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Research paper

Prevalence and genotyping of *Giardia duodenalis* isolated from sheep in Henan Province, central China

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ABSTRACT

Giardia duodenalis is a gastrointestinal protozoan that infects sheep. It is a well-known zoonotic pathogen and sheep have been implicated as a source of human infection. However, there have been few studies of its potential threat to public health in China. We used a multilocus analysis of the beta-giardin (*bg*), glutamate dehydrogenase (*gdh*), and triose phosphate isomerase (*tpi*) genes to examine the occurrence and genotype distribution of *G. duodenalis* in sheep in China. In total, 716 fresh faecal specimens, including 89 from pre-weaned lambs (<3 months old) and 627 from post-weaned sheep (>3 months old) from nine intensive sheep farms in Henan Province, China, were examined for *Giardia* cysts with microscopy. Of these specimens, 6.65% (47/716; 99% CI: 6.2%–6.92%) from five farms were positive for *G. duodenalis*. The infection rate was significantly higher in pre-weaned lambs than in post-weaned sheep (12.36% versus 5.74%, respectively; $P < 0.05$). Infection rates ranged from 2.8% to 17.2% on the *G. duodenalis*-positive farms. All *G. duodenalis*-positive samples were assayed with PCR followed by sequencing at the three gene loci investigated in this study. As a result, two assemblages were detected: assemblage A ($n = 5$) and assemblage E ($n = 31$), with some mixed E and A infections ($n = 3$). The ratios of assemblage A to assemblage E on the different successfully sequenced *G. duodenalis*-positive farms were 0:1, 3:14, 1:4, and 1:12. Two new *tpi* sequences and one new *gdh* sequence were identified. Multilocus genotyping yielded seven multilocus genotypes (MLGs): one new assemblage A MLG and six assemblage E MLGs. In the phylogenetic analysis, the assemblage A MLG was more closely related to AI than to AII or AIII. The detection of *G. duodenalis* assemblage A in sheep has public health implications, although *G. duodenalis* assemblage E was predominant. The data provide basic information for control of giardiasis in human and sheep in Henan province, central China.

1. Introduction

Giardia duodenalis (syn. *Giardia lamblia*, *Giardia intestinalis*) is a gastrointestinal protozoan that infects sheep. The most common clinical symptoms associated with *G. duodenalis* are enteritis, including the excretion of malodorous, loose to diarrhoeic faeces, reduced weight gain, and impaired feeding efficiency, especially in lambs (Aloisio et al., 2006). As well as its veterinary relevance, this parasite is a zoonotic pathogen and animals can contribute significantly to the contamination of watersheds. Because of the unexpectedly high levels of infection in sheep, sheep have long been considered a reservoir of human infections (Giangaspero et al., 2005; Ryan et al., 2005; Ozmen et al., 2006; van der Giessen et al., 2006).

To date, eight major morphologically similar but genetically distinct assemblages of *G. duodenalis* have been identified, assemblages A–H, two of which (A and B) are found in both humans and animals, whereas the remaining six (C–H) seem to specifically infect nonhuman host species. However, assemblages C, D, E, and F have also been isolated from infected humans, but at very low prevalence (Feng and Xiao, 2011; Ryan and Caccio, 2013). Studies of sheep have identified a predominance of *G. duodenalis* assemblage E, with assemblage A occurring infrequently (Geurden et al., 2008; Giangaspero et al., 2005; Monis and Thompson, 2003; Ryan et al., 2005; Santin et al., 2007; Van der Giessen et al., 2006, and assemblage B is rarely found in sheep (Aloisio et al., 2006; Castro-Hermida et al., 2007a; Lebbad et al., 2010; Robertson et al., 2010).

Studies of the prevalence and genotypes of *G. duodenalis* in sheep have been performed worldwide. However, there have been only three reports of genotyping data for sheep giardiasis in China (Liu et al., 2014; Ye et al., 2015; Zhang et al., 2012), so data are lacking for China, where sheep rearing is an important zootechnical activity. Previous investigations based only on a single locus or two loci provide insufficient information on the characterization of the transmission of human giardiasis (Wielinga and Thompson, 2007; Caccio and Ryan, 2008). Thus far, multilocus genotyping (MLG) tools are increasingly

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used to characterize *G. duodenalis* isolates from humans and animals. The present study was aimed to determine the distribution of *G. duodenalis* genotypes and subtypes based on multi-locus analysis of the *tpi*, *gdh* and *bg* genes to better understand the endemic genetic characteristics of sheep-derived *G. duodenalis* isolates in Henan Province, central China.

2. Materials and methods

2.1. Ethics statement

This study was performed strictly according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. Our protocol was reviewed and approved by the Research Ethics Committee of Henan Agricultural University (approval no. LVRIAEC 2014-011). All faecal specimens were collected from animals after permission was obtained from the farm owners, and no specific permits were required to authorize the collection of these specimens.

