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Research brief

Cryptosporidium parvum and *Enterocytozoon bieneusi* in American Mustangs and Chincoteague ponies

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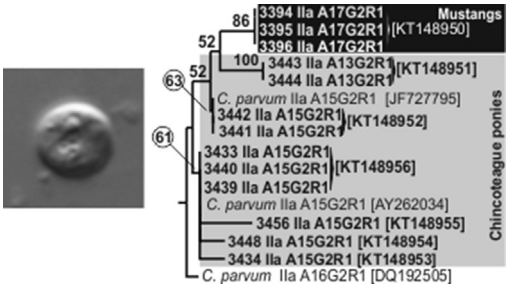
HIGHLIGHTS

- Burden of *Cryptosporidium* and microsporidia in feral horses was determined by PCR.
- Feral horses host the zoonotic species *Cryptosporidium parvum*.
- Feral horses host horse-specific *E. bieneusi* genotype horse 1.
- These species from feral horses also are found in horses managed closely by humans.

GRAPHICAL ABSTRACT



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ABSTRACT

The prevalence of *Cryptosporidium* and microsporidia in feral horses, which have minimal contact with livestock and humans, is not currently known. We report the findings of a study on *Cryptosporidium* and microsporidia in 34 Mustangs and 50 Chincoteague ponies in the USA. Fecal samples were screened for presence of *Cryptosporidium* spp. by analysis of the small-subunit rRNA (SSU) and 60-kDa glycoprotein (gp60) genes, and *Enterocytozoon bieneusi* and *Encephalitozoon* spp. by analysis of the ribosomal internal transcribed spacer region (ITS). *Cryptosporidium* spp. and *E. bieneusi* were detected in 28/84 (33.3%) and 7/84 (8.3%) samples, respectively. Sequence analysis of SSU and ITS revealed the presence of *Cryptosporidium parvum* (n = 20) and *E. bieneusi* genotype horse 1 (n = 7), respectively. Subtyping of *C. parvum* isolates at the gp60 locus showed the presence of subtype IIaA17G2R1 in Mustangs and subtypes IIaA13G2R1 and IIaA15G2R1 in Chincoteague ponies. *Enterocytozoon bieneusi* genotype horse 1 was detected in Mustangs (n = 2) and Chincoteague ponies (n = 5). No *Cryptosporidium* or *E. bieneusi* positive animals had diarrhea. The finding that Mustangs and Chincoteague ponies are host to the zoonotic pathogen *C. parvum* suggests that their infrequent contact with humans and livestock is sufficient to maintain transmission; however, we should also consider the possibility that *C. parvum* is an established

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parasite of Mustangs and Chincoteague ponies that persists in these animals independently of contact with humans or livestock.

1. Introduction

The extant horse species are *Equus ferus caballus* (domestic horse) and *E. ferus przewalskii* (Przewalski's horse or wild horse). Most domestic horses are closely managed by humans and are used for work and recreation, but some horses of domestic ancestry became feral through escape and release from human settlements and they subsequently adopted behaviors more similar to wild horses. These feral horses are minimally managed by humans.

Cryptosporidium parasitizes cells of the gastrointestinal epithelium, causing a diarrheal disease that can be chronic and fatal in immunocompromised individuals. *Cryptosporidium* horse genotype was originally identified in a Przewalski's horse at a zoo in the Czech Republic (Ryan et al., 2003) and subsequently in domestic horses in the Czech Republic (Wagnerová et al., 2015), USA (Burton et al., 2010), and Italy (Caffara et al., 2013). Domestic horses also host *Cryptosporidium parvum*, a major zoonotic species, and rarely *Cryptosporidium hominis*, *Cryptosporidium muris*, *Cryptosporidium tyzzeri*, *Cryptosporidium erinacei*, and *Cryptosporidium felis* (Guo et al., 2014; Laatamna et al., 2013, 2015; Wagnerová et al., 2015). Although there have been more than 30 reports worldwide describing the natural occurrence of *Cryptosporidium* spp. in domestic horses (Burton et al., 2010; Imhasly et al., 2009; Laatamna et al., 2013, 2015; Majewska et al., 2004; Majewska et al., 1999; Wagnerová et al., 2015; Xiao and Herd, 1994), there is no record of the occurrence of this parasite in feral horses.

