40 within the genus can all be assigned to either non-, uni-, quadri- or octonucleated cyst-  
41 producing morphological groups. Uninucleated cyst-producing entamoebae have been  
42 isolated from humans, non-human primates, other mammals and birds (e.g. Noble and Noble,  
43 1952; Kingston and Stabler, 1978; Silberman et al., 1999; Verweij et al., 2001; Clark et al.,  
44 2006; Skirnisson and Hansson, 2006). Ruminants such as cattle and sheep appear to be  
45 common hosts of uninucleate cyst-producing entamoebae (Noble and Noble, 1952;  
46 Skirnisson and Hansson, 2006) (Table 1). The differentiation between species and the  
47 assignment of species names in this group have depended largely on morphological data and  
48 the host in which organisms were identified. However, since cyst morphology varies  
49 substantially within as well as between uninucleated cyst-producing species from different  
50 ruminant hosts (Table 1), morphological data alone are not enough to distinguish between  
51 some of the named *Entamoeba* spp. (Noble and Noble, 1952). To date species names have  
52 been used mainly to indicate the host from which a particular isolate was recovered, for  
53 instance *Entamoeba bovis* from cattle and *Entamoeba ovis* from sheep. Uninucleated cysts  
54 from goats, however, have been attributed to *Entamoeba debliciecki* or *Entamoeba polecki*  
55 (Noble and Noble, 1952) (Table 1), both of which are also hosted by pigs. Genetic  
56 characterisation of *Entamoeba suis* separated this from *E. polecki* (Clark et al., 2006),  
57 showing that pigs can host at least two distantly related species of uninucleated entamoebae.  
58 Hence, molecular data are needed not only in order to establish definitive identification but  
59 also clarify the epidemiology and pathobiological significance – if any – of *Entamoeba* spp.  
60 isolated from ruminants, and to improve our understanding of the evolution in the genus  
61 *Entamoeba*.

62 Data from the genetic characterisation of *Entamoeba* spp. from ruminants have not yet  
63 been published. The aim of this study was to provide such data on *Entamoeba* isolated from  
64 sheep, cattle, roe deer and reindeer, and to identify their evolutionary and taxonomic status  
65 based on phylogenetic inferences using *ssrRNA* gene sequences.

66 Cysts were isolated from fresh faecal samples from sheep, cattle, a roe deer and a  
67 reindeer by sucrose gradient purification (Lebbad et al., 2008) (Table 2). Sheep samples were  
68 also positive by microscopy for *Giardia*. One hundred cysts from each of Sheep310, Cow349  
69 and Roedeer352 were measured by light microscopy and frequency distributions of cyst  
70 diameter were compared by *t*-test analysis; *P*-values of  $< 0.01$  were considered statistically  
71 significant.

72 Purified *Entamoeba* cysts from sheep, cattle and roe deer isolates were studied using  
73 IFAT and a monoclonal antibody (mAb) originally shown to react with *Entamoeba*  
74 *histolytica* and *Entamoeba dispar* cysts, but not with *Entamoeba hartmanni*, *Entamoeba coli*,  
75 or *E. polecki* (unpublished observations). All IFAT stainings were combined with DAPI  
76 staining and, in addition, some samples were also stained with 0.5% Calcofluor.

77 DNA was extracted from cyst suspensions using the QIAamp DNA mini kit (Qiagen,  
78 Hilden, Germany) after an initial disruption of the purified cysts with a Mini-BeadBeater  
79 (Biospec Products Inc. , USA) (Lebbad et al., 2008). After PCR amplification and sequencing  
80 using the ENTAM1/ENTAM2 primers (Verweij et al., 2001) and others of more general  
81 eukaryotic specificity (Clark et al., 2006), specific primers were designed and combined with  
82 the general eukaryotic primers to fully sequence the *ssrRNA* gene (Table 3). PCR products  
83 were purified and sequenced directly in both directions using an ABI 3730 capillary  
84 sequencer. Sequences were edited manually by the use of CHROMAS (Technelysium Pty  
85 Ltd, Queensland, Australia) and entered into isolate-specific databases using the Staden

86 Package (<http://staden.sourceforge.net/>). The six sequences were deposited in the NCBI  
87 nucleotide database with accession nos. **FN666248-FN666253**.

