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Short Communication

Morphological and molecular characterization of an uninucleated cyst-producing *Entamoeba* spp. in captured Rangeland goats in Western Australia

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Highlights

- First report of *Entamoeba* from wild Rangeland goats in Western Australia
- Molecular characterisation at 2 loci
- Genetically closest to an *E. bovis* isolate from a sheep from Sweden
- First study to produce actin sequences from *E. bovis*-like *Entamoeba* sp.

Abstract

Uninucleated *Entamoeba* cysts measuring 7.3 x 7.7 μm were detected in faecal samples collected from wild Rangeland goats (*Capra hircus*) after arrival at a commercial goat depot near Geraldton, Western Australia at a prevalence of 6.4% (8/125). Sequences were obtained at the 18S rRNA (n=8) and actin (n=5) loci following PCR amplification. At the 18S locus, phylogenetic analysis grouped the isolates closest with an *E. bovis* isolate (FN666250) from a sheep from Sweden with 99% similarity. At the actin locus, no *E. bovis* sequences were available, and the isolates shared 94.0% genetic similarity with *E. suis* from a pig in Western Japan. This is the first report to describe the morphology and molecular characterisation of *Entamoeba* from Rangeland goats in Western Australia and the first study to produce actin sequences from *E. bovis*-like *Entamoeba* sp.

Keywords: *Entamoeba*; 18S rRNA; actin gene; Rangeland goats

1. Introduction

Organisms of the genus *Entamoeba* have adapted to live as parasites or commensals in the digestive tract of humans and other mammals, birds, amphibians, fish and reptiles (Skirnisson and Hansoon, 2006; Stensvold et al., 2010). Species within the genus can all be assigned to either uni-, quadri- or octo-nucleated and non-cyst-producing morphological groups (Stensvold et al., 2010): 1) species without cysts (*E. gingivalis*-like group), 2) species
with uninucleated cysts (*E. bovis*-like group), 3) species with quadrinucleated cysts (*E. histolytica*-like group), 4) octonucleated cysts (*E. coli*-like group). Several species are found in humans and animals with the quadrinucleate *E. histolytica* responsible for invasive 'amoebiasis' (which includes amoebic dysentery and amoebic liver abscesses) in humans.

Uninucleated cyst-producing Entamoebae have been isolated from a range of hosts including humans, non-human primates, other mammals and birds (Noble and Noble, 1952; Skirnisson and Hansson, 2006; Stensvold et al., 2010). Ruminants such as cattle and sheep are common hosts of uninucleate cyst-producing Entamoebae (Noble and Noble, 1952; Jacob et al., 1990; Hampton et al., 2006; Skirnisson and Hansson, 2006; Kanyari et al., 2009; Stensvold et al., 2010; Stensvold et al., 2011) and unidentified *Entamoeba* species have been reported in goats in Kenya (Kanyari et al., 2009), Thailand (Sangvaranond et al., 2010), Tanzania (Mhoma et al., 2011), Cameroon (Ntonifor et al., 2013) and Brazil (Radavelli et al., 2014).

Until recently, the detection, identification and assignment of *Entamoeba* organisms to species relied mainly on morphology and the host in which parasites were identified (Stensvold et al., 2010; Stensvold et al., 2011). However, morphology is not a reliable tool for delimiting *Entamoeba* species as cyst morphology varies substantially within as well as between uninucleated cyst-producing species from different ruminant hosts (Noble and Noble, 1952; Pillai and Kain, 1999; Stensvold et al., 2010). The use of molecular tools is therefore essential to resolve the identification, taxonomy, epidemiology and clinical significance of *Entamoeba* species without reliance on parasite cultures or experimental infections (Stensvold et al., 2011; Jacob et al., 2016).

Rangeland goats are an introduced animal species in Australia. They can be legally trapped and reared by licensed operators (goat depots) for the domestic and export meat markets, which was worth approximately $AUS242 million in 2014 (MLA, 2015). Few
studies have conducted genetic characterisation of *Entamoeba* species from ruminants (Stensvold et al., 2010; Jacob et al., 2016), which is important for understanding their evolutionary and taxonomic relationships. In the present study, uninucleate *Entamoeba* cysts were identified in the faeces of Rangeland goats in Western Australia by microscopy and were characterised at the 18S ribosomal RNA (rRNA) and actin loci.

