Severe amoebic placentitis in a horse caused by *Acanthamoeba hatchetti* identified using next generation sequencing

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**RUNNING HEAD:** *Acanthamoeba hatchetti* amoebic placentitis

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A case of amoebic placentitis in a mare from eastern Australia was diagnosed post-partum by histopathological examination of the placenta. Identity of the aetiological agent was confirmed as *Acanthamoeba hatchetti* by use of a diversity profiling based on next generation sequencing approach.
CASE REPORT

A normal underweight term foal was born to an 11-year-old multiparous Thoroughbred mare in the Hunter region of New South Wales, Australia. A thick mucopurulent tan discharge was covering the body of the placenta encompassing the pregnant horn (Figure 1A). Routine culture of the discharge grew mixed organisms comprised of *Escherichia coli*, *Streptococcus agalactiae*, *Bacillus cereus*, and an unidentifiable Gram-negative bacillus (identification by matrix-assisted laser desorption/ionization time of flight mass spectrometry) (1). The mare had been present on the same farm for 6 years with no history of reproductive problems, placentitis or abortion with her previous six pregnancies.

Histologically, the allantochorion of the pregnant horn was extensively effaced by a severe chronic active placentitis (Figure 1B). The chorion was primarily involved with limited extension through the extra embryonic coelom into the allantois. There was extensive chorionic villous loss and shortening with variable trophoblastic epithelial hyperplasia and squamous metaplasia. A thick adherent layer of amorphous eosinophilic material with extensive multifocal mineralisation and foci of necrotic inflammatory cells, primarily neutrophils, covered the surface. The residual villi and adjacent chorionic stroma were expanded by large numbers of plasma cells, macrophages, some multinucleated giant cells and neutrophils with multifocal active villous necrosis (Figure 1B). Acute villous necrosis and suppurative inflammation was evident in less affected areas with large numbers of 3-15 µm diameter round to irregular amoeboid organisms (presumed trophozoites) with a characteristic large dark endosome. These organisms were primarily located within trophoblastic cells of the chorionic villi (Figure 1C). Large numbers of degenerate encysted stages up to 24 µm in diameter were present in the intervillous spaces and superficial necrotic debris (Figure 1DE).
Diagnostic material was submitted to the Veterinary Pathology Diagnostic Services (University of Sydney) for characterisation and identification. Initially, Calcofluor White (Fluka, Sigma-Aldrich) was used on unstained histological sections (Figure 1FG) to confirm the presence of cellulose and chitin in pathogen cell walls, including cysts of *Acanthamoeba* spp., microsporidia or yeast (2, 3). Calcofluor White staining was confirmed to be the same structures as those identified in the stained histological section (Figure 1CDE). The morphology and size of the Calcofluor White stained structures was not consistent with known morphology of microsporidia (1-3 µm cysts) or yeasts (budding).

To identify the potential intracellular and free eukaryotes present in the diagnostic specimen, we used a community profiling based amplification 454 sequencing approach of conserved eukaryotic SSU rDNA (4). Genomic DNA (gDNA) was isolated from approximately 25 mg of frozen (-20°C) allantochorion using the Isolate II Genomic DNA kit (BioLine, Australia). The gDNA was submitted to the diversity profiling service at the Australian Genomic Research Facility, Australia. The SSU rDNA assay applied was based on primer pair Euk1A (5'-CTGGTTGATCCTGCCAG-3’) and Euk516 (5’-CCAGACTTGCCCTCC-3’), amplifying approximately 500-550 bp of the 5’-end of the eukaryotic SSU rDNA. The barcoded PCR amplicon was pooled and sequenced on the 454 GS-FLX platform. 8,634 reads were obtained, subsequently reduced to 2,294 high quality reads with an average read length of 501 bp. We removed homopolymers (>8) and sequences <150 bp in length and kept only sequence reads with a cut-off quality score of 20 across 80% of the sequence read. The high quality reads were clustered using CD-HIT (cd-hit.org) into pools of >97% identity sequence clusters. 18 clusters (98.8% duplicates) and 6 singleton sequences were identified and used as queries in BLASTN within CLC Main Workbench 6.9 (Qiagen, CLC bin, Denmark). A single cluster represented by two sequences and two singletons was most closely related to *Acanthamoeba hatchetti* (AF019068). A remaining...
singleton was most closely related to a fungus *Cystofilobasidium infirmominiatum* (DQ645524) suspected to be a contaminant with no direct relevance to the case. Horse SSU rDNA matched 17 of the clusters and 5 of the singletons. Querying of all reads (including the low quality reads) using BLASTN with *A. hatchetti* (AF019068) confirmed presence of 4 hits in the high quality pool of reads as well as additional 5 sequence reads in the previously discarded poor quality reads. Phylogenetic tree revealed monophyly of the horse *Acanthamoeba* sequences with *A. hatchetti*, together forming a sister group to *A. stevensoni* (Figure 2A). Pairwise nucleotide comparison revealed over 98.5% percent identity with available SSU rDNA sequences of *A. hatchetti* strains BH2 and 4RE (Figure 2B). Strain BH-2 (ATCC® 30730™) is the type species *A. hatchetti* and forms a distinct SSU rDNA clade T11 together with *A. stevensoni* (5). BH2 was isolated from brackish sediment from Brewerton Channel in Baltimore Harbour, Maryland (6). 4RE was derived from a contact lens storage case in Austria (7). Further querying (BLASTN) revealed high (>98%) similarity with a phylotypes from faecally contaminated water from Equatorial Guinea (KF433820) and a sink plughole in Japan (AB859622).