88 Full *ssrRNA* gene sequences from each of the six isolates were incorporated into the  
89 alignment produced by Clark et al. (2006). After removal of ambiguously aligned bases the  
90 alignment included 1,572 positions. Phylogenetic analyses were performed using distance  
91 (Neighbor joining as implemented in MEGA 4.0; Kumar et al., 2008), maximum likelihood  
92 (PHYML 2.4.5; Guindon and Gascuel, 2003) and Bayesian (MrBayes 3.1.2; Huelsenbeck and  
93 Ronquist, 2001) methods. Bayesian and maximum likelihood analysis used a General Time  
94 Reversible (GTR) model of nucleotide substitution with four categories of among-site rate  
95 variation and the proportion of invariant sites. Statistical support for distance and maximum  
96 likelihood trees was evaluated using bootstrapping (1,000 replicates). Bayesian analysis used  
97 four Markov chain Monte Carlo (MCMC) strands, 1,000,000 generations, with trees sampled  
98 every 100 generations. A consensus tree was produced after excluding an initial burn-in of  
99 25% of the samples, as recommended.

100 Cyst diameter frequency counts for all three samples analysed gave unimodal  
101 distributions, and cysts from the sheep and the cow exhibited very similar average cyst  
102 diameter ranges ( $P > 0.01$ ). However, cysts from the roe deer had a narrower size range and  
103 were significantly larger on average than cysts from the other two hosts ( $P < 0.0001$ ) (Table  
104 2).

105 By DAPI staining the vast majority of the *Entamoeba* cysts screened by microscopy  
106 were found to be uninucleate; a very few were binucleate and none were quadri- or  
107 octonucleate. Calcofluor staining revealed that all organisms observed had a cyst wall and  
108 therefore that none of them could be trophozoites.

109 Uninucleate cysts from all samples reacted with the mAb, visualised by FITC-  
110 staining. However, since by microscopy 4-6% of the cysts from the sheep samples were seen

111 not to react with the mAb, PCR using specific primers (unpublished data) for identification of  
112 *E. polecki* (all subtypes) was performed but no amplification of the DNA was seen.  
113 Moreover, sequencing of PCR amplicons produced by the ENTAM 1/2 primers of broader  
114 specificity did not provide any evidence of mixed *Entamoeba* infection. It is possible that  
115 non-reacting cysts are immature and the antigen to which the mAb binds might only be  
116 produced late in the maturation process.

117 The analysis of *Entamoeba* sequences from sheep, reindeer and roe deer required very  
118 little manual editing, if any, whereas analysis of the sequences from cattle isolates was more  
119 complicated, since a few base calls were ambiguous despite multiple amplification and  
120 sequencing efforts. Therefore, a few base positions are annotated as degenerate bases in both  
121 of the sequences from cattle (accession nos. FN666248 and FN666249), and may represent  
122 differences between individual gene copies in these isolates.

123 Phylogenetic analysis revealed that the six sequences from the four ruminant hosts  
124 belong to a separate clade, branching within the previously described group of quadrinucleate  
125 cyst-producing species (Fig. 1). High bootstrap values separated the group consisting of the  
126 ruminant isolates plus the quadrinucleate cyst-producing species from groups of non-, other  
127 uni- or octonucleate cyst-producing species, which implies that the ruminant entamoebae  
128 studied here are descended from a quadrinucleated cyst-producing ancestor. These amoebae  
129 are most closely related to organisms such as *E. histolytica*, and only remotely related to  
130 other uninucleated cyst producers such as *E. polecki* (Fig. 1). The roe deer sequence was the  
131 basal lineage within the ruminant clade, supported by a bootstrap value of 100% and a  
132 posterior probability value of 1.0. The cattle, sheep and reindeer isolates clustered together  
133 closely and, interestingly, the two sheep sequences were not specifically related within this  
134 clade, being separated by the reindeer sequence, high posterior probabilities and moderate  
135 bootstrap values.

