Detection of the "Tim" gene of sheep *Giardia* using "Tim" Gene primers of *Giardia* with human origin

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Abstract

Giardiasis is an important human parasitic disease. *Giardia* is a genus composed of binuclear flagellate protozoa. *Giardia duodenalis* is a parasitic species for a wide range of vertebrates, including humans. Heterogeneity in *G. duodenalis* has been shown by serological, biochemical, and molecular analysis. In the present study, the possible genetic similarity between *Giardia* in sheep and humansand their probable zoonosis was investigated. Direct examination and formalin ether concentration technique were performed on the contents and tissues of sheep intestines. The gradient sucrose method was applied to collect and purify cysts, and DNA extraction was performed by the phenol-chloroform method. Only very small amounts of DNA could be extracted after repeated freezing, thawing and suspension with lysis buffer, after which polymerase chain reaction (PCR) was performed for DNA amplification by primers that were designed for *Giardia* of human origin. The gene, "triose phosphate isomerase" (tim or tpi), was selected as the molecular marker and two sets of primers (PM290, PM924) were used. We examined 308 sheep stool samples in our study, including 21 positive samples. Cultures for *Giardia* were negative. Three sheep isolates were determined by a 290 base pair (bp) amplicon that were similar to certain human types. The similarity of the sheep and human genomic characters of *Giardia* implies the possibility that there is transmission of these protozoa between humans and sheep.

Introduction

The Giardia genus is a flagellate binuclear protozoa parasite for many vertebrates, including human beings (Mayrhofer *et al*., 1995). This parasite has two phases in its life cycle, in which the flagellate trophozoite is the active form and is responsible for the clinical manifestations. The second form is infectious cysts that are excreted with stools and persist in the environment for several weeks (Slavin *et al*., 2002). Infections occur via contaminated food and water. Therefore, this is potentially the most important causative agent for childhood diarrhea, traveler's diarrhea and diarrhea in homosexuals (Lujan *et al*., 1998). Giardiasis accounts for an important parasitic disease of humans and extensive studies on different aspects of this disease have been conducted so far.

Approximately 200 million cases worldwide are infected by this parasite and there are in the region of 500,000 new cases diagnosed each year. Therefore, the precise and systematic understanding of *Giardia* will be very helpful for the more accurate recognition of its disease pattern, drug sensitivity monitoring and treatment, virulence and infectivity, zoonotic identification, and disease control. However, major defects still exist in our knowledge about biological characteristics of this organism and its consequences in disease. One of the most important unresolved issues in this field is the role of animals as carriers, and whether animal-specific species of *Giardia* are similar to species that infect humans (Robertson, 2006). Recently, attention has become focused on the determination of these species. To date, the determination of the genus based on genetic approaches is difficult and the identification of the species is based on morphology (Thompson and Lymbery, 1990). In 1952, based on morphological characters, Filice confirmed only three species within the genus:

1) *G. agilis* in cold-blooded animals with a club-like median body parallel to its longitudinal axis.
2) *G. muris* in rodents with two small globular median bodies.
3) *G.duodenalis* in mammals with one or two transverse hammer-like median bodies.

Recently, two new species of bird parasites (*G.*

psittaci in budgerigars and *G. ardeae* in*the* great blue heron) have been detected. *G. duodenalis* has been found in mammals and some birds and is therefore important in medical and veterinary sciences (Thompson and Reynoldson, 1993). Genetic diversity has been demonstrated in *G. duodenalis* by means of serological, biochemical, and molecular genetic analyses (Baruch *et al*., 1996). Methods based on genetic diversity, including restriction fragment linked polymorphisms (RFLP) and sequence analysis, have categorized *G. duodenalis* into genotypes 1, 2, and 3 (Nash, 1992). Homan (1992) classified *G. duodenalis in*to "Belgian" and "Polish" isolates by means of isoenzyme electrophoresis and DNA probes (Homan *et al*., 1992). On this basis, Mayrhofer (1995) categorized *G. duodenalis* isolates into two major assemblages that included four genetic groups (Mayrhofer *et al*., 1995). Based on the last reports, *G. duodenalis* have been classified to three groups: group 1 (WB), group 2 (JH), and group 3 (GS) (Siqi *et al*., 2002).

