Detection of Assemblage A, *Giardia duodenalis* and *Eimeria* spp. in alpacas on two Maryland farms

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**Abstract**

Sixty-one fecal samples were collected from adult alpacas and crias (ages 10 weeks to 10 years) on two farms in central Maryland. The farms raised both suri (silky-haired) and huacaya (crimpy-haired) breeds. Females and crias were housed together on pasture, whereas older/breeding males were maintained on separate pastures. Samples were subjected to a density gradient centrifugation protocol to concentrate parasites and remove fecal debris and were examined by immuno-fluorescent and differential interference contrast microscopy. Oocysts of *Eimeria* spp. were noted in 14 fecal samples, 6 on MD-1 and 8 on MD-2. Based on oocyst morphometrics two species of *Eimeria* were present: *E. punoensis* (19.2 μm x 16.5 μm) and *E. alpacae* (23.7 μm x 19.5 μm). Five animals shed exclusively *E. punoensis*, seven shed exclusively *E. alpacae*, and two had mixed infections. The *Eimeria* infections were not associated with obvious clinical signs. To determine the presence of *Cryptosporidium* and *Giardia* species and genotypes, DNA was extracted from feces and subjected to PCR utilizing specific primers for the ssu-rRNA gene for both parasites. All PCR positive samples were further analyzed by DNA sequencing to identify the species or genotypes that were present. Assemblage A, *G. duodenalis* was detected in fecal samples from two alpacas on MD-1 and in one alpaca on MD-2. Assemblage E, *G. duodenalis* and *Cryptosporidium* spp. were not detected on either farm. Although the prevalence on these two farms was low, alpacas can harbor zoonotic *G. duodenalis*, and this should be borne in mind by persons interacting with the animals.

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**Keywords**: *Giardia; Cryptosporidium; Eimeria; Alpaca; Camelid; Prevalence*

1. Introduction

Despite the widespread importance of the domesticated camelids, alpacas (*Lama pacos*, syn. *Alpaca pacus*) and llamas (*Lama glama*) in South America, and their growing numbers in North America, there are relatively few reports on the prevalence of economically important protozoan parasites in these animals. Conversely, for other large domesticated animals such as cattle, sheep, and pigs, the presence of pathogenic and zoonotic species of *Cryptosporidium* and *Giardia* (Fayer et al., 2000; Thompson et al., 2000), as well as *Eimeria* has been studied to a much greater extent. However, identification of *Giardia* or *Cryptosporidium* has been complicated by the fact that many species and genotypes within these genera are morphologically indistinguishable (Fayer et al., 2000; Thompson et al., 2000). Molecular analysis is essential for identifying species and genotypes and characterizing their host ranges (Monis et al., 1999, 2003; Xiao et al., 2004). For South American Camelids (SAC) only a few studies have applied molecular methods to identify *Cryptosporidium* and *Giardia*, whereas somewhat more...
is known about the prevalence of *Eimeria* species which can be readily identified microscopically by the size and shape of the oocyst stage. Seven genotypes (assemblages) of *G. duodenalis* are currently recognized. Assemblages A and B have been reported in humans and a wide range of other mammals (Monis et al., 2003). Assemblage E appears to be restricted to hoofed-livestock and is highly prevalent in cattle (Monis et al., 2003; Trout et al., 2004, 2005, 2006). Although six species of *Cryptosporidium* have been identified in human infections, the majority are caused by *C. parvum* and *C. hominis* (Xiao et al., 2004; Caccio et al., 2005; Leoni et al., 2006). *C. parvum* is zoonotic and highly prevalent in young dairy calves, whereas *C. hominis* typically exhibits a human to human cycle, although animal infections have been reported (Morgan-Ryan et al., 2002; Santín et al., 2004; Smith et al., 2005; Park et al., 2006).