136 For a long time, evidence supported the hypothesis that morphological characteristics  
137 of *Entamoeba* cysts mirrored the genetic diversity of the genus *Entamoeba* and that the  
138 placement of species into separate clusters could be predicted from the number of nuclei  
139 within the mature cyst (Clark et al., 2006). However, the uninucleate cyst-producing species  
140 *E. suis* was recently shown to branch at the base of the quadrinucleate cyst-producing clade,  
141 although it is most closely related to the non-cyst-producing species *Entamoeba gingivalis*  
142 (Clark et al., 2006). The sequences obtained in the present study were all derived from  
143 uninucleated amoebic cysts yet clearly emerge from within the quadrinucleate cyst-producing  
144 clade, which means that grouping species based on cyst nuclei number does not always  
145 reflect genetic relationships.

146 The ENTAM1/2 primers were designed to target all species of the genus *Entamoeba*  
147 and no other organisms (Verweij et al., 2001). Analysis of the chromatogram obtained from  
148 sequencing the ENTAM1/2 PCR product for Sheep310 apparently revealed a mixed infection  
149 as seen by the presence of double peaks. However, analysis of the underlying minor sequence  
150 disclosed the presence of a *Candida* PCR product in the sample and not another species of  
151 *Entamoeba*. This means that the cysts that did not react with the mAb most likely did not  
152 represent another *Entamoeba* sp. It also means that the ENTAM1/2 primers are not as  
153 specific for *Entamoeba* as originally thought.

154 The morphological data collected in this study resemble closely those reported by  
155 Noble and Noble (1952) on sheep and cattle entamoebae. In the present study, most cysts  
156 were found to be uninucleate. In very few instances, binucleate cysts were seen. However, so-  
157 called supranucleate cysts have also been reported for other parasites such as *E.coli*,  
158 *Endolimax nana*, *Iodamoeba bütschlii* (Dobell, 1919) and *E. polecki* (Levin and Armstrong,  
159 1970).

160 Kingston and Stabler (1978) reported finding *E. bovis* and *E. coli* in white-tailed deer.



161 In the present study, it was observed that the *Entamoeba* isolate obtained from a roe deer was  
162 genetically distinct from the *Entamoeba* found in the cattle, and that the two sheep sequences,  
163 although different, were more related to the cattle *Entamoeba* than was the deer sequence.  
164 Examination of cysts from the roe deer revealed a narrower cyst diameter range than for the  
165 cysts from cattle and sheep, and although the maximum diameter recorded in the present  
166 study was only 12.0  $\mu\text{m}$ , the cysts from the roe deer were larger on average than those found  
167 in cattle and sheep (Table 1). Unfortunately, cyst measurements were not available for the  
168 reindeer isolate. However, this study showed that the roe deer isolate differed significantly  
169 from the cattle and sheep isolates not only phylogenetically, but also morphologically, and  
170 may represent a distinct species.

171 The nomenclature of *Entamoeba* species found in ungulates (ruminants plus pigs) in  
172 general has been very confused. In the absence of molecular data, the assignment of species  
173 names to a given isolate has relied on parasite morphology and host species. There are  
174 probably several reasons why confusion has prevailed. Firstly, Noble and Noble (1952)  
175 stressed that various physical and chemical factors pertaining to the processing and analysis  
176 of cyst preparations affect cyst morphology. Secondly, we know that multiple *Entamoeba*  
177 spp. can be isolated from pigs (Clark et al., 2006) and cattle (unpublished observations).  
178 Moreover, we know that many *Entamoeba* spp. have been isolated from more than one host  
179 species, and it is likely that different species of ungulates may be infected by (variants of) the  
180 same *Entamoeba* species (Mackinnon and Dibbs, 1938). Finally, we know that there is a  
181 considerable range of cyst diameter within isolates and a considerable overlap among isolates  
182 from different hosts. The presence of bimodal cyst diameter frequency distributions of  
183 *Entamoeba* identified in pigs, as observed by for example Noble and Noble (1952),  
184 complicates interpretation of data, since this could be due to a true variation in size within a  
185 species or to the presence of a mixed species infection. Interestingly, Noble and Noble (1952)

