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MOLECULAR DETECTION OF *GIARDIA* SPECIES IN CATS AND HANDLERS AT WASIT PROVINCE, IRAQ

Bassim J.H. Al Mosawy*, Jenan Mahmod Khalaf and Abbas Ali Al-Kinany

Department of Zoonotic Diseases, Veterinary Medicine, University of Baghdad, Iraq.

Abstract

This study designed to detect the infection of *Giardia intestinalis* in the stool of cats and handlers in the Wasit province, Iraq, by using the traditional method and molecular technique (nested PCR) based on gene triosephosphate isomerase (tpi) specific to genotyping of *Giardia intestinalis.* Study the special effects of risk factors (ages, gender, and months) on the infection rates, the virulence of recognized isolate and genotype of strains was pointed and there are the study that were done. Molecular study was done to detect the prevalence of infection and genotyping of *G. intestinalis.* The overall prevalence of giardiasis by nested PCR in cats and handlers in this study 58%, 57% respectively. A high significant relationship between PCR and microscopic examination was obtained (P=0.000). Incidence of infection in the present study showed that the percentage of infection in female cat higher than male was 82.8% in female and 17.2% in contrast of human was 70.1% in male human more than female was 29.9%, according to age group in the human high rate of infection was showed between 20-29 years old was 22%.while high percentage in young cats 34% more than adult cat 24%. The infection rate was influenced by environmental temperature, the percentage of infection in humans was 49.6% and there's an equal percentage of infection in April and it reported 15.7% in both cats and handlers, there is no significant relationship with the season (P>0.05). The distribution of genes shows a highly significant relationship between the frequency distribution of genes at a p-value of 0.000 by Cochran's Q test. A, B, and F genes reported 75, 53, 25 respectively. This study was successfully identified the *G. intestinalis* assemblage of six isolates from cat and human using sequencing of the PCR amplified Giardia triosephosphate isomerase (tpi) gene. Only zoonotic *G. intestinalis* genotypes A, B and F were detected in the six isolates. And reported for the first time in the Wasit province middle of Iraq in cats and humans.

Key words: *Giardia*; cats and handlers; Wasit provinces; nested PCR; sequencing.

Introduction

Giardia is a protozoan that has been acknowledged as an important cause of diarrhea (Cotton *et al.,* 2015), the main manner of transmission is coverage to contamination water and food by Giardia cysts in human and animals (Singh *et al.,* 2018).

Based on geno- and phenotyping changes and range of host specificities, the genus of *Giardia* is presently classified into 6 species, by smallest six extra variants or assemblages within *G. duodenalis*, which are likely to characterize different species (Monis *et al.,* 2009) (Feng & Xiao, 2011).

Since a public health perception, the comprehension of the epidemiology of giardiasis and the transmission passageways that donate to the disease weight is important for evaluating the zoonotic probable and *Giardia* pathogenesis ; classifying animal populations that can assist as a reservoir of disease for human and also domestic animals (Ali & Hill, 2003).

For that reason, the epidemiology and spread dynamic forces of *Giardia* have been investigated in different situations involving the environmental medium (Carmena *et al.,* 2014); human populaces (Cacciò *et al.,* 2005), production (Geurden *et al.,* 2010); (Robertson, 2009) and domestic animals (Thompson *et al.,* 2008) and wildlife.

The species multifaceted *Giardia duodenalis* host-adapted and zoonotic genotypes/species (Soares *et al.,* 2011). At least 8 individual Assemblages (A-H) of *G. duodenalis* have been testified based on genetic examines (Lasek-Nesselquist *et al.,* 2010); (Monis *et al.,* 2009); (Scorza *et al.,* 2012). Assemblages A and B have been noticed in a wide range of hosts containing humans, other primates, dogs, cats, livestock and wildlife

Author for correspondence : E-mail : bassimj89@gmail.com

(Ballweber *et al.,* 2010), (Monis *et al.,* 2009); (Scorza *et al.,* 2012). Assemblages C to H are well-thought-out host-adapted in dogs (Assemblages C and D), cats (Assemblage F), livestock (Assemblage E), rats (Assemblage G) and marine mammals (Assemblage H) (Heyworth, 2016); (Zahedi *et al.,* 2017); (Pipiková *et al.,* 2020).