*Giardia* spp., isolated from an asymptomatic young llama in Wisconsin, was established in culture and was infectious for Mongolian gerbils (Kiorpes et al., 1987). In California 3.4% of 354 llamas were positive for *Giardia* spp.; most were 1–4-month-old crias (Rulofson et al., 2001). Analysis of diarrheic feces collected from un-weaned SAC crias in Oregon revealed 18% were *Giardia* spp. positive and 9% were *Cryptosporidium* spp. positive (Cebra et al., 2003). In Ohio, data from 58 clinical cases of diarrhea in alpacas less than 4 months of age revealed that 26% and 33% of the crias were positive for *Cryptosporidium* spp. and *Giardia* spp., respectively (Whitehead and Anderson, 2006). In England, *Cryptosporidium* sp. infection in alpaca crias was associated with diarrhea and in three cases, with development of a fatal enteritis, although asymptomatic individuals were also identified (Bidewell and Cattell, 1998; Twomey et al., 2007). Although such infections have been documented by microscopic analysis, molecular data on the species and genotypes of these parasites in alpacas is lacking. Data obtained from isolated cases suggest that alpacas can harbor both Assemblage A and E, *G. duodenalis* (Ey et al., 1997) as well as *C. parvum* (Morgan et al., 1998; Ryan et al., 2003; Starkey et al., 2007). Additionally, a recent report of a *Cryptosporidium* outbreak in alpacas and their handlers identified *C. parvum* in four crias and provided evidence of zoonotic transmission (Starkey et al., 2007).

Six *Eimeria* species have been reported in SAC: *E. alpacae*, *E. ivitaensis*, *E. lamae*, *E. macusaniensis*, *E. punoensis*, and *E. peruviana* (Yamikoff, 1934; Guerrero, 1967; Guerrero et al., 1971; Rickard and Bishop, 1988; Schrey et al., 1991; Leguía and Casas, 1998; Jarvinen, 1999). *Eimeria lamae* and *E. macusaniensis* have been reported to cause clinical pathology in SAC (Guerrero et al., 1970; Cebra et al., 2007), with *E. macusaniensis* causing severe infections in crias (Cebra et al., 2007). Although the pathogenic potential of *E. ivitaensis* is unclear, co-infection with *E. macusaniensis* resulted in the deaths of seven young alpacas in Peru (Palacios et al., 2006). *Eimeria punoensis* and *E. alpacae* have not been associated with any pathology in experimental infections of llamas greater than 1 year of age (Foreyt and Lagerquist, 1992), however, clinical disease attributed to *E. punoensis* infection has been reported in British alpacas (Schock et al., 2007).

The current study was undertaken to determine the prevalence of these three economically important genera of protozoan parasites in alpacas on two farms in Maryland using microscopy and molecular methods to detect and identify the species and genotypes.

2. Materials and methods

2.1. Alpaca farms

Two alpaca farms in Maryland were selected, based on their willingness to participate and the ability to provide a minimum of 25 animals for sample collection. The alpacas from which samples were collected ranged from 10 weeks to 10 years of age on farm MD-1 and from 12 weeks to 10 years of age on farm MD-2. The farms raised both suri (silk-haired and huacaya (crimpy-haired) breeds. Females and crias were housed together on pasture, whereas older/breeding males were maintained on separate pastures. All animals had free access to barns for shelter. Alpacas were routinely treated with Dectomax®, Safe-Guard®, and Ivomec Plus®.

2.2. Sample collection and processing

The owners preferred not to perform direct fecal collections. Therefore, animals were observed and upon defecation, the sample was immediately collected from the ground into a screw top specimen cup and labeled with the animal’s identification. Specimens were placed on ice and held until all samples had been obtained. After all samples had been collected, they were transported on ice to the laboratory. Prior to processing, samples were examined and any abnormalities (e.g. diarrhea) were noted. Samples were processed as previously described (Trout et al., 2004). Briefly, 15 g of feces from each sample were placed into a 50-ml screw top tube and 35 ml of dH2O were added. The contents were thoroughly mixed to disperse the fecal material.
and the contents were passed through a 45-μm screen. The screened material was collected into a second 50-ml tube and the volume adjusted to 50 ml with dH2O. The tubes were centrifuged at 1800 × g for 15 min; the supernatant was discarded and the pellet was suspended in 25 ml of dH2O. Twenty-five milliliters of CsCl (1.4 g/ml) was added to each tube, the contents were mixed thoroughly and the tubes were centrifuged at 300 × g for 20 min. Following centrifugation, the top 4 ml of supernatant were aspirated from each tube and transferred into a 15-ml tube and 11 ml of dH2O was added. Portions of this pellet were examined by immuno-fluorescence and differential interference contrast (DIC/Nomarski) microscopy and molecular analysis as described below.