2. Materials and methods

Faecal samples were collected by rectal palpation from 125 male Australian Rangeland goats (*Capra hircus*) on arrival at a commercial goat depot near Geraldton, Western Australia, after capture and transport from a sheep and cattle extensive rangeland grazing property, North Wooramel station, located 78 km east of Denham and 113 km south east of Carnarvon in the Gascoyne region of Western Australia. On arrival, the goats weighed on average 30.7 ± 0.3 kg (±SEM), and the estimated age of the goats, based on dentition, was between 9 – 12 months. Faecal samples were immediately placed on ice until transported to the lab, where microscopy work was performed on the same day of collection and then stored in the refrigerator (4.0°C) until DNA extraction was performed. All sample collection methods used were approved by the Murdoch University Animal Ethics Committee (approval number R2617/13).

Direct microscopic examination of faecal suspensions in saline and wet mounted with 0.9% saline and Lugol's iodine was conducted. *Entamoeba* cysts were concentrated using zinc-sulfate gradient floatation (Faust’s method) (Ramos et al., 2005) and observed under Nomarski contrast with a 100× oil immersion objective lens in combination with an ocular micrometre on an Olympus DP71 digital micro-imaging camera. The diameters of cysts (n=35) (isolated from the eight positive samples) and trophozoites (n=15) (isolated from two positive samples) were measured and averages and ranges were calculated.
Genomic DNA was extracted from 200 mg of each faecal sample using a Power Soil DNA Kit (MolBio, Carlsbad, California) with some modifications. Briefly, samples were subjected to four cycles of freeze/thaw by liquid nitrogen and boiling water to ensure efficient lysis of cysts before being processed using the manufacturer’s protocol. A negative control (no faecal sample) was used in each extraction group.

Samples that were positive for *Entamoeba* by microscopy were examined by PCR using the eukaryotic primers RD5 (5’-ATCTGGTTGATCCTGCCAGT-3’) and RD3 (5’-ATCCTTCCGCAGGTTACCTAC-3’) as previously described (Clark et al., 2006). An approx. 1,950 bp PCR product was amplified, which was initially sequenced with the RD5 or RD3 primers in both directions. Based on the partial sequences obtained, a set of new *Entamoeba* specific primers ENF2 (5’-AAGCATGGGACAATATCGAGG) and ENR2 (5’-GTCCCTTTAAGAAGTGATGC) were designed to conduct sequence walking to obtain the full length sequence of the 1,950 bp PCR product. The primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). All microscopy positives were also analysed at the actin locus (~1,100 bp amplicon) as described by Sulaiman et al. (2002), as although these primers were originally designed for *Cryptosporidium*, they also amplify *Entamoeba* (Matsubayashi et al. 2014).

The amplified DNA fragments from the PCR products were gel purified using an in-house filter tip method as previously described (Yang et al., 2013) and sequenced using forward and reverse primers in duplicate using amplicons from different PCR runs. An ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) was used for Sanger sequencing according to the manufacturer's instructions.

The results of the sequencing reactions were analysed and edited using FinchTV (Version 1.4), compared to existing *Entamoeba* spp. 18S rRNA and actin sequences on
GenBank using BLAST searches and aligned with reference sequences from GenBank using Clustal W in BioEdit (V7.2.5).

Phylogenetic trees were constructed for the 18S and actin sequences obtained and including additional sequences available in GenBank. Parsimony, Maximum likelihood (ML) and Neighbor-joining (NJ) analyses were conducted using MEGA 6 (Tamura et al., 2013). ML and NJ analyses were conducted using Tamura-Nei based on the most appropriate model selection using ModelTest in MEGA 6. Bootstrap analyses were conducted using 1000 replicates to assess the reliability of inferred tree topologies.

3. Results

Entamoeba was identified by microscopy in eight faecal samples at a prevalence of 6.4% (8/125) (2.1-10.7 95 CI). Cysts (n=35) were uninucleate and spherical with a single-layered cyst wall and within the nucleus, a centrally located karyosome (condensed zone of chromatin filaments) was seen (Table 1, Fig. 1a and b). Glycogen was diffuse and vacuoles were noted in most examined cysts which measured 7.3 (6.5-11.4) x 7.7 (6.6-12.3) μm with a width to length ratio of 1.05 (1.01-1.11). The diameter of the single nucleus averaged 1.7 μm (1.3-2.9) (Table 1). Trophozoites were identified in two faecal samples and measured 15.8 μm (14.2-18.2 μm) in diameter and and within the nucleus, a centrally located and irregular shaped karyosome was seen (Fig. 2, a and b).

