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DETERMINE THE ZOONOTIC GIARDIA BETWEEN HUMAN AND COWS BY NESTED PCR TECHNIQUE IN BASRAH, IRAQ

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ABSTRACT

The aims of present study were to detection of zoonotic potential of Giardia parasite and to evaluate the risks of transmission to human because it was most hazardous to human health and animal in Basrah province. During this study examination of 66 fecal samples of infected cows and 40 stool samples of infected humans with Giardia parasite were utilized. Amplification of 292bp fragment of 16S rRNA ribosomal unit from elute DNA of 66 cows and 40 human Giardia isolated using nested PCR technique, revealed that 25.7% of cows and 72.5% of human isolated were belonging to the zoonotic Giardia. These give an indicative about giardiasis transmission from human to animals and vice versa. Also this level of zoonotic Giardia demonstrates the possibility of cows to play an important role as a reservoir for human giardiasis in this province. This study is belonging as a first study of its kind to take place in Basrah, Iraq.

KEY WORDS: Giardia, Nested, PCR, Zoonotic, 16S Rrna, DNA Extraction, Assemblage.

INTRODUCTION

The word Protozoa was once a phylum name. Today, however, the term is used colloquially as a common noun to refer to a number of phyla. Several other nouns, such as Archaezoa, Protoctista, and Protista, have been used to refer to this highly diverse group of microscopic creatures [1]. Flagellated protozoa also have members that are parasitic. These flagellates are likely to be found in every kind of animal, from cockroaches to humans. A few of them are structurally complex, and some, such as Giardia duodenalis [1]. Giardia was single celled microscopic protozoan parasites that cause enteric disease in humans and other mammals [2]. It was seen under microscope first time by Antony Van Leeuwenhock during 1681 [3]. In both humans and animals, the clinical outcome of a Giardia infection in animals is highly variable and infection can result in either acute or chronic diarrhea, nausea, weight loss, and hypersensitivity but asymptomatic infections are also known to occur frequently [4].

It has been reported worldwide in farm animals, although prevalence data are mainly available for cattle, and to a lesser extent for other ruminants [4]. It was a cosmopolitan parasite and classified by the WHO as a zoonosis [5]. This intestinal protozoan has been found in a wide range of mammals and has been accepted as a zoonotic agent [6, 7]. Human giardiasis is caused by the intestinal flagellate Giardia duodenalis and is considered a zoonotic infection because of these subgenotypes of Giardia, namely A and B, were found to be associated with infections of humans, dogs and calves, which supported the role of these animals as a source of infection for humans [8]. Giardiasis is a major diarrheal disease worldwide and diarrheal diseases account for a quarter of deaths under the age of five [9].

Zoonotic transmission of G. duodenalis from food producing animals and pets also provides a less common means of infection [10]. It remains controversial, but animals have been recognized as a potential source of infection due to the existence of zoonotic genotypes amongst the population [10]. It can occur either from direct contact or through their contamination of water sources [10]. In 1979 the WHO recognized the zoonotic potential of Giardia [10]. Cross transmission experiments [11, 12, 13], and immune-blotting of sera from both human and animal carriers [14] later confirmed the zoonotic transmission. On the other side, research carried out in the National Park of Uganda has shown that men were a source for giardiasis for wild animals and dairy cows [15].

MATHODOLOGY

Stool samples : All samples taken from patients complain of diarrhea, abdominal discomfort, nausea and abdominal cramp whose attended to general hospital, centers of sector for primary health care , and all samples collected on sterilized cups and taken up to laboratory of Parasitology / College of Veterinary Medicine - University of Basrah for confirmed diagnosis.

Faecal samples: All fecal samples were collected freshly , directly from rectum or as soon as after defecation, collected samples stored in sterilized cups were used for each animal and all these cups were labeled by information with number, date of collection ,animal condition , strength of feces ,age of animal ,address and name of owner. After collecting samples, transported to laboratory of Parasitology / College of Veterinary Medicine - University of Basrah for confirmed diagnosis.

DIAGNOSIS

All samples collected on sterilized cups and taken up to laboratory and diagnosis by:

1- Direct smear: [16]

a) Lugol's iodine.

b) Normal saline: to detection for trophozoite.

2- Concentration methods: formol-ether sedimentation [16].

MOLECULAR DIAGNOSIS

Polymerase Chain Reaction

This test is designed to include the examination 106 samples, 40 human stool samples and 66 cow faecal samples taken from the humans and cows infected with giardiasis, after diagnosis by previous above methods, DNA from stool samples obtained then tested by PCR technique depend on two types of primer, the first primer Gia2029 and Gia2150c to diagnosed Giardia spp and specific second primer RH11 and RH4 for genotyping of G. lamblia, the 16s rRNA gene was amplified in a nested PCR reaction.