Materials and Methods

Collection of Samples

One hundred samples were selected from cats and 100 from handlers for Nest-PCR screening. The triose phosphate isomerase (tpi) gene-based Nest-PCR was aimed at the detection of *Giardia spp* from the fecal samples of cates and handlers and as per the method used by (Yu *et al.,* 2009 and Ruecker *et al.,* 2013).

Stool DNA Extraction

Genomic parasite DNA was extracted from stool samples were extracted by using Accu Prep® stool DNA Extraction Kit, Bioneer. Korea, and done according to company instructions.

Primers

The Nested PCR for recognition G lamblia genotype A and B were planned by (Minvielle *et al.,* 2008). The Nested PCR primer G lamblia genotype F was planned in this study using NCBI Genbank database and Primer3plus online. Then these primers were on condition that from Macrogen Company, Korea as following tables:

-Design: LC341570.1 Giardia intestinalis TPI gene for triose phosphate isomerase, partial cds, genotype: assemblage F.

Nested PCR primer Giardia intestinalis assemblage A

Primer		Sequence 5'-3'	Amplicon
TPI gene PCR	F	CGAGACAAGTGTTGAGATG	576bp
	R	GGTCAAGAGCTTACAACACG	
TPI gene nPCR	F	CCAAGAAGGCTAAGCGTGC	476bp
	R	GGTCAAGAGCTTACAACACG	

Nested PCR primer Giardia intestinalis assemblage B

Primer		Sequence 5'-3'	Amplicon
TPI gene PCR	F	GTTGCTCCCTCCTTTGTGC	208bp
	R	CTCTGCTCATTGGTCTCGC	
TPI gene nPCR	F	GCACAGAACGTGTATCTGG	140bp
	R	CTCTGCTCATTGGTCTCGC	

Nested PCR primer Giardia intestinalis assemblage F

Primer		Sequence 5'-3'	Amplicon
TPI gene PCR	F	AACGGCTCGCTCGACTTTAT	471bp
	R	GGGCTCGTAGGCAATAACGA	
TPI gene nPCR	F	GGCCATTGCTGCCCACAAG	401bp
	R	TCTTCGACTCTCCAAGCTCC	

Nested PCR technique

CR technique was achieved for recognition G. lamblia A , B and F genotype centered on triosephosphate isomerase gene (TPI). This technique was carried out according to defined by (Minvielle *et al.,* 2008).

Nested PCR master mix preparation

Nested PCR master mix was by using Accu Power® PCR PreMix (Bioneer. Korea), and done according to company teachings

Results and Discussion

Molecular detection

The application of PCR procedure for the amplification of defined DNA sequences has shown considerable promise for the development of highly sensitive and specific diagnostic test polymerase chain reaction highlighted the sensitivity because of multi-copy nature of the gen as of interpretation and the potential to further differentiate to the spices and genotype level (Eze & Eze, 2015).

The use of molecular technique to confirm the *Giardia lamblia* is also important ephology (Boontanom *et al.,* 2010). Molecular detection method included in the present study nosing *Giardia lamblia* infection and species identification.

Nested PCR product analysis

The overall prevalence of giardiasis in cats and handlers in this study 58%, 57% respectively. This finding was comparable with the results of (Basrah, 2014) in Al Basrah who recorded infection rate in human was 30.1%. However, the results were higher than that recorded in Iraq (Al muthanna) (Turki *et al.,* 2015) who recorded infection rate in human by PCR was 25%. In addition the results of (khudair Hussein, 2010) was 23.7% in Thi Qar southern Iraq.

Many researchers diagnosed of *Giardia lamblia* by PCR such as (Rahimian *et al.,* 2018) which recorded 23% of *Giardia lamblia* in human in Tehran. (Hijjawi *et al.,* 2018) recorded 64.6% in Jordan. (Al-mohammed, 2011) was recorded 41.2% in Saudi Arabia.