The influence of zoonotic pathogens in animal on human health as a direct or indirect cause of human enteric illness is examined. Available international data have considered that the recent application of molecular characterisation procedures based on PCR has made an enormous contribution to understanding the genetic structure of the *Giardia* populations. The aim of the present pilot study was to establish if isolates of *Giardia* from sheep were similar to *Giardia* from humans with regards to the tim gene.

Materials and Methods

The contents of the small intestine, including duodenal tissue, of 308 slaughtered sheep (aged 6-12 months, average weight 25±15) were collected from the slaughterhouse in order to obtain trophozoites and cysts. In addition, the contents of the distal large intestine were collected to obtain cyst forms of parasite.

Microscopic examination was performed immediately after sampling in order to isolate viable parasites. At first, duodenal tissue was scraped to increase the likelihood of observing the trophozoites. Then, a direct smear was prepared using a drop of Ringers and Lugol's solution before direct examination. Negative samples from the direct examination were then tested using the formalin-ether concentration method (Allen *et al.*, 1970).

The determination of parasite viability was performed using 1% eosin (Vital stain). Samples with 10-15 cysts in each field of 40x magnification and a high rate of viable cysts were suitable for culture.

The collection and purification of cysts was performed using the sucrose gradient method. To inhibit bacterial contamination, a mixture of antibiotics (Penicillin G :100-500 IU/ml , Streptomycin:100-500 Mg/ml ,Gentamycin:50 Mg/ml) was added and then the samples were washed twice daily during a 48 hr period according to previously published reports (Meyer, 1976). Slides of all isolates were then prepared by means of trichrome staining.

Unsuitable samples for culture were collected in microtubes and kept at -70ºC after the washing phase. Samples for culture were incubated in 0.1 M HCl (pH=2) in 1/9 ratio or trypsin for some cases 60 min at 37ºC and 5 min at 56ºC to prepare cysts for excystation. After washing, cysts were added to TYI-S-33 culture media in sterile conditions and after two hours we examined them under an inverted microscope(Zeiss, Axiovert 35M) for excystation. Daily examinations continued for up to two weeks (Phillips et al., 1984). In case of parasite proliferation, the tubes containing parasites were placed in ice in order to detach the trophozoites from tube walls. Samples were washed and kept in 70ºC for further molecular analysis.

For the first stage of the molecular phase, we extracted trophozoites with the use of the phenol-chloroform method (Sambook *et al*., 1989). For cysts, DNA extraction was accomplished by repeated cycles of freezing and thawing with lysis buffer for the suspension preparation. To confirm the success of DNA extraction, we ran some of the samples on agarose gels. In this investigation, the tim gene was assessed using polymerase chain reaction (PCR). PM290, a specific primer, was used to amplify a 290 bp amplicon (Table 1). In all cases, samples of human *Giardia* were used as positive controls. A gradient thermal cycler (Corbet Research, Australia) was used for PCR, and the program for these primers is shown in Table 2 (Zare Bavani., 2006). PCR products underwent electrophoresis on an agarose gel that was stained with ethidium bromide, and products were assessed by an ultraviolet transilluminator.

Table 1: Sequences of primers (PM290, PM924).

Primers	Forward (F)/Reverse (R)	Sequence (5'→3')
PM290	F	GCC ATT GCT GCC CAC AAG AT
PM290	R	GTC ATC CCC TTT TCT AGA GT
PM924	F	TCA TGC ACC GTG ATT TGG AC
PM924	R	AGT TGC TTC CAT TGG CCG AT

Table 2: PCR program for primer amplification in the thermal cycler.

Phase	Temperature/ ºC	Duration
1. Primary denaturing	94	240 seconds
2. PCR cycles (35 cycles):		
• Denaturing	94	30 seconds
• Annealing		
-PM290	52.5	30 cycles
-PM924	58.5	30 cycles
• Extension 72	30	
3. Terminal extension	72	30 seconds

Results

We examined 308 samples in our study, including 21 positive samples and three cases in which *Giardia* infection was suspected. Thick wall cysts were seen by trichrome staining. Excystation only occurred in one case Ovine cyst- eighth positive sample (OC-8) and even this case could not be established in culture medium. However, the viability of cultured cysts was 90-100%, but excystation did not occur.