18S sequences (1,800 bp) were obtained from all eight microscopy positives. Phylogenetic analyses using Parsimony, NJ and ML analyses produced similar results (Fig. 3- NJ tree shown) and grouped the 18S Entamoeba sequences from Rangeland goats in a clade with Entamoeba bovis and were most closely related (99% similarity) to an E. bovis isolate (FN666250) from a sheep (Ovis aries) isolate from Sweden (Fig. 3). Amongst all E. bovis isolates, the genetic similarity ranged from 96% to 99%.
At the actin locus, a 1,066 bp PCR product was successfully amplified from 5 isolates. Some sequence variation was observed in the actin sequences with 1-6 single nucleotide polymorphisms (SNP’s) observed between sequences. There were fewer Entamoeba spp. actin sequences available in the GenBank database compared to the 18S rRNA locus and E. bovis sequences were not available. Phylogenetic analysis of available sequences grouped the Entamoeba actin sequences from Rangeland goats in a separate clade with the highest similarity (94%) with Entamoeba suis from pigs (Sus scrofa domesticus) from Western Japan (AB914739) (Fig. 4). Representative 18S and actin sequences have been deposited in GenBank under accession numbers KY012746, KY012747, KY012748 and KX363870.

4. Discussion

In the present study, uninucleate Entamoeba cysts identified in the faeces of Rangeland goats in Western Australia were analysed morphologically and molecularly. It is difficult to compare the Entamoeba detected in the present study with Entamoeba species identified in goats and other ruminants in previous studies, as few microscopic studies are available and no molecular characterisation has been conducted on Entamoeba from goats. A non-encysting species, E. caprae has been described in goats (Fantham, 1923) and within the uninucleate E. bovis-like group, E. dilimani and E. debliecki (syn. E. ovis) has been described in goats (Noble and Noble, 1952; Noble, 1954). The size of the cysts (7.3 x 7.7 μm) in the present study were smaller than those of E. bovis from cattle (diameter of 8.8 μm) previously reported (Noble and Noble, 1952) and slightly bigger than the average cyst diameters reported in sheep (7.2 μm) (5.4-13.8 μm) and cattle (6.6 μm) (3.9-14.4 μm) in Sweden (Stensvold et al., 2010). The Entamoeba cysts analysed in the present study also differed from Entamoeba cysts isolated from an English goat, believed to be E. debliecki, 6.6μm (4.7
to 13.3 μm) and also differed in karyosome location, which was centrally located in the present study and eccentric (off centre) in the cysts from an English goat (Hoare, 1940). The cysts in Rangeland goats also differed from the round/oval cysts 6.4 (4-12) μm thought to be \textit{E. debliecki} (Noble and Noble, 1952). Cysts of \textit{E. dilimani} were reported to be 9.7 (5-16) μm in diameter (Noble, 1954). Further research is required to clarify the taxonomy of caprine \textit{Entamoeba} sp.

Sequence comparison and phylogenetic analysis at the 18S locus revealed that the \textit{Entamoeba} isolated from Rangeland goats in the present study shared 99% similarity with \textit{E. bovis} (FN666250) from a sheep in Sweden. Amongst the five identified \textit{E. bovis} 18S sequences available in GenBank, the genetic similarity ranged between 96-99% and therefore the \textit{Entamoeba} sequences detected in Rangeland goats are within this range. At the actin locus, unfortunately no actin sequences were available from \textit{E. bovis} in GenBank and the \textit{Entamoeba} sp. from Rangeland goats grouped (94% similarity) with \textit{E. suis} from a pig in Western Japan (AB914740), the only other actin sequence available from a uninucleate \textit{Entamoeba} sp. The actin gene is a conserved locus but with sufficient single nucleotide polymorphisms (SNPs) to delimit species in a genus and therefore is a common locus for taxonomic studies of microorganisms (Reisler and Egelman 2007; Lahr et al., 2011). Similar to the 18S rRNA PCR, no mixed chromatograms were detected, suggesting that only one \textit{Entamoeba} sp. was present in the Rangeland goats.