2.3. Microscopic examination

A 100-μl aliquot of the fecal suspension was transferred to a microcentrifuge tube and washed once with dH2O; the resulting pellet was suspended in 50 μl of premixed MerIFluor® reagents (Meridian Bioscience, Cincinnati, OH) and 2 μl was transferred to a three-well (11-mm diameter) glass microscope slide (Erie Scientific, Portsmouth, NH). The slide was covered with a 24 mm × 50 mm glass coverslip and examined at 400× on a Ziess Axioskop equipped with epifluorescence and a FITC-TexasRed® dual wavelength filter. The samples were further examined at 400× using DIC optics on the same microscope to determine if *Eimeria* spp. were present. Five *Eimeria* spp. oocysts were measured from each positive sample using an ocular micrometer and brightfield optics. If different sized oocysts were noted a minimum of five of each size were measured per sample.

2.4. DNA extraction

Total DNA was extracted from each CsCl-cleaned fecal sample using a DNeasy Tissue Kit (Qiagen, Valencia, CA) with a slightly modified protocol. The protocol, described below, utilized reagents provided by the manufacturer. A total of 50 μl of processed feces were suspended in 180 μl of ATL buffer and thoroughly mixed by vortexing. To this suspension, 20 μl of proteinase K (20 mg/ml) was added, and the sample was thoroughly mixed. Following an overnight incubation of the mixture at 55 °C, 200 μl of AL buffer was added. The remaining protocol followed manufacturer’s instructions with one exception. To increase the quantity of recovered DNA, the nucleic acid was eluted in 100 μl of AE buffer.

2.5. Polymerase chain reaction and DNA sequence analysis

Samples were subjected to PCR directed at fragments of the ssu-rRNA gene for both *Cryptosporidium* and *Giardia*, using primers and protocols previously described (Hopkins et al., 1997; Xiao et al., 1999; Santin et al., 2007). The PCR products were subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. Negative and positive controls were included for all PCR runs.

PCR products were purified using EXO-SAP-IT™ (USB Corporation, Cleveland, OH). Purified products were sequenced with the same PCR primers used for the original amplification in 10 μl reactions, Big Dye™ chemistries, and an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, CA). Each sample was sequenced in both directions. Sequence chromatograms from each strand were inspected and aligned using Lasergene software (DNASTAR, Inc., Madison, WI).

3. Results

Twenty-six and 35 fecal samples were obtained from farms MD-1 and MD-2, respectively. MD-1 had 6 animals <1 year of age, 8 animals 1–3 years of age and 12 animals >3 years of age; MD-2 had 8 animals <1 year of age, 10 animals 1–3 years of age and 17 animals >3 years of age. The results of the fecal analysis are presented in Table 1. Neither *Cryptosporidium* nor

<table>
<thead>
<tr>
<th>Farm</th>
<th>E. alpaca</th>
<th>E. punoensis</th>
<th>Mixed Ea./Ep.</th>
<th><em>Cryptosporidium</em> spp.</th>
<th><em>Gi. duodenalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>MD-1</td>
<td>26</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>MD-2</td>
<td>35</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers represent number of animals.
Giardia was detected by immuno-fluorescent microscopy. PCR analysis detected three samples that were positive for Giardia, but none that were Cryptosporidium positive. DNA sequence analysis revealed that the three Giardia positive samples were Assemblage A, G. duodenalis. These positive samples were obtained from 14- and 27-month-old females on MD-1 and from a 13-week-old male on MD-2. The nucleotide sequence of the ssu-rRNA gene Assemblage A, G. duodenalis has been deposited in GenBank under the accession number EU562195.