DNA extraction and purification

The genomic DNA was extracted from 106 samples, 40 human stool and 66 cow faecal infected with giardiasis by using (QIAamp® DNA Stool Purification Kit /QIAGEN / Germany).

Procedure

A 180–220 mg stool was weighed in a 2 ml micro-centrifuge tube. Then added 1.4 ml Buffer ASL to each stool sample. Vortex continuously for 1 min or until the stool sample was thoroughly homogenized. Heated the suspension for 5 min at 70°C. Vortex for 15 second and centrifuged sample at full speed for 1 min to pellet stool particles. Pipetted 1.2 ml of the supernatant into a new 2 ml micro-centrifuge tube and discarded the pellet. Added 1 Inhibit EX Tablet to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubated suspension for 1 minute room temperature to allowed inhibitors to adsorbed to the Inhibit EX matrix. Centrifuged sample at full speed for 3 min to pellet inhibitors bound to Inhibited EX matrix. Pipetted all the supernatant into a new 1.5 ml micro-centrifuge tube and discarded the pellet. Centrifuged the sample at full speed for 3 min. Pipetted 15 µl proteinase K into a new 1.5 ml micro-centrifuge tube. Pipetted 200 µl supernatant from previous step into the 1.5 ml micro-centrifuge tube containing proteinase K. Added 200 µl Buffer AL and vortex for 15 s. Incubated at 70°C for 10 min. Added 200 µl of ethanol (96–100%) to the lysate, and mixed by vortexing. Labeled

the lid of a new QIAamp spin column placed in a 2 ml collection tube. Carefully applied the complete lysate from previous step to the QIAamp spin column without moistening the rim. Closed the cap and centrifuged at full speed for 1 min. Placed the QIAamp spin column in a new 2 ml collection tube, and discarded the tube containing the filtrate. Closed each spin column in order to avoided aerosol formation during centrifugation. If the lysate has not completely passed through the column after centrifugation, centrifuged again until the QIAamp spin column is empty. Added 500 µl Buffer AW1. Closed the cap and centrifuged at full speed for 1 min. Placed the QIAamp spin column in a new 2 ml collection tube, and discarded the collection tube containing the filtrate. Opened the QIAamp spin column and added 500 µl Buffer AW2. Closed the cap and centrifuged at full speed for 3 min. then discarded the collection tube containing the filtrate. Placed the QIAamp spin column in a new 2 ml collection tube and discarded the old collection tube with the filtrate. Centrifuged at full speed for 1 min. transferred the QIAamp spin column into a new, labeled 1.5 ml micro-centrifuge tube. Carefully opened the QIAamp spin column and pipetted 200 µl Buffer AE directly onto the QIAamp membrane. Closed the cap and incubated for 1 min at room temperature, then centrifuged at full speed for 1 min to elute DNA.

DNA concentration measurement

All elute DNA samples were measured by Nano-drop. By taking 0.5 µl of each eluted DNA by micro-pipette , put in well, closed the apparatus, then measured concentration of elute DNA, and all concentration were recorded using Microsoft Excel 2007 and labeled for each samples.

Amplification of 16S rRNA gene

The genomic DNA was amplified by using a fragment of 16s-rRNA ribosomal unit was amplified using nested PCR protocol was used the initial primers Gia2029 (5`-AAGTGTGGTGCAGACGGACTC-3) and Gia2150c (5`-CTGCTGCCGTCCTTGGATGT-3) to detected Giardia spp were used to produce a 497bp product and secondary primers RH11 (5`-CATCCGGTCGATCCTGCC-3) and RH4 (5`-AGTCGAACCCTGATTCTCCGCCAGG-3) a292bp. The PCR reaction consist of master mix (20 µml) consist of following: Top DNA polymerase 1 U, each: dNTP (dATP, dCTP, dGTP, dTTP) 250 µM, Tris-HCl (pH 9.0) 10 mM, KCl 30 mM, MgCl2 1.5 mM , forward primer 2.0 µl, reverse primer 2.0 µl, DNA template, 5.0 µl., nuclease free water11.0 µl (Bioneer).

Table (1): PCR program.