In conclusion, a uninucleated \textit{E. bovis}-like \textit{Entamoeba} spp. was identified from Rangeland goats, which we believe is a genotype of \textit{E. bovis}. In addition, for the first time an \textit{E. bovis}-like actin sequence was produced, which will facilitate future phylogenetic analysis of \textit{Entamoeba} in ruminants and other ungulates. The clinical impact of the \textit{E. bovis} isolate in Rangeland goats is unknown, but given the economic importance of Rangeland goat meat,
further studies should be conducted to examine the prevalence and clinical impact (if any) on Rangeland goats.

Acknowledgements

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References


Fig 1. Nomarski interference-contrast photomicrographs of *Entamoeba* cysts isolated from rangeland goats showing centrally located karyosome; 1a: cyst stained with iodine, 1b: cyst in saline mount. Scale bar = 10 μm.
Fig 2. Nomarski interference-contrast photomicrographs of *Entamoeba* trophozoites isolated from rangeland goats showing centrally located karyosome and diffuse glycogen, 1a: trophozoite stained with iodine, 1b: trophozoite in saline mount. Scale bar = 20 μm.
Fig 3. Evolutionary relationships of *Entamoeba* sp. inferred by distance analysis of using 18S rRNA gene sequences. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node (- = value was <50%).
Fig 4. Evolutionary relationships of *Entamoeba* sp. inferred by distance analysis of using partial actin gene sequences. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node (- = value was <50%).
Table 1 Morphometric characteristics of uninucleated cyst-producing *Entamoeba spp.* reported from livestock compared with the *Entamoeba* cysts isolated from Rangeland goats in Western Australia in the present study.

*The non-cyst species *E. caprae*, has been reported in a goat (Fantham, 1923), however as only trophozoite and its nucleus measurements were given, this species was not included.

bBased on morphological data, *Entamoeba ovis*, *E. suis*, *E. polecki* and *E. dilimani* were previously considered synonymous with *E. debliecki* (Levine, 1985). However, molecular data has shown that *E. polecki* and *E. suis* are not synonymous (Clark et al., 2006).

cThis is the median of 800 cysts measured in English goats and believed to be *E. debliecki* (Hoare, 1940).

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Cyst diagnostic characters</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Entamoeba bovis</em></td>
<td>Cattle (Bos taurus)</td>
<td>Cyst diagnostic characters</td>
<td>Noble and Noble (1952)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyst shape</td>
<td>Cyst size (mean)</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>4–12μm (8.8μm)</td>
<td>1.5–5.5μm (3.0μm)</td>
</tr>
<tr>
<td><em>White-tailed deer</em> (Odocoileus virginianus)</td>
<td>N/A</td>
<td>6–11 μm (8.2 μm)</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Goat</em> (Capra hircus)</td>
<td>N/A</td>
<td>6–13 μm (9.0 μm)</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Bay Duiker</em> (Cephalophus dorsalis)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Cattle</em> (Bos taurus)</td>
<td>N/A</td>
<td>3.9–14.4μm (6.6μm)</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Sheep</em> (Ovis aries)</td>
<td>N/A</td>
<td>5.4–13.8μm (7.2μm)</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Entamoeba ovis</em></td>
<td>Sheep (Ovis aries)</td>
<td>N/A</td>
<td>4–13 μm (7.2 μm)</td>
</tr>
<tr>
<td><em>Entamoeba debliecki</em></td>
<td>Goat (Capra hircus)</td>
<td>round/ oval</td>
<td>4–12 μm (6.42 μm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spherical/ ovoid/ ellipsoid</td>
<td>4.75–13.3</td>
</tr>
<tr>
<td><em>Entamoeba dilimani</em></td>
<td>Goat (Capra hircus)</td>
<td>N/A</td>
<td>5–16μm (9.7μm)</td>
</tr>
<tr>
<td><em>Entamoeba suis</em></td>
<td>Pig (Sus domesticus)</td>
<td>N/A</td>
<td>9.5–15.5μm (12.85μm)</td>
</tr>
<tr>
<td><em>Entamoeba polecki</em></td>
<td>Pig (Sus domesticus)</td>
<td>N/A</td>
<td>4–17μm (8.09 μm) large</td>
</tr>
<tr>
<td><em>Entamoeba from rangeland goats</em></td>
<td>Goat (Capra hircus)</td>
<td>spherical</td>
<td>6.5–12.3 μm (7.3×7.7μm)</td>
</tr>
</tbody>
</table>