Ahmed. Z.A. (2018) was recorded the rate of infection diagnosed by PCR in cat was 10% in Baghdad , this percentage disagree with present study. (Bouzid *et al.,* 2015) agreement with present study which recorded the percentage of *Giardia lamblia* in cat by PCR technique 56% in USA. (Robertson *et al.,* 2007) who recorded rate of infection in cat by PCR 80% in Australia. Other researchers showed the high percentage of

infection such as (Volotão *et al.,* 2007) in Brazil recorded 100%. However other studies showed lower percentage of infection such as (Tangtrongsup *et al.,* 2020) recorded 27.3%.

The difference in the proportions results due to primers, method and type of PCR technique. And the difference in the prevalence of Giardia lamblia infection reported in different studies is due to the differences in the time, geographical living areas and the hygiene habits and lifestyles in which the samples were obtained (Abbasi *et al.,* 2020).

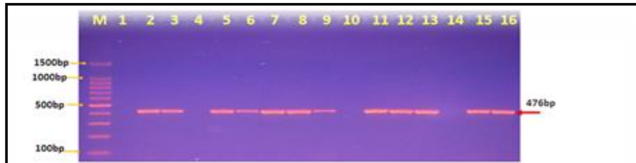

Figure (1): Agarose gel electrophoresis image that showed Nested PCR product analysis of TPI gene in Giardia intestinalis assemblage A of cat feces samples. (M) Marker ladder (2000-100bp). Lane (1-16) Giardia intestinalis assemblage A at 476bp Nested PCR product.

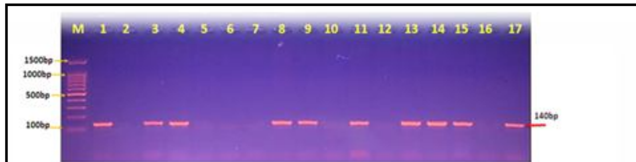

Figure (2): Agarose gel electrophoresis image that showed Nested PCR product analysis of TPI gene in Giardia intestinalis assemblage B of cat feces samples. (M) Marker ladder

Figure (3): Agarose gel electrophoresis image that showed Nested PCR product analysis of TPI gene in Giardia intestinalis assemblage F of cat feces samples. (M) Marker ladder (2000-100bp). Lane (1-14) Giardia intestinalis assemblage F at 401bp Nested PCR product.

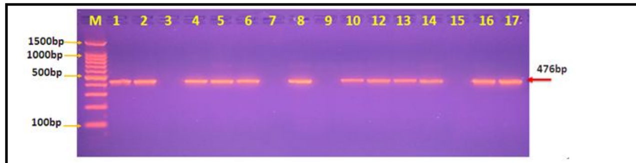

Figure (4): Agarose gel electrophoresis image that showed Nested PCR product analysis of TPI gene in Giardia intestinalis assemblage A of Human stool samples. (M) Marker ladder (2000-100bp). Lane (1-17) Giardia intestinalis assemblage A at 476bp Nested PCR product.

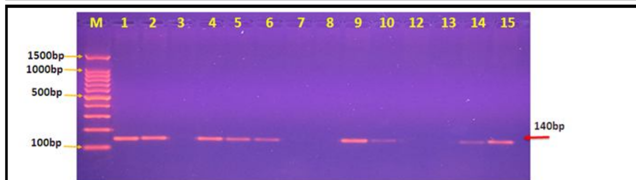

Figure (5): Agarose gel electrophoresis image that showed Nested PCR product analysis of TPI gene in Giardia intestinalis assemblage B of Human stool samples. (M) Marker ladder (2000-100bp). Lane (1-15) Giardia intestinalis assemblage B at 140bp Nested PCR product.

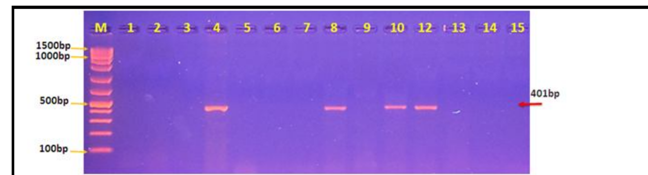

Figure (6): Agarose gel electrophoresis image that showed Nested PCR product analysis of TPI gene in Giardia intestinalis assemblage F of Human stool feces samples. (M) Marker ladder (2000-100bp). Lane (1-15) Giardia intestinalis assemblage F at 401bp Nested PCR product.