186 concluded that uninucleate entamoebae isolated from the faeces of cattle, goats, sheep, and  
187 pigs were morphologically indistinguishable.

188         Although we did not obtain the same sequence from cysts in two different hosts, the  
189 phylogenetic analysis in the present study provides indirect evidence that the same host  
190 species can be infected with different variants of the same *Entamoeba* species, since the two  
191 sequences from sheep are interspersed with cattle and reindeer isolates. The segregation of  
192 the two sheep isolates (Fig. 1) renders *E. ovis* a paraphyletic taxon in the event that the  
193 species name *E. bovis* is retained for the other isolates. Since the genetic distance between the  
194 cattle, sheep and reindeer isolates is relatively small (2-3%), we believe that these isolates  
195 represent genotypes of the same species and that the cattle, sheep and reindeer isolates all  
196 belong to what should be termed *E. bovis* (Liebetanz, 1905). Whether roe deer are indeed  
197 infected with a separate species, as suggested here, remains to be established. This will  
198 require more extensive sampling of entamoebae in ruminants and other ungulates.

199

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207

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253 of uninucleated cyst-producing *Entamoeba* species. J. Clin. Microbiol. 39,  
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- 255

256 **Figure legend**

257

258 Fig. 1. Phylogenetic analysis of the *Entamoeba* ssrRNA gene sequences. The six new  
259 sequences from this study (Cow349, Cow351, Sheep297, Sheep310, Reindeer100 and  
260 Roedeer352) were incorporated into an existing alignment consisting of 1,572  
261 unambiguously aligned positions (Clark et al., 2006). The tree from the Bayesian analysis is  
262 shown. Bootstrap support and posterior probabilities are shown at each node in the order:  
263 maximum-likelihood/distance/Bayesian analysis. An asterisk indicates that support for a node  
264 by one method was less than 50% and unlabelled nodes indicate that two or more analyses  
265 gave less than 50% support. Bar: 0.05 substitutions per site.

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269 Table 1. Uninucleated cyst-producing *Entamoeba* spp. reported from ungulates. For a detailed morphological description of the various  
 270 cysts and species, please refer to works by Levine (1973) and Noble and Noble (1952).

Species/name <sup>a</sup>	Host	Cyst size (mean) <sup>b</sup>	Reference
<i>Entamoeba bovis</i>	Cattle ( <i>Bos taurus</i> )	4—15 µm (8.8 µm)	Noble and Noble, 1952
	White-tailed deer ( <i>Odocoileus virginianus</i> )	6—11 µm (8.2 µm)	Kingston and Stabler, 1978
	Gnu ( <i>Connochaetes taurinus</i> )	6—13 µm (9.0 µm)	Mackinnon and Dibb, 1938
	Bay Duiker ( <i>Cephalophus dorsalis</i> )	N/A	Bray, 1964
<i>Entamoeba ovis</i>	Sheep ( <i>Ovis aries</i> )	4—13 µm (7.2 µm)	Noble and Noble, 1952
	Sable antelope ( <i>Hippotragus niger</i> )	N/A	Triffitt, 1926
	Water buck ( <i>Cobus ellipsiprymus</i> )	N/A	Triffitt, 1926
	Eland ( <i>Oreas canna</i> )	5—12 µm (N/A)	Triffitt, 1926
<i>Entamoeba deblickei</i> <sup>c</sup>	Goat ( <i>Capra hircus</i> )	4—12 µm (6.4 µm)	Noble and Noble, 1952
<i>Entamoeba dilimani</i>	Goat ( <i>Capra hircus</i> )	5—16 µm (9.7 µm)	Noble, 1954
<i>Entamoeba bubalus</i>	Carabao	5—9 µm (8.0 µm)	Noble, 1955
	( <i>Bubalus bubalis carabanensis</i> )		