No	steps	temperature	time	Cycles
I	Initial Denaturation	96 °C	4 min	1
II	Denaturation	96 °C	45 sec	35
	Annealing	55 °C	30 sec	
	Extension	72 °C	45 sec	
III	Final Extension	72 °C	4 min	1

Nested PCR

The PCR are mix for the nested reaction was consisting of new master mix tube with addition the following in below:

Table (2): The reaction mixture (20 µl) for Giardia lamblia.

	Forward primer (RH11)	2.0 µl.
	Reverse primer (RH4)	2.0 µl
	First PCR product	5.0 µl
	Nuclease free water	11.0 µl.

As previous protocol of amplificated PCR, tubes were transferred to preheated thermocycler and start the program.

Table (3): Nested PCR program.

No	steps	temperature	time	Cycles
I	Initial Denaturation	96 °C	4 min	1
II	Denaturation	96 °C	45 sec	35
	Annealing	59 °C	30 sec	
	Extension	72 °C	45 sec	
III	Final Extension	72 °C	4 min	1

Detection of the nested PCR amplified product of 292bp was done by electrophoresis as mention in primary PCR.

RESULTS

After microscopically examination of freshly 40 human stool sample and freshly 66 cow faecal samples contain living Giardia trophozoites. DNA extraction and purification successfully by used QIAamp DNA Stool Mini Kit (Qiagen / Germany), all samples contain whole elute DNA, fig (1). Each elutes DNA samples were measured by Nano-drop which measuring were in human (16.8 - 184) ng/µl and in cow (5.3 - 343.9) ng/µl. The 106 specimen were positive for Giardia spp. infection examined by using of PCR technique. The PCR technique detection done by using a fragment of the 16S rRNA ribosomal unit was amplified using to diagnose of Giardia spp. infection. The initial primers for the first amplification of the PCR (Gia2029 and Gia2150c, which generated a 497bp product), the results showen all 40 human and 66 cow were positive in microscopically examination were also positive 100% to these technique, see table (4). All 40 human and 66 cow were positive to the initial primers examination examined by the secondary primers for the final amplification of nested PCR (RH11 and RH4, which generated a 292bp fragment), using to diagnosed Giardia lamblia infection. The results , see table (5), of this technique showen human 72.5% was positive to G. lamblia , fig.(2), and 27.5% was negative to G. lamblia infection but were infected by other Giardia spp. While the results in cows were showen 74.3% negative to G. lamblia infection but were infected by Giardia spp other than lamblia, and otherwise 25.7% positive to infection with G. lamblia , fig.(3).

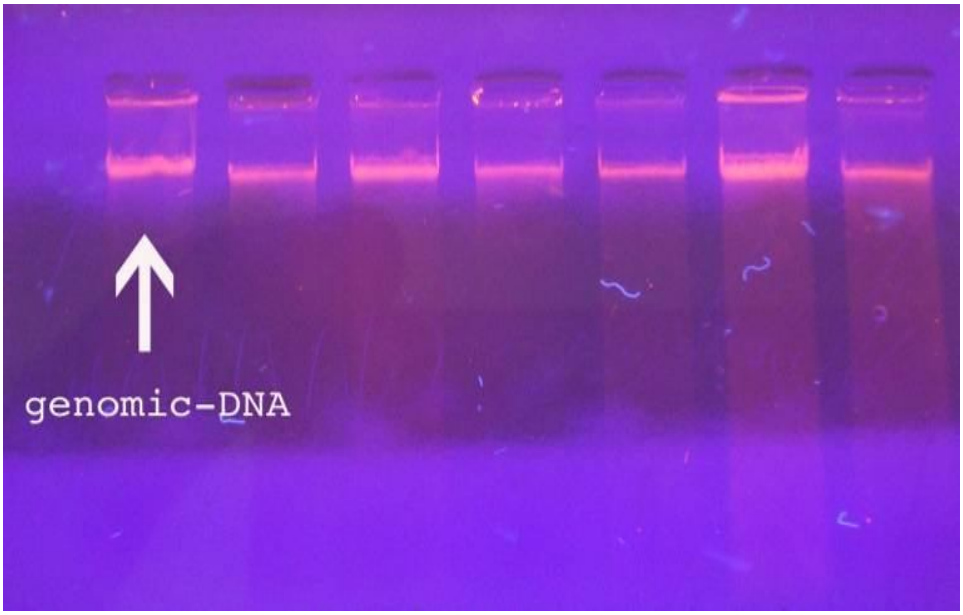

Fig. (1): Agar gel electrophoresis of amplified whole DNA.