Microsporidia are obligate intracellular parasites that infect a wide range of invertebrate and vertebrate hosts (Didier and Weiss, 2006). There are more than 1200 microsporidian species and several are important parasites of vertebrates, including *Encephalitozoon hellem* in birds, *E. intestinalis* in humans, and the broadly-specific *Encephalitozoon cuniculi* and *Enterocytozoon bieneusi* (Cacciò and Pozio, 2001; Camero et al., 1999). Although microsporidia are known to cause abortion in horses (Patterson-Kane et al., 2003; Szeredi et al., 2007; van Rensburg et al., 1991), there have been few studies describing the prevalence of these parasites in horses (Laatamna et al., 2015; Santín et al., 2010; Wagnerová et al., 2012, 2013).

The objective of this study was to determine the prevalence and diversity of *Cryptosporidium* spp. and microsporidia in feral Mustangs and Chincoteague ponies the USA.

2. Material and methods

2.1. Origin of samples and sampling

The research was performed at two locations in the USA. Thirty-four Mustangs were sampled from Oregon. Fifty Chincoteague ponies were sampled from Chincoteague National Wildlife Refuge in Virginia. Mustangs and Chincoteague ponies graze throughout the year and use several natural freshwater sources for drinking water. Mustangs, unlike ponies, occasionally drink from the same sources and graze the same grasslands as cattle and wild animals such deer and elk. Supplemental feed (certified weed free hay/forage) and drinking water (watering troughs filled from tankers) is supplied to Chincoteague ponies during weather extremes such as heat and drought, strong coastal storms and tidal flooding, and snow and ice storms. Mustangs are not provided with supplementary feed or drinking water.

Each fecal sample was collected from the ground after defecation, placed into a separate plastic container, and transported to the laboratory for examination. The fecal consistency was noted at the time of sampling.

2.2. Parasite genotyping

Total DNA was extracted from 0.2 g faecal samples by phenol–chloroform extraction and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) as previously described (Feltus et al., 2006; Peng et al., 2003).

Nested PCR protocols were used to amplify fragments of the *Cryptosporidium* small-subunit (SSU) rRNA (Jiang et al., 2005) and 60-kDa glycoprotein (gp60) genes (Alves et al., 2003). Only samples that were positive for SSU were screened for the gp60 gene. All samples were analyzed in duplicate. A nested PCR protocol was used to amplify the internal transcribed spacer (ITS) region of the rRNA gene of *E. bieneusi*, as previously described (Buckholt et al., 2002). Negative (water instead of DNA) and positive (DNA of *C. muris* for SSU, *C. hominis* for gp60 and *E. bieneusi* genotype D for ITS) controls were used in each reaction.

PCR products were visualized following electrophoresis on a 2% agarose gel containing 0.2 g/ml ethidium bromide. Sequencing was performed on an ABI 3730XL sequence analyser (Applied Biosystems, Foster City, CA, USA). The sequences were assembled by using ChromasPro 1.7.6. (www.technelysium.com.au/ChromasPro.html), edited by using BioEdit 7.04 (www.mbio.ncsu.edu/BioEdit/bioedit.html), and aligned using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) online server with automatic selection of alignment mode. The optimal nucleotide substitution model was selected and phylogenetic trees were inferred by the Neighbor-Joining (NJ) method using MEGA6 (http://www.megasoftware.net/) (Tamura et al., 2011). Bootstrap support for branching was based on 1000 replications. Phylograms were edited for style using CorelDrawX7 (Corel Corporation, Ottawa, Ontario, Canada). Sequences have been deposited in GenBank under the accession numbers KT148950-KT148960.

2.3. Statistical analyses

We used the chi-squared test of independence to test relationship between the variables. In some cases we used Generalized Fisher exact test due to small cell counts in analyzed contingency tables. All computations were performed by programming environment R 3.0.2. (http://www.r-project.org/).

3. Results

Seven out of eight-four (8.3%) samples from horses in Oregon and Virginia were positive for *E. bieneusi* DNA, and isolates from five positive samples were successfully genotyped. Phylogenetic analysis revealed the presence of *E. bieneusi* genotype horse 1 in all samples (Table 1).

Twenty-eight out of eight-four (33.3%) samples were positive for *Cryptosporidium* spp. DNA (Table 1), and isolates from 20 to 13