Coprologic and molecular prevalence of *Giardia duodenalis* from children in Tabuk City, Saudi Arabia

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Abstract. *Giardia* infection in children has been recognized as a significant cause of diarrhea in school children around the world. The objective of the present study was to determine the prevalence and genotypes of *Giardia duodenalis* in school children in Tabuk City, Saudi Arabia. Fecal samples from 200 children with ages ranging from 9 to 12 years (106 boys and 94 girls) were examined for the presence of *Giardia* cysts using direct microscopy and ELISA. DNA was extracted from fecal samples and subjected to polymerase chain reaction (PCR) using primers which amplified the partial 18S rRNA locus. Direct microscopy revealed that 9 (4.5%) were positive for *Giardia* cysts. ELISA and PCR revealed 20 (10%) and 44 (22%) of the samples were positive respectively. Assemblage A was detected through genotyping of *Giardia duodenalis* isolates prevalent in Tabuk City through sequencing the fragment obtained as a result of amplifying partial 18S rRNA locus.

INTRODUCTION

Giardia duodenalis is the most prevalent cause of parasitic diarrhea in the developed world, and this infection is also very common in developing countries (Auerbach, 2012). There is an association of *Giardia* species and strain assemblages with diarrhea (Haque, 2007). Transmission of *G. duodenalis* cyst to humans occurs mainly after ingestion of contaminated water, autoinfection, and person-to-person contact. In 2013, there were about 280 million people worldwide with symptomatic giardiasis (Esch & Peterson, 2013). Clinical manifestations of symptomatic giardiasis include greasy stools, flatulence, diarrhea, abdominal cramps, epigastric tenderness, and steatorrhea accompanied by full-blown malabsorption syndrome (Gardner & Hill, 2001). The majority of cases are asymptomatic or minimally symptomatic in immuno-competent individuals (Furness *et al.*, 2000).

There are at least 6 species of *Giardia* recognized based on morphology as well as ultrastructural and genetic characteristics. These species include; *G. intestinalis (G. lamblia)*, *G. agilis*, *G. muris*, *G. ardeae*, *G. psittaci*, and *G. microti* (Monis *et al.*, 1999). Among the 6 species, only *G. intestinalis* infects humans and numerous other mammals (Thompson *et al.*, 2000). Isolates of *G. intestinalis* are classified into 7 assemblages, based on the characterization of the glutamate dehydrogenase, small-subunit rRNA, and triosephosphate isomerase (tpi) genes (Hopkins *et al.*, 1997; Monis *et al.*, 1999; Sulaiman *et al.*, 2003; Read *et al.*, 2004). Assemblage A isolates have been further placed into subgroups I and II. Assemblage B isolates have been separated into subgroups III and IV (Thompson *et al.*, 1994; Monis *et al.*, 1999). The issue of reclassifying assemblages A to G as separate *Giardia* species, based on genetics and host specificity data, which was proposed has not

been resolved yet (Monis *et al.*, 2009). It was assumed that sub-assemblage AI was the only zoonotic and sub-assemblage AII occured only in humans, however, recently it has been found that sub-assemblage AII has a wider host range as well (Sprong *et al.*, 2009). An additional assemblage AIII has recently been characterised as non zoonotic strictly infecting wild angulates (Lalle *et al.*, 2007). Other assemblages C, D, E, F, and G seem to be restricted to domestic animals, livestock, and wild animals (Adam, 2001). At present, antigen detection immunoassays for *Giardia* species are used as a diagnostic procedure (Zimmerman and Needham, 1995; Garcia and Shimizu, 1997; Johnston *et al.*, 2003), but these methods are unable to differentiate between the genetic assemblages of *G. intestinalis*. However, molecular detection methods based on polymerase chain reaction (PCR) have been developed to detect *G. intestinalis* cysts in faeces (Nash *et al.*, 1985). These molecular techniques allow for the genotyping of *G. intestinalis* cysts (McGlade *et al.*, 2003).