The prevalence of giardiasis by nested PCR according genders

Incidence of infection in present study showed that the percentage of infection in female cat higher than male was 82.8% in female and 17.2% in male in disagreement with (Tangtrongsup *et al.,* 2020) was reported 52% in Thailand. This lead to variation of hormonal affection than male, this due to some hormones in female lead to decrease of immune defense mechanism.

The incidence of infection was 70.1% in male human more than female was 29.9% in agreement with previously reported (Rahimian *et al.,* 2018) (Ismail, 2016) (Skhal *et al.,* 2016). These variation may be due to the male greatly they raise pet cats more than female in Wasit province.

The prevalence of giardiasis by nested PCR according to age group

The percentage rate of Molecular nested PCR test showed that 57% positive in total of human, high rate of infection were showed between 20-29 years old was 22%. This percentage disagreed with (Al., 2016) in Iraq was showed 4% and (Mahmoudi *et al.,* 2020) in Iran was 14%; the reason for this may be due to climate change from one region to another, as well as the difference in temperature, which led to a change in the infection rates.

Molecular nested PCR test showed that in present study was 58% positive in total, and the high percentage in young cats 34% more than adult cat 24%. The higher infection in young than old cats was due to variant immune status of animals, the maternal immunity lowered significantly in small cats after 6 months. Giardia infections in dogs and cats have been investigated worldwide. The infection is most common in younger animals, and is higher in kennel and shelter populations than in households (Mundim *et al.,* 2007); (Gates & Nolan, 2009)

The prevalence of giardiasis by nested PCR according to season

In present study by molecular nested PCR showed the total percentage rate was 50.4%, while the total percentage of infection in humans was 49.6% and there's

an equal percentage of infection in April and it reported 15.7% in both cats and handlers. In another study conducted slightly high percentage in Al Basrah was 28%. The finding from the current study suggests that giardiasis prevalence differs even within different governorates of the same country. The differences are probably an indication of differences in population densities, overcrowding, unhygienic conditions and local environmental factors inherent in each governorate (Saheb, 2018). Regarding seasonal in the prevalence of *Giardia*, seasonal effects on the infection rate may reflect climatic changes on the parasite, host physiology or in the photoperiod (Mosallanejad *et al.,* 2010).

Molecular distribution of gene for Human and cats

Distribution of gene Positive results for Human and cats**,** in present study recorded gene assemblage A in human and cat (51%, 49%) respectively , this percentage disagreed with another study in Al Muthanna 2015 was showed lower than present study 30.14% in human (Turki *et al.,* 2015). And in agreement with (Rahimian *et al.,* 2018) in Iran was recorded rate 58% in human. In other study in China founded higher percentage than present study in cat was 80% (Li *et al.,* 2017). While agreement with [40] was showed the percentage (35%).

And assemblage B was recorded in total (75) in both human and cat (38%, 52%) respectively. This percentage was lower than (Al., 2016) in Al Muthanna for human was showed 26%. And disagree with Skhal in Syria was recorded 10% , Egypt 19% and agreed with study found in Saudi Arabia was conducted rate 37% and Ethiopia 22% (Skhal *et al.,* 2016). While in cat disagree with study in china was recorded 28.5% (Li *et al.,* 2017). This variant in result study may depend on different molecular technique in each country.

Assemblage F was recorded in present study as total (25) in both human and cat (40%, 60%) respectively. The present study disagree with (Li *et al.,* 2017) that was recorded percentage of assemblage F gene in human was 23.29% and agreed with another study showed assemblage F in cat was 64% (Vasilopulos *et al.,* 2007). And other study in Germany 2018 for cats was conducted rate 75% (Sommer *et al.,* 2018). And assemblage both A&B conducted in both cat and human in present study (42.8%, 57.2%) respectively. This percentage agreed with study in Syria founded 22.5% in human (Skhal *et al.,* 2016).