Initial attempts at direct pathogen PCR identification with *Acanthamoeba* spp. specific SSU rDNA primers ACAN18SF0 (5′-TCCTGCCAGTAGTCATATGC-3′) and ACAN18SR0 (5′-CTTCTCCTTCTCTAAATGGT-3′) (8), were unsuccessful. Subsequently, successful amplification was achieved with primers targeting partial SSU rDNA fragment JDP1 (5′-GGCCCAATCGTATTACGTGGA-3′) and JDP2 (5′-TTCACAAAGCTGAGGAGCTCA-3′) (9). PCR used MyTaq™ Red Mix (BioLine, Australia) using the following cycling conditions: 95 °C for 15 s, 57 °C for 15 s, and 72 °C for 15s for 35 cycles in a Veriti PCR cycler (Life Sciences, Australia). PCR product band of expected was bidirectionally sequenced using amplification primers at Macrogen Ltd. (Seoul,
Korea) and analysed in CLC Main Workbench. The obtained sequence was 100% and 99.5% identical to *A. hatchetti* strain 4RE and BH2 strains.

The identity *A. hatchetti* was further supported by cyst morphology. Calcofluor White visualization of the placenta revealed myriad 14.0 µm (12.4-15.7 µm) diameter cysts morphologically compatible with those for the BH-2 isolate *A. hatchetti*, originally reported to form cysts that measured 13.1 (11.5-16.1) µm in diameter (6).

*Acanthamoeba* spp. are ubiquitous free-living amoebae that received a bad reputation as the cause of a sight-threatening keratitis in contact-lens wearers and rare but fatal granulomatous encephalitis (10). In humans *Acanthamoeba* spp. enter CNS either through respiratory track or skin injury followed by amoebae invasion into the blood vessels or through the nasal passage (10, 11). Infections are generally diagnosed by visualisation of typical cysts, culture and PCR (10). Free-living amoebae have rarely been documented to cause encephalitis in animals including horses; this is most likely due to underreporting (12). Animals as sentinels for human *Acanthamoeba* spp. exposure are scarcely explored (13). In domestic animals, only encephalitis is recognised as *Acanthamoeba*-associated disease.

We report a unique placentitis in a mare with documented presence of *A. hatchetti* associated with histopathological lesions of focal chronic active chorioallantoitis. To our knowledge, this is the first opportunistic *Acanthamoeba*-infection of the reproductive tract of any species, and confirms the experimental potential of *A. hatchetti* to cause significant disease (6). *A. hatchetti* is a common free-living amoebae that has not previously been documented to cause significant pathology under natural conditions (14). However, experimental intranasal inoculation of laboratory mice with *A. hatchetti* BH-2 results in death.
due to extensive inflammation and necrosis of the brain (6). This case report of amoebic equine placentitis is the first documented case of clinical disease caused by *A. hatchetti*.

Several opportunities exist for *A. hatchetti* to enter into the horse reproductive system. A vascular route of transmission (the route for CNS infection) is supported by literature if respiratory or skin injury were present (10, 11). However, the mare was clinically healthy prior to and throughout the pregnancy. Recently, setae (hairs) of the Processionary caterpillar (*Ochrogaster lunifer*) were documented to migrate from the gastrointestinal tract of experimentally exposed mares into the uterus and foetal membranes ultimately causing abortion (15). Mechanical transport of the *A. hatchetti* by the setae into the uterus may be possible due to the hollow interior of the setae or unknown adherence factors related to biofilm or any setae-associated bacteria. Alternatively, *A. hatchetti* may have localized in the uterus after vascular invasion or via cervical incompetence with a bacterial focal placentitis. This lesion can occur as sequelae to caterpillar exoskeleton ingestion in early gestation and any bacteria may have served as a food source for *A. hatchetti* in the role of opportunistic pathogen (16). *Acantamoeba*-cysts themselves carry a myriad of pathogens within their cysts, including the infamous *Legionella* (10, 11). Therefore, if evidenced that *Acantamoeba*-cysts were carried into the uterus on caterpillar hairs, it would represent one of the most remarkable cases of pathogen transmission.

The diagnosis of *Acantamoeba* spp. is challenging because of cryptic morphology of their trophozoites resembling macrophages. The case demonstrated the advantages of the use of diversity profiling approaches to identification of eukaryotic pathogens, including emerging pathogens for which routine diagnostic approaches are not available.

**Nucleotide sequence accession numbers.**
Nucleotide sequence data from this study are available in the GenBank database under accession number KJ801938 and SRA: SRP041013.

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Legends to figures

FIGURE 1. Horse placenta with *Acanthamoeba hatchetti*. Mucopurulent orange-brown discharge in the placenta (A). Numerous *A. hatchetti* within the allantochorion (Hematoxylin and eosin stain, H&E) (B-C). Detailed view of the cyst of *A. hatchetti* (D, H&E; E, Grocott’s methenamine silver stain). Calcofluor White labelled *A. hatchetti* cysts (green) under fluorescence microscopy (FITC filter set) (F-G).

FIGURE 2. Evolutionary relationships of *Acanthamoeba hatchetti* from horse placentatis based on SSU rDNA. A) The tree was inferred using the Minimum Evolution (ME) method. The bootstrap supports in percentages are shown next to the branches (1000 replicates for ME and 100 replicates for Maximum Likelihood). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and the ME tree was searched using the CNI algorithm in MEGA6 (www.megasoftware.net). All ambiguous positions were removed for each sequence pair. There were 589 positions spanning partial *A. hatchetti* SSU rDNA. B) Pairwise comparison of nucleotide distance (uncorrected) for published *A. hatchetti* SSU rDNA sequences and the sequences obtained using 454 sequencing, a Cluster 16 (represented by IJMZ0LK01BAKMG and IJMZ0LK01AO988 reads) and two singletons (IJMZ0LK01ATWF7, IJMZ0LK01AKNTK). *A. stevensoni* is the closest sister species to *A. hatchetti*. 