In Saudi Arabia, *Giardia* infection was among the common gastro-intestinal parasites detected from children from different regions in Saudi Arabia (Kasim and Elhelu, 1983; Omar *et al.*, 1996). Ahmad and Bolbol (1989) detected trophozoites of *G. duodenalis* in healthy and symptomatic children in Riyadh city, while Alharthi and Jamjoom (2007) reported prevalence of 6% in Makka city. El-Badry *et al.* (2010) and Abas *et al.* (2011) found 11.9% and 1.9% prevalence of *G. duodenalis* in children in Almadinah, respectively using the antigen ELISA method. In the south western region Alayed *et al.* (2013) reported a prevalence of the organism in 0.9% of the organism from Najran city. Al-Mohammed (2011) studied the prevalence of *Giardia* in children in Alhasa region and he characterised assemblages A and B from humans using intergenic spacer region (IGS) and he incriminated assemblage B as causing the disease in humans. Despite several studies conducted on *G. duodenalis* in different regions in Saudi Arabia, none has been dealt with the prevalence of the organism in Tabuk city in the northern Saudi Arabia. Therefore, the present study was conducted in order to determine the prevalence of *G. duodenalis* in school children from Tabuk City, Saudi Arabia. Furthermore the genotype of the *Giardia* isolates detected in the present study was also determined.

MATERIALS AND METHODS

Sample collection

Faecal samples were collected from 200 (106 boys and 94 girls) school children aged 9-12 years at Tabuk City in the northern part of Saudi Arabia. All samples were screened immediately for cysts at Tabuk General Hospital by direct microscopy using the direct wet smear stained with Lugol's iodine followed by formalin acetyl acetate concentration technique (Farthing, 1996; Garcia, 2001). The microscopic examination was performed 3 times on each sample for confirmation. The criteria for positive *Giardia* were the recovery of active motile flagellated trophozoites and thick hyaline wall of cyst stages. Part of each sample was then frozen and transported to the laboratory at the Department of Zoology, College of science, Girls Section, King Saud University. Samples were kept frozen at -20ºC for ELISA and Polymerase Chain Reaction (PCR) analyses.

The ethical considerations were addressed by prescribing appropriate medication for the children with positive results under the supervision of doctor and nurse working at the hospital. The objective of the present study was explained carefully to the children guardians; and oral consent was sought from parents or guardians of the selected individuals for stool sample collection. The study was approved by the research committee of the Department of Zoology, College of Science, King Saud University.

Faecal immunoassay

In the laboratory, the antigen (Ag)-antibody (Ab) reaction used in the current study is an immunoassay for the qualitative determination of *Giardia* antigen in feces. It is a double Ab (sandwich) ELISA (Novom Diagnostica, Germany) using an anti-*Giardia* Ab to capture the Ag from the stool

supernatant. Faecal samples were diluted 1 in 4 and used for the ELISA alongside with the positive and negative controls provided with the kit. The reaction was left to incubate at room temperature ($23º - 25ºC$) for 30 minutes before the microtitre plates were washed and the *Giardia* polyclonal antibodies were added and the reaction was incubated for 5 minutes before it was washed again, the chromogen (TMB) was added and incubated for another 5 minutes, the reaction was then stopped by adding 1M phosphoric acid. The reaction was read on microplate reader at 450 nm. The interpretation of results was dependant on the optical densities (OD) of the positive and negative controls. The positive control OD value must not be less than 0.5 units while that of the negative control must be lower than 0.15 units. Any sample giving OD value more than 0.15 units was considered positive and lower than 0.15 units was considered negative.

DNA extraction and Polymerase Chain Reaction (PCR)

DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGen, Hilden, Germany) according to the manufacturer's instructions with slight modifications. The template used for DNA extraction was the suspension prepared for the ELISA test. A nested PCR protocols were used for the amplification of the 18S ribosomal DNA (rDNA) region (Hopkins *et al.*, 1997; Applebee *et al.*, 2003) from *Giardia*.