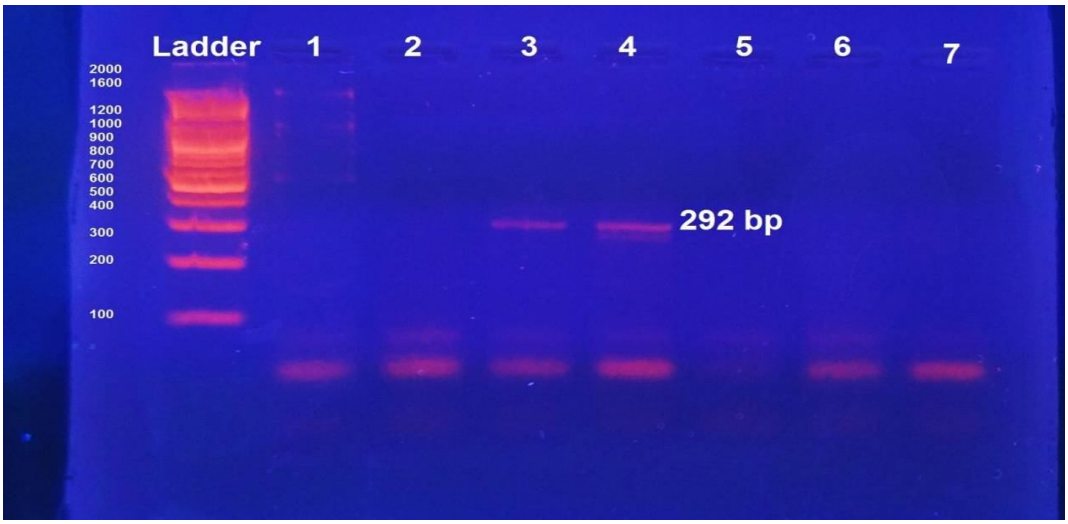

Fig. (2): Agar gel electrophoresis of amplified human DNA from G. lamblia by using primer set RH11 and RH4 of 16S rRNA. Lane 0 DNA ladder. Lane 3, 4 positive to (292bp).

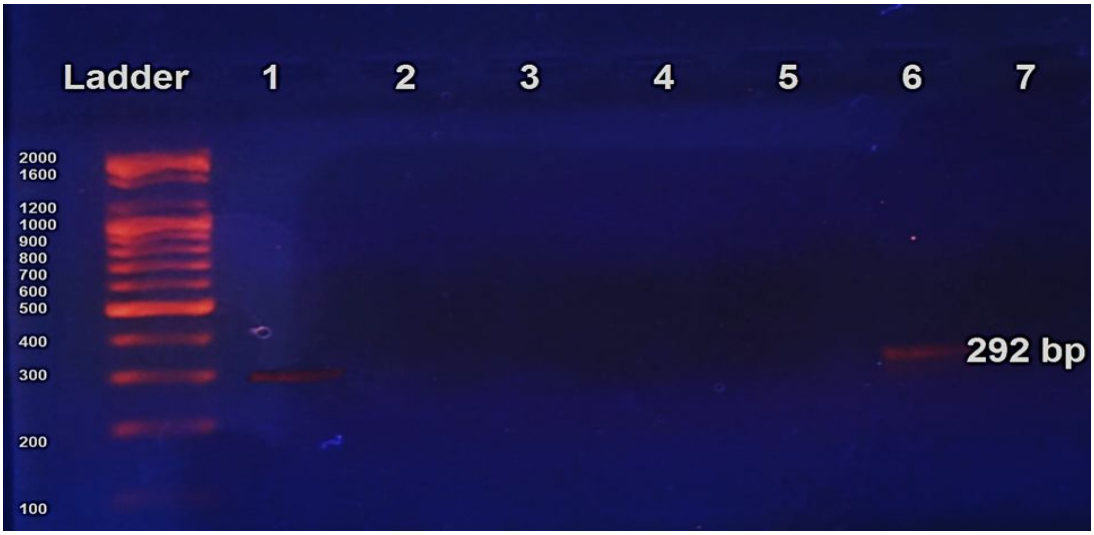

Fig. (23): Agar gel electrophoresis of amplified cows DNA from G. *lamblia* by using primer set RH11 and RH4 of 16S rRNA. Lane 0 DNA ladder. Lane 1, 6 positive to (292bp).

Table (4): Results of molecular diagnosis: first set of primers.

Host	No of samples infected	Positive for initial PCR	Percentage%
Human	40	40	100
cows	66	66	100

Table (5): Results of molecular diagnosis: second set of primers.