<i>Entamoeba suis</i>	Pig ( <i>Sus domesticus</i> )	9.5—15.5 $\mu\text{m}$ (12.85 $\mu\text{m}$ )	Clark et al., 2006
<i>Entamoeba polecki</i>	Pig ( <i>Sus domesticus</i> )	10—12 $\mu\text{m}$ (N/A)	Prowazek, 1912
	Pig ( <i>Sus domesticus</i> )	4—17 $\mu\text{m}$ (8.09 $\mu\text{m}$ )	Noble and Noble, 1952
	Wild boar ( <i>Sus scrofa</i> )	9—15 $\mu\text{m}$ (N/A)	Mackinnon and Dibb, 1938
	Indian boar ( <i>Sus cristatus</i> )	9—15 $\mu\text{m}$ (N/A)	Mackinnon and Dibb, 1938
	Giant Forest hog ( <i>Hylochoerus meinertzhageni</i> )	9—15 $\mu\text{m}$ (N/A)	Mackinnon and Dibb, 1938

271 <sup>a</sup>*Entamoeba gedoelsti* and *Entamoeba caprae* have been found in horses and a goat, respectively, but since cysts have not been reported,  
272 these species are not included in the table.

273 <sup>b</sup>Rounded figures.

274 <sup>c</sup>*Entamoeba deblickei* has been considered a small variant of *E. suis* or synonymous with *E. polecki* (Noble and Noble, 1952; Burrows and  
275 Klink, 1955).

276 N/A, data not available.

277

278 Table 2. *Entamoeba* samples included in this study. See text for details.

Sample ID	Host	Country of origin	Cyst diameter of 100 cysts (mean) [S.D.]	Accession number of corresponding sequence
Cow349	<i>Bos taurus</i>	Sweden	3.9—14.4 $\mu\text{m}$ (6.6 $\mu\text{m}$ ) [1.80]	<b><u>FN666248</u></b>
Cow351	<i>Bos taurus</i>	Sweden	N/A	<b><u>FN666249</u></b>
Sheep310	<i>Ovis aries</i>	Sweden	5.4—13.8 $\mu\text{m}$ (7.2 $\mu\text{m}$ ) [1.36]	<b><u>FN666250</u></b>
Sheep297	<i>Ovis aries</i>	Sweden	N/A	<b><u>FN666251</u></b>
Reindeer100	<i>Rangifer tarandus</i>	Iceland	N/A	<b><u>FN666252</u></b>
Roe Deer352	<i>Capreolus capreolus</i>	Sweden	7.2—12.0 $\mu\text{m}$ (9.3 $\mu\text{m}$ ) [0.77]	<b><u>FN666253</u></b>

279 N/A, data not available.



280 Table 3. Primers used to obtain the full sequence of the *Entamoeba* *ssrRNA* gene from all ruminant isolates.

Primer ID	Sequence (5'-3')	Reference
RD5	ATCTGGTTGATCCTGCCAGT	Clark et al., 2006
RD3	ATCCTTCCGCAGGTTACCTAC	Clark et al., 2006
AEMH3.1	AAGGGCATCACGGACCTGTT	Clark et al., 2006
EntOv_430F	GTAGTGACGACAAATAACTCTTG	Present study
EntOv_1200F	GAAAACTTACCAAGACCGAACAG	Present study
EntUng_500R	CCTCCAATTGATTTCTTTAGAG	Present study
EntUng_900R	TTTCGTTCTTGATTAATGAACG	Present study

281

282

Figure 1