Assemblage both A & F in cat and human (72.7%, 27.3%) respectively and Assemblage both B & F in cat and human (55.5%, 54.5%) respectively and there are no other study showed these percentages such as in present study.

DNA sequencing

DNA sequencing has become the most commonly used approach for typing *Giardia* isolates from different geographical and host origins (Suzuki *et al.,* 2011).

DNA sequencing based on small subunit DNA in the present study was identical to previous studies.

Six samples of DNA sequencing were purified by PCR technique and analyzed by sequencing to get nucleotide sets of *Giardia lamblia* gene (476 pb) for assemblage A, gene (140pb) for Assemblage B and gene (401pb) for assemblage F for *Giardia lamblia* isolated from cats and handlers in the middle of Iraq and recorded in gene bank with accession numbers: MT747424, MT747425, MT747426, MT747427, MT747428, MT747429. The analysis of phylogenetic tree based on sequences of triose phosphate isomerase (tpi) gene (476 pb) for assemblage A, gene (140pb) for Assemblage B and gene (401pb) for assemblage F for *Giardia lamblia.*

Phylogenetic tree analysis based on triose phosphate isomerase (tpi) gene partial sequence in local Giardia intestinalis IQ Cat and Human Isolates that used for genetic relationship analysis. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local Giardia intestinalis IQ. No. 1 Cat and Human - IQ.No.1 isolate were showed closed related to NCBI-BLAST Giardia intestinalis isolate A: assemblage A (LC329330.1). The local Giardia intestinalis IQ. No. 2 Cat and Human - IQ. No. 2 isolate were showed closed related to NCBI-BLAST Giardia intestinalis isolate A: assemblage B (KY444789. 1). The local Giardia intestinalis IQ. No. 3 Cat and Human - IQ. No. 3 isolate were showed closed related to NCBI-BLAST Giardia intestinalis isolate A: assemblage F (KU378639.1) at total genetic changes (0.020%). The analysis result showed that the homology of triose phosphate isomerase (tpi) gene sequences between local isolate of Iraq *Giardia intestinalis*. Was nearly closed to Iran isolates (LC329330.1) of assemblage A gene with homology sequence identity (99.87) %, Nearly closed to Iran isolate (KY444789) of assemblage B gene with homology sequence identity (99.14)%. And nearly closed to Iraq isolate (KY444789) of assemblage F gene with homology sequence identity (99.78)%.

Phylogenic analysis

The results of the analysis showed that the maximum homology of the triose phosphate isomerase (tpi) gene sequences between the local *Giardia intestinalis* isolated

strain ranged from (99.13 _99.87 %). While the maximum homology of triose phosphate isomerase (tpi) gene sequences between the Iraq *Giardia intestinalis* isolated strain and world strains ranged from (99_100%).

Phylogenetic analysis confirmed low differences between Iraqi strains of *Giardia intestinalis* and other countries. This genetic variation may be due to differences in area size of the reference sequence and differences in geographical the areas where isolates collected. Using different methods like PCR based on gene sequencing of partial or complete genes have been used for genetic analysis. When the environment of parasite change, genetic diversity play an important role in the survival and makes accurate analysis of variation applicable for studies on taxonomy, biology, epidemiology and pathogenesis of parasites. Result of this study agreed with some studies that reported the existence of genetic variation and phylogenetic relationships based on mitochondrial and nuclear gene sequences among populations of parasite that analyze genetic variations of Giardiasis protozoa in the world.