2.2. Sample collection and microscopy

During the period from July 2009 to June 2010, 716 faecal samples were randomly collected from nine sheep farms in Henan Province, China: 89 from pre-weaned lambs (<3 months old) and 627 from post-weaned sheep (>3 months old). The pre-weaned lambs and post-weaned sheep were bred in different stalls, and every stall had its own automatic water supply system. All the sheep sampled were native Hu sheep. Only intensive commercial farms were included. The nine farms were from various cities in which sheep-rearing activities are focused: Xinxiang (farms 4, 8, and 9), Jiaozuo (farm 7), Pingdingshan (farm 6), Zhengzhou (farms 1 and 2), Anyang (farm 5), and Nanyang (farm 3) (Table 1), with 100–1000 sheep per farm. The farms were visited on a single occasion and the faecal samples were randomly collected from at least 20% of the animals. The samples were collected directly and immediately from fresh faeces deposited on the ground after defecation by the animals. Care was taken to avoid sampling faecal material that had contacted the ground. At the time of faecal collection, no diarrhoea was apparent in the herds. The fresh faeces were placed into clean plastic bags marked with the date, age of the sheep, and city of origin, and were then transported immediately to the laboratory and stored at 4 °C. The faecal samples were examined microscopically within 24 h of collection using the following procedure. A subsample (5–10 g) of faeces was suspended in distilled water (30–40 mL) and strained through a layer of surgical gauze to remove large debris. After centrifugation three times at 3000 ×*g* for 10 min, the sediment was resuspended in 1 mL of distilled water. Each faecal specimen was directly smeared onto a clean slide for iodine wet-mount staining. The wet smears were examined for *G. duodenalis* cysts (12–14 μm in length and 9–11 μm in width, with an axostyle and nucleus) with microscopy at 400× magnification. An animal was considered positive if a *G. duodenalis* cyst with the correct morphology was detected in the sample. All *G. duodenalis*-positive whole faecal specimens were stored at 4 °C in less than 24 h until molecular characterization.

2.3. Molecular characterization

DNA was extracted from all *G. duodenalis*-positive faecal specimens using the E.Z.N.A.R® Stool DNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA), according to the manufacturer's instructions. To increase the quantity of DNA recovered, the extracted DNA was eluted in 100 μL of AE elution buffer and stored at −20 °C.

Fragments of ~511 bp of *bg* (Lalle et al., 2005), ~520 bp of *gdh* (Caccio et al., 2008), and ~530 bp of *tpi* (Sulaiman et al., 2003) were individually amplified with the protocols cited above. The PCR reactions for the *tpi* and *gdh* loci were conducted in 25 μL reaction mixtures containing 1× rPCR buffer (Takara Shuzo Co., Ltd., Otsu, Japan), 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Takara Shuzo Co., Ltd), 0.4 μM each primer, 1 unit of rTaq DNA polymerase (Takara Shuzo Co., Ltd), 2 μL of DNA sample, and 1 μL of bovine serum albumin. To amplify the *bg* locus, Ex Taq DNA Polymerase (Takara Shuzo Co., Ltd) was used. Positive (cattle DNA) and negative (distilled water) control samples were included with each PCR reaction. Amplification was performed in triplicate, and the amplification products were separated with electrophoresis in 1.5% agarose gels with ethidium bromide and visualized on a UV transilluminator.

2.4. Sequence and phylogenetic analyses

All secondary PCR amplicons were sent to Beijing Nuosai Biological Engineering Biotechnology Company for two-directional sequencing using an ABI PRISM™ 3730 XL DNA Analyser (Applied Biosystems, Foster City, CA, USA). The sequences obtained were genotyped and subtyped with a multiple-sequence alignment analysis together with reference sequences retrieved from the GenBank database, using ClustalX 1.83 (http://www.clustal.org/).

For the phylogenetic analysis, the sequences from isolates with complete data at all three loci were concatenated to form one multilocus sequence for each isolate, and compared with the multilocus sequences from reference isolates downloaded from GenBank. A neighbour-joining (NJ) analysis was performed in MEGA 5.0 based on the Tamura–Nei model (Tamura et al., 2011). The consensus trees were constructed after bootstrap analyses with 1000 replications.

2.5. Statistical analysis

Differences in infection rates between age groups were compared with the χ^2 test in SPSS for Windows (Release 13.0 standard version; SPSS Inc., Chicago, IL, USA). Differences with $P < 0.05$ were considered significant.

2.6. Nucleotide sequence accession numbers

All the nucleotide sequences determined here were submitted to the National Center for Biotechnology Information GenBank database under the following accession numbers: KP635099–KP635106 for the *tpi* gene, KP635107–KP635111 for the *gdh* gene, and KP635112–KP635115 for the *bg* gene.

3. Results

3.1. Prevalence of G. duodenalis

Of the 716 faeces sample tested, 47 (6.56%, 99% CI: 6.2%–6.92%) were positive for *G. duodenalis* on microscopic examination, and infected animals were detected on five of the nine sheep farms examined. Table 1 presents the different prevalence rates of *G. duodenalis* infection on the nine intensive sheep farms. Pre-weaned lambs were more often infected with *G. duodenalis* than were post-weaned sheep, with infection rates of 12.36% (11/89, 99% CI: 10.38%–14.34%) and 5.74% (36/627, 99% CI: 5.36%–6.12%), respectively ($P < 0.05$).

3.2. Sequence analysis

A total of 77 sequences were obtained and analysed in this study: 32 of the *tpi* gene, 25 of the *gdh* gene, and 20 of the *bg* gene. The sequence analysis revealed the presence of two assemblages of *G. duodenalis*: assemblage A in five isolates and assemblage E in thirty-one isolates.