Host	No of samples infected	Positive for secondary PCR	Percentage%	Negative for secondary PCR	Percentage%
Human	40	29	72.5	11	27.5
Cows	66	17	25.7	49	74.3

DISCUSSION

For assurance and discrimination of zoonotic potential of giardiasis, the PCR technique was used. In current study showed high sensitivity and specificity of PCR technique in diagnosis of Giardia spp., these agreement with most studies [7, 17, 18, 19, 20, 21], whom recorded this difference in assay sensitivity is supported by a recent blinded trial which showed Cryptosporidium and Giardia spp., were detected 22 times more often by PCR than by conventional microscopic examination of human faecal specimens and it is a well-known tool for the detection of G. duodenalis and Cryptosporidium spp.

In this study the PCR technique detection done by using a fragment of the 16S rRNA ribosomal unit was amplified using to diagnose of Giardia spp. infection and this is the best procedure as mentioned [22], who's reported that this protocol has been the most sensitive PCR method compared to other protocols. This superior sensitivity could be attributed to the high copy number of the 16S rRNA gene in the organism, which reported by [23], that said the approximately 60 to 130 copies of 16S rRNA gene are present per nucleus of G. intestinalis, arranged in tandem repeats. Furthermore, this 16S rRNA has widely been used as a single genetic locus for Giardia characterization and is still considered valid [7, 15, 24, 25, 26, 27]. Monis et al., (1999) mentioned that species of Giardia can be distinguished on the basis of morphology, ultra structural features, or the 16S rRNA sequence and there are at least 6 species, i.e., G. intestinalis (5lamblia), G. agilis, G. muris, G. ardeae, G. psittaci, and G. microti [12].

The initial primers for the first amplification of the PCR (Gia2029 and Gia2150c, which generated a 497bp product), the results showen all 40 human and 66 cow were positive in microscopically examination were also positive 100% to these technique. These reflected that Basrah province is an endemic area for giardiasis among man and cows.

Miller and Sterling, (2007) detected the sensitivity of nested PCR in the different sets (consisting of 1, 2, 3, 4, 5, 7, and 10 cysts) of G. lamblia cysts isolated, in replicates of 50, the amplification results were as follows: 100% for 10, 7, 5, and 4 cysts; 94% for 3 cysts;

90% for 2 cysts; and 80% for 1 cyst [28]. Amplification of a target gene by nested PCR could detect one Giardia cyst [29, 30].

All 40 human and 66 cow were positive to the initial primers examined by the secondary primers for the final amplification of nested PCR (RH11 and RH4, which generated a 292bp fragment), using to diagnosed Giardia lamblia infection. In human the results of this technique showen 72.5% was positive to G. lamblia and 27.5% was negative to G. lamblia infection but were infected by other Giardia spp. The percentage 72.5% referred that most giardiasis cases in human due to G. lamblia assemblage A or B whereas the remaining ratio 27.5% belong to other Giardia assemblages these agree with study in Egypt by [31] that found the assemblage B was the most prevalent (80%) genotype, with another 15% of the positive samples belonging to assemblage E, and 5% to assemblage A. This also corresponding to the findings of an Italian study that reported 80% assemblage A caused the majority of giardiasis isolates [27]. Sprong et al, (2009) explained that G. intestinalis assemblages A and B are responsible for both human infection as well as infections in a broad range of other mammals [32]. The research performed in Australia showed that infections with Giardia from Assemblage B were more common (70%) as compared to those from Assemblage A (30%) [33].

While in cows the results were showen 74.3% was negative to G. lamblia infection but were infected by Giardia spp other than lamblia, and otherwise cows 25.7% were positive to infection with G. lamblia. These rates indicated that the most Giardia assemblage among cows is hoofed livestock assemblage E and to lesser extent the zoonotic assemblage A and this going with results of [17, 34, 35], whom showed that in cattle the livestock specific assemblage E is most prevalent, although up to 20% zoonotic assemblage A isolates have been reported. However, even a low prevalence of assemblage A or B isolates could pose a significant public health risk, since infected animals tend to excrete a large number of cysts [36].

CONCLUSION

We concluded that the Basrah province was an endemic area for giardiasis. Our data suggests that calves may represent an important biological reservoir and a potential risk for environmental contamination by Giardia. The study was showen that the population had 2 cycles of Giardia transmission, both zoonotic cycle and specific cycle in the same community. The PCR technique was a high sensitive and specific technique for detection and diagnosis of Giardia. The molecular diagnosis by nested PCR was recorded high percentage of infection by G. lamblia in human and low percentage with other Giardia spp.

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