The 18S rDNA PCR protocol utilized the initial primers Gia2029 (5'-AAGTGT GGTGCAGACGGACTC-3') and Gia2150c (5'-CTGCTGCCGTCCTTGGATGT-3') which amplify a DNA fragment of 497 bp amplicon, and secondary primers RH11 (5'-CATCCGG TGGATCCTGCC-3') and RH4 (5'-AGTCGAA CCCTGATTCTCCGCCAGG-3') generating a DNA fragment of 292 bp. The PCR mix consisted of 1× buffer containing 2.0 mM $MgCl_2$, 200 µM of each dNTP, 5% DMSO, 10 pmol of each primer, 5 units of Taq DNA polymerase (Bioline, London, UK), and 2–5 µl of purified DNA in a final volume of 50 µl. PCR was performed as follows: after an initial denaturation step of 2 min at 92ºC, a set of 35 cycles was run, each consisting of 30s at 92ºC, 30s at 58ºC and 45 s at 72ºC, followed by a final extension of 5 min at 72ºC. The sequential nested-PCR cycle was the same except in the second round PCR, the annealing temperature was reduced to 55ºC. The second round PCR product (292 bp) was visualised on 2% agrose gel electrophoresis after staining with ethidium bromide after exposed to UV light from a transilluminator.

DNA sequencing and sequence alignment

PCR products from 10 samples of the second round were cleaned after cutting from the gel using Qiagen gel extraction kit (QIAgen, Hilden, Germany). DNA sequencing was performed by Macrogen (Seoul, South Korea) using an ABI 3130 DNA sequencer. DNA Sequences were aligned using (BioEdit), biological sequence alignment editor (Hall, 1999). A representative DNA sequence was deposited at the Genbank (Accession number KT844470).

Calculation of sensitivity and specificity of tests

Specificity and sensitivity of the tests were calculated according to the following formulae:

Sensitivity %:
(Positive samples on direct microscopy and ELISA/Positive samples on ELISA) X100

Specificity %:
(Negative samples on direct microscopy and ELISA/Negative samples on ELISA) X100

Specificity and sensitivity of the PCR was also calculated according to the same formulae by including the PCR as variable.

Statistical analysis

Results were analyzed using Chi square test (X^2) using the computer program SPSS (Ver 15). Significant differences were indicated when the *p* value is less that 0.01 or 0.001.

RESULTS

Trophozoites of *Giardia* were detected in 9 (4.5%) samples by direct microscopy while the antigen of *Giardia* was detected in 20

(10%) of the samples in screened. The PCR products of *Giardia* DNA, however, was detected in 44 (22%) of the samples studied (Table 1). There was a significant difference between the results obtained using different tests (X^2 = 29.98, $p<0.0001$) with the PCR yielding the highest results. In direct microscopy, 6 of the positive samples were obtained from boys while 3 were obtained from girls, on antigen ELISA 10 from each of the boys and girls yielded positive results. Based on PCR results, 23 samples were from boys while 21 samples from girls. However, there was no significant difference in the prevalence of *Giardia* between boys and girls screened during this study (Table 1).

The sensitivity of the antigen ELISA with direct microscopy was found to be 94% and the specificity was 100%. Whereas the sensitivity of the PCR compared to the direct microscopy and the antigen elisa tests was found to be 82% and 65% respectively while the specificty was 100% for the PCR compared with both the direct microscopy and ELISA (Table 2).

The second round PCR revealed the desired product which was around 292 bp. Results obtained from the sequencing of 10 positive samples resulted in indetical sequences in all sequences genearted. Searching for identical sequences in the Genbank revealed that, the query sequences were identical to those obtained from sequenceing of the 18S rDNA of *Giardia duodenalis* assmelbage A (i.e sequences AF1113902, AF199446). They were different from sequences of *G. duodenalis* assemblages B, C and D (AF113897, AF113899 and AF113900) respectively.