DNA extraction from trophozoites that were directly collected from intestinal contents was performed easily. PCR was then accomplished and a 290 bp band was visible, which was the same as the human positive control in Ovine trophozoite- 11th positive sample (OT-11). DNA extraction from some of the cysts used for PCR was difficult to accomplish, and the OC-13 and OC-5 isolates had bands that were almost identical to the positive control with a 290 bp band.

However, for most cysts, we extracted either very little DNA or extraction could not be carried out at all. Even in the samples that DNA extraction could be performed, PCR with relevant primers was not possible.

Discussion

Although the parasites of *G. duodenalis* species are morphologically indistinguishable (Buret and Den Hollander, 1990), the slides prepared by means of trichrome staining in our study showed *Giardia* organisms with thicker walls and larger sizes in sheep isolates when compared to isolates from human.

Although cultures of sheep *Giardia samples* have been successful in some studies (Buret and DenHollander, 1990), there are limited reports of successful in vitro *Giardia* culture from sheep. In this study, we did not succeed in culturing these samples, which suggests that a suitable number of viable cysts is necessary for this to be successful. Appropriate changes are required in modified-TYI-S-33 media, which is used for culturing *Giardia* samples from humans. After DNA extraction, we ran samples on agarose gels to confirm DNA extraction, but in most cases there was either a paucity of DNA or DNA was absent. In these circumstances, the causative agent could have low parasite numbers from sheep isolates (infection in sheep is usually much milder than human cases), cyst walls are resistant to DNA extraction, therefore DNA extraction was unsuccessful. This became possible with repeated freezing and thawing and using lysis buffer. However, DNA extraction from trophozoites was performed easily when the same technique was used in human isolates. Some of cyst samples used for PCR had few cysts, with approximately one found per each field (x40); PCR could not be performed for them (OC-1, -3, -8). Some samples had many cysts (three or more in each field; x40) but it was not possible to extract DNA (OC-2, -4, -16, -20). It is possible that DNA was extracted, but the fecal material contained nucleic acid inhibitors that interfered with PCR.

DNA extractions from the OT-11 isolate, in which trophozoites had been collected directly from intestinal contents, was accomplished easily. However, in the first phase, high DNA levels prevented PCR and because of inhibitor existence in direct samples of sheep *Giardia* we had to decrease the concentration of template DNA to achieve a successful PCR.

In cases of weak bands (OC-5) obtained during electrophoresis we could also obtain *Giardia* amplicon by means of double PCR (Performing PCR from first PCR amplicon). All cases of successful PCR in isolates were amplified with PM290 and the PCR product was a 290 bp amplicon. Based on studies on human giardiasis (Zare Bavani, 2006), 46 out of 78 human cyst isolates could not be amplified by primers, which was probably because of DNA extraction insufficiency or the presence of an inhibitor; 16 isolates amplified by both primers, eight cases amplified only with PM924 primer, eight cases amplified only with the use of PM290 primer, but most of the latter cases amplified only once and their second amplification was not visible (Zare Bavani, 2006). On the basis of this information, the molecular evaluation in our study on 15 isolates was performed, and three cases amplified only with the use of the PM290 primer; 12 cases did not amplify and no amplification was seen by PM924. Therefore, three noted sheep isolates amplified with the PM290 primer; based on studies on human isolates and after referring to GenBank, these can be placed in assemblage A with accession numbers AC#L02120, AC#U57897. Therefore, it appears that some sheep isolates are similar to human isolates with regards to the tim gene (Santin *et al*., 2007).

This pilot study indicates that the "tim" gene of certain isolates of sheep *Giardia* is similar to human *Giardia*. In order to clarify the assemblage to which it belongs, RFLP is required. This study needs to be performed on a larger scale and the culture conditions should be optimized in order to obtain a higher concentration of *Giardia* for DNA extraction. Additionally, since the clinical symptoms of sheep and human are similar (Kiorpes *et al*., 1987) and because of it is a repeated finding to locate *Giardia* with a human origin from animals (usually domestic) with molecular methods, here we propose that there is a higher possibility of transmission of this parasite from animals to humans and vice versa (Van Keulen *et al*., 2002).

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