Eimeria spp. oocysts were detected in six animals on MD-1 and in eight animals on MD-2. The oocysts could be grouped into two size ranges, the smaller oocysts averaged 19.2 \( \mu \text{m} \times 16.5 \mu \text{m} \) and the larger oocysts averaged 23.7 \( \mu \text{m} \times 19.5 \mu \text{m} \). These sizes correspond to the descriptions of E. punoensis (19.9 \( \mu \text{m} \times 16.4 \mu \text{m} \)) and E. alpacae (24.1 \( \mu \text{m} \times 19.6 \mu \text{m} \)), respectively (Guerrero, 1967). Three alpacas on MD-1 had exclusively E. punoensis; two had exclusively E. alpacae, and one had a mixed infection; infected animals ranged in age from 12 weeks to 27 months. One of the alpacas infected with E. punoensis was also infected with Assemblage A Giardia. On MD-2, two alpacas had exclusively E. punoensis; five had exclusively E. alpacae, and one had a mixed infection; infected animals ranged in age from 13 weeks to 5 years, although 6 of the 8 were <2 years old. Only one alpaca on either farm had diarrhea, a 9-year-old female, however, this animal was negative for the presence of these parasites, and the etiology of the diarrhea was unknown.

4. Discussion

In the current study, E. alpacae and E. punoensis were detected in 13.1% and 9.8%, respectively, of 61 alpacas ranging in age from 12 weeks to 5 years; two animals (3.3%) were concurrently infected with both species. Although five of the infected animals were less than 6 months of age, neither these juveniles nor the adults had any clinical signs associated with the infection. This supports previous data suggesting that E. punoensis and E. alpacae do not appear to be pathogenic, even in younger animals (Foreyt and Lagerquist, 1992). Conversely, another recent report suggests that E. punoensis infection resulted in clinical disease in British alpacas (Schock et al., 2007).

Giardia sp. and Cryptosporidium sp. have been reported in llamas and alpacas (Kiorpes et al., 1987; Rulofson et al., 2001; Cebra et al., 2003), occasionally resulting in fatal infections (Bidewell and Cattell, 1998). Previously, both Assemblage A and E, G. duodenalis have been reported in alpacas (Ey et al., 1997); additionally, the Giardia sp. isolate by Kiorpes et al. (1987) was infectious for Mongolian gerbils, suggesting it was a zoonotic genotype. In the current study Assemblage A, G. duodenalis was detected in 3 of 61 animals (4.9%); Cryptosporidium spp. was not detected on either farm nor was Assemblage E, G. duodenalis. Additionally, the lack of clinical signs in the infected animals suggests that Giardia is not necessarily pathogenic in alpacas, although both Giardia and Cryptosporidium infections accompanied by diarrhea have been documented (Bidewell and Cattell, 1998; Cebra et al., 2003).

The alpaca farms in the current study were not in close proximity to other livestock operations, which could have limited exposure to potential sources of parasites. These alpacas were also treated with Safeguard (fenbendazole) at least twice annually; fenbendazole has been reported to have anti-giardial activity in cattle (O’Handley et al., 1997, 2000) and thus could have suppressed Giardia infections in these animals. Previous studies also suggested that infections were more common in young alpacas and llamas (Rulofson et al., 2001; Cebra et al., 2003); this is true of Giardia and Cryptosporidium infections in cattle as well (Santín et al., 2004; Trout et al., 2004, 2005, 2006). In cattle, C. parvum infections peak at about 2 weeks of age and are virtually absent after 8 weeks of age (Santín et al., 2004); Giardia infections exhibit a broader peak between 4 weeks and 4 months of age (Trout et al., 2004, 2005, 2006). The youngest alpaca in the current study was 10 weeks of age, thus it is possible that the observed absence of C. parvum infection and the low levels of Giardia infection were due to age-related changes in prevalence. Clearly, additional studies sampling more farms and including younger animals are needed to obtain accurate prevalence data.

Alpacas are raised in the U.S. primarily for fiber production and thus are routinely handled by humans in order to clip and collect the coats. Such close association could result in passage of Assemblage A, G. duodenalis from the animals to their handlers. Thus although the current study did not find high number of infected animals, alpacas should be considered potential sources of human-infective G. duodenalis, and appropriate precautions should be considered when interacting with the animals. Although it does not appear that alpacas are likely to represent a large source of infectious Cryptosporidium or Giardia for humans or other livestock, until molecular data on younger crias is obtained, the potential for SAC to serve as significant
reservoirs for these parasites cannot be determined with certainty.

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