DISCUSSION

In the present study the prevalence *Giardia* infection among children in Tabuk City was found to be 22% using the polymerase chain reaction (PCR). The prevalence was found to be much lower using direct microscopy and antigen ELISA techniques. The genetic identity of the *Giardia* assemblage was also determined in the present study and was found to be assemblage A. Previous reports dealt with *Giardia* in Saudi Arabia detected prevalence rates which are comparable to the present study (Kasim and Elhelu, 1983; Ahmad and Bolbol, 1989; Omar *et al.*, 1995; Al-Shammary *et al.*, 2001). In all these previous investigations, direct microscopy

Table 1. Results of the prevalence of *Giardia duodenalis* using different tests on the faecal samples from school children in Tabuk City

Test	Males			Females		
	+ve	-ve	%	+ve	-ve	%
Direct Microscopy	6	100	6.36	3	91	3.19
ELISA	10	96	9.43	10	84	10.64
PCR	23	83	31.7	21	73	22.34

Table 2. Senstivity and specificty of different tests used for the dignosis of *Giardia duodenlais* in faecal samples from school children in Tabuk city

Test	Sensitivity (%)		Specificity (%)	
	DM	ELISA	DM	ELISA
Direct Microscopy (DM)	–	94	–	100
Antigen ELISA (ELISA)	94	–	100	–
Polymerase Chair Reaction (PCR)	82	65	100	100

method was mainly employed. In the present study, however, in addition to direct microscopy, antigen ELISA as well as PCR methods were used to diagnose *Giardia* infection in school children in Tabuk City. Direct microscopy is the method used routinely in hospitals for the detection of *Giardia* infections and this method is labour intensive and it requires experienced personnel. Antigen ELISA is more accurate and rapid method for screening large number of samples at once but relatively expensive compared to direct microscopy. The sensitivity and specificity of the PCR was found to be high when compared with direct microscopy and ELISA test. The specificity of the PCR was found to be 82% and 65% versus the direct microscopy and ELISA respectively. That was certainly due to the fact that PCR detects the DNA of the organism and even if you have one cyst and DNA was extracted from it then it will be detected. While the direct microscopy is highly subjective and it requires experienced personnel. Based on the results of the present study, the Polymerase Chain Reaction was found to be more accurate and sensitive and gave even further information about the assemblage of *Giardia duodenalis* involved but it is very expensive to perform and it requires a well equipped laboratory and trained personnel.

There was no significant difference in the prevalence of infection between males and females as has been detected earlier (Omar, 1973). Unlike what has been reported by Kasim and Elhelu (1983) who reported significantly high prevalence in males compared to females and this can be attributed to the fact that males are more exposed to contaminated environments than females according to their nature as has been suggested by (Rabbani and Islam, 1994).

Apart from the work conducted by Al-Mohammed (2011) from Alhasa, and Shalaby *et al.* (2011), no work has characterized the genotype of *Giardia* prevalent in Saudi Arabia. The work by Shalaby *et al.* (2011) has employed a less sensitive method for molecular characterization of *Giardia* infections in children and adults. They have used the PCR-RAPD which resulted in two groups of organisms were found and it was not possible to show which assemblage was involved. However, Al-Mohammed (2011) has detected both assemblages A and B when studying intergentic spacer region (IGS). It is interesting that assemblages A and B are both of zoonotic potential. It is likely that the source of infection with *Giardia* in children in Alhasa and in Tabuk is probably of animal origin. Unlike what has been detected by Al-Mohammed (2011), assemblage B was absent in the present study which has not been involved in causing *Giardia* infection in children in Tabuk, however, the prevalence of assemblage A was comparable to what has been reported in the present study. Al-Mohammed (2011) detected a very high rate infection by assemblage B in symptomatic children and he attributed the cause of disease in Alhasa to this assemblage. These differences could be attributed to variation in the climatic conditions of both areas as Alhassa is an oasis where the conditions may be more suitable for more than one assemblage to propagate.

In conclusion, this study has established that there is a high prevalence of *G duodenalis* in children in Tabuk city using the PCR technique in contrast to the direct microscopy and the immunological ELISA test. *G. duodenlais* assemblage involved in Tabuk city was determined as assemblage A which is known to be zoonotic and infection in humans may have been contracted from animals.

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