Phylogenetic analysis of *G. intestinalis*, sequence was conducted using BLAST search tool. A dataset was assembled using sequences retrieved from this study and GenBank at the National Centre for Biotechnology Information representing the diversity of *Tpi* sequences. The six isolates from the present study had (97.17-99.87%) similarity to sequences deposited in GenBank. The six isolates detected in present study were *G. intestinalis* assemblage A, B and F depended on cluster of others in

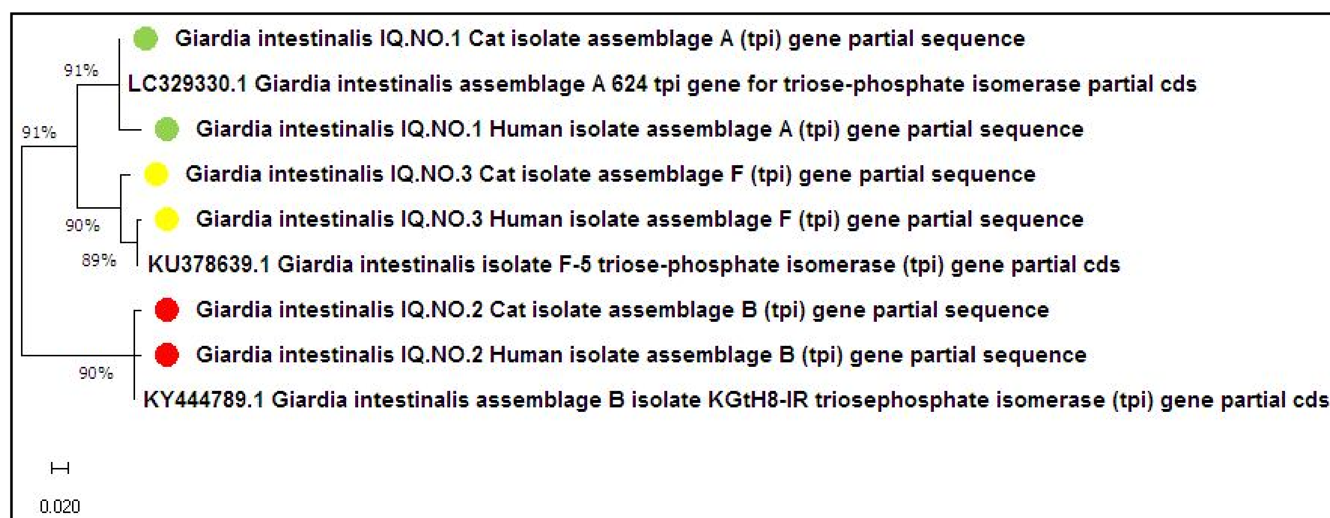

Figure (8): Phylogenetic tree analysis based on triose phosphate isomerase (tpi) gene partial sequence in local Giardia intestinalis IQ Cat and Human Isolates that used for genetic relationship analysis . The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local Giardia intestinalis IQ.No.1 Cat and Human - IQ.No.1 isolate were showed closed related to NCBI-BLAST Giardia intestinalis isolate A: assemblage A (LC329330.1). The local Giardia intestinalis IQ.No.2 Cat and Human - IQ.No.2 isolate were showed closed related to NCBI-BLAST Giardia intestinalis isolate A: assemblage B (KY444789.1). The local Giardia intestinalis IQ.No.3 Cat and Human - IQ.No.3 isolate were showed closed related to NCBI-BLAST Giardia intestinalis isolate A: assemblage F (KU378639.1) at total genetic changes (0.020%).

Table 3-2: The NCBI-BLAST Homology Sequence identity (%) between local Giardia intestinalis IQS-No.1isolates and NCBI-BLAST submitted Giardia intestinalis isolates.

G.intestinalis isolate No.	Genbank Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical NCBI genotype	Genbank Accession number	County	Identity (%)
G. intestinalis IQ No.1 Cat isolate	MT747424	Assemblage A	LC329330.1	Iran	99.87%
G. intestinalis IQ No.1 Human isolate	MT747425	Assemblage A	LC329330.1	Iran	97.62%
G. intestinalis IQ No.2 Cat isolate	MT747426	Assemblage B	KY444789	Iran	99.14%
G. intestinalis IQ No.2 Human isolate	MT747427	Assemblage B	KY444789	Iran	99.13%
G. intestinalis IQ No.3 Cat isolate	MT747428	Assemblage F	KU378639.1	Iraq	99.78%
G. intestinalis IQ No.3 Human isolate	MT747429	Assemblage F	KU378639.1	Iraq	97.17%

GenBank (Shown in following figures).

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