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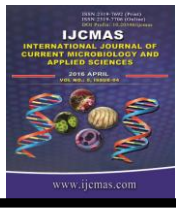

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Detection and Genotyping of *Giardia lamblia* in Clinical and Environmental Samples in Some Regions of Baghdad city

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A B S T R A C T

The present study was conducted to estimate the prevalence of *Giardia lamblia* infection in some regions of Baghdad city, the relationship of source of drinking water with the prevalence of this intestinal protozoal parasite had been evaluate, The study revealed that the total rate of *G.lamblia* infection in humans was 68/375 (18.13%) according to three diagnostic methods, there was significant relation ($p \leq 0.01$) between infectivity rate (25%) of this parasite and persons who consumed tap water as a source of drinking water as compared to other source of drinking water. RT-PCR *tpi* gene application was able to detected and genotyping *G.lamblia* to assemblage A and B, since mix infection of assemblage (A+B) are more prevalent in fecal samples than others, while in environmental samples the assemblage A, assemblage B showed in 1/4 (25%), 2/4(50%) respectively and mixed assemblage (A+B) showed in 1/4 (25%) of samples.

Introduction

Giardiasis is the most common parasitic infection of the human intestine worldwide, caused by infection with flagellated, bi-nucleated protozoan parasite *G.lamblia*, disease ranges in seriousness from asymptomatic, to acute or chronic diarrheal disease associated with abdominal pain and nausea (Halliez and Buret, 2013). *G.lamblia* (also known as *G. duodenalis* and *G.intestinalis*) (Siripattanapipong *et al*., 2011), First discovered in 1681 by Antonie van Leeuwenhoek (Raza *et al*., 2013). Genus *Giardia* contains at least six species that infect animals and/or humans (Ankarklev, 2012), *G. lamblia* is the only recognized species found in humans and most other mammals including cats, dogs, cattle, pigs, sheep and horses (Raza *et al*., 2013). A variety of molecular tools, including PCR-RFLP and sequence analysis of housekeeping genes, have shown that *G.lamblia* is a species complex made up of morphologically indistinguishable isolates that are classified into 7 assemblages based on the characterization of the glutamate dehydrogenasa (*gdh*), small subunit (ssu rRNA), and triose phosphate isomerase (*tpi*) genes (Nahavandi *et al*., 2011), these

assemblages include A and B, which are potentially zoonotic assemblage A consists of isolates that can be divided into 2 distinct clusters, I and II, assemblage B has been divided into clusters III and IV (Helmy *et al*., 2009).

Life cycle of *G.lamblia* has a simple two stages: Infective cysts are ingested with contaminated food or water or by the direct fecal/oral route (Adam, 2001), and trophozoites is vegetative stage live in the upper small intestine where they adhere (Raza *et al*., 2013). This study aimed to determined the prevalence of giardiasis in some regions of Baghdad, the relationship between giardiasis, the source of drinking water and using molecular approach in detection and genotyping of *G.lamblia* in fecal and environmental samples.

Materials and Methods

Clinical Samples were collected from: AL-Khadimya Teaching Hospital, Al-Noaaman Hospital and the Laboratories of Medical City for outpatients during the period from the beginning of April 2014 until the end of March 2015, stool samples were collected from 375 patients with gastroenteritis, of both sexes at different ages, and each sample put in a clean screw cup container, labeled with the number, date of collection and request forms which filled for each patient including information about the age, sex and the source of drinking water.

Detection of *G.lamblia* in Clinical Samples

The screening for *G.lamblia* in stool samples under an optical microscope using Lugol'iodine-stained preparation and fresh normal saline smear (Yakoob *et al*., 2005; Al-Saeed and Issa, 2010), Zinc sulfate flotation method and chromatographic immunological detection (Certest *Giardia* rapid strip test). The positive samples transfer directly to the molecular lab to conduct the Real Time - polymerase Chain Reaction RT-PCR.

Environmental Samples

This part of study was performed during the period from beginning of March 2015 till the end of August 2015. Eighteen water samples were collected randomly from some parts of Tigris River located in the study area using polyethylene containers; water samples were taken directly from the surface of the river, the date of collection is recorded for each sample.

Treatment of River Water Samples

Approximately Twenty liters of river water sample was taken, the sample is left overnight, to precipitate, then got rid the supernatant and took the precipitate which was conducted by a serious of filtration process: filtered through a membrane filter with a pore size of 0.45µm. by vacuum pumping system. The solids captured on the filter were removed by rinsed in Phosphate Buffer Saline PBS each 100 ml of PBS contained (20 mg Sodium Dodecyl Sulphate SDS and 20µl Tween 80). The rinse water was collected in 50 ml conical centrifuge tube and then centrifuged at 1000 rpm for 10 min, then separated the supernatant about sediment in collection tubes (Nikaeen *et al*., 2005).

Preservation of Water Sample

The sample of water (supernatant and precipitate) diluted in 2.5% potassium dichromate solution and kept at 4C° until subsequent methods.

Molecular Approach

DNA was extracted from each *Giardia*-positive stool sample using the QIAamp®

Stool mini kit (Qiagen, Germany) following the manufacturer's instructions and according to the manufacturer's protocol of Wizard® Genomic DNA Purification Kit supplied by Promega / USA from environmental samples. The purity of DNA was estimated using a Nanodrop Spectrometer. The RT-PCR was used for targeting the *tpi* gene to detect and genotype *G. lamblia* in clinical samples and environmental samples, the sequence of primers of *tpi* gene that used in RT- PCR which provided by Alpha DNA, Canada, is *tpi* A primer, forword (5'- CGA GAC AAG TGT TGA GAT G-3') and reverse (5'- GGT CAA GAG CTT ACA ACA CG-3').The *tpi* B primer , forword (5'- GTT GCT CCC TCC TTT GTG C-3') and reverse (5'-CTC TGC TCA TTG GTC TCG C-3'). The amplification reactions (25µl) contained (10µl) of DNA template, PCR working solution which consist of GoTaq® RT-PCR Master Mix is provided as a simple to use, stabilized 2X master mix that includes all components for quantitative PCR at volume (12.5µl), *G.lamblia* forward Primer 10 µM(1µl) *G.lamblia* reverse Primer (1µl), nuclease free water (0.5 µl). Annealing temperature were 55 C^o for both primers. The sizes of the DNA amplicons were 576bp region and 208bp region from the *tpi* A and *tpi* B genes respectively. Following amplification, the PCR products were subjected to melting curve analysis by raising the temperature from 60 to 95°C at a rate of 0.2°C/s.

Results and Discussion

The prevalence of *G.lamblia* estimated in this study at 18.13%(68/375). Many studies considered *G.lamblia* as one of the most common identified causes of waterborne outbreaks (Nikaeen *et al*., 2003; Nygård *et al*., 2006), Giardia cysts have been isolated from water supplies in different parts of the world, over the past 10 years, the contamination of drinking water with *Giardia* spp. had been recognized (Younas *et al*., 2008). Study in Baghdad city by Al-Saqur *et al*. (2015) demonstrated scientifically the presence of *G.lamblia* in tap water which consider the source of drinking water in this city. Therefore, this study focused on the source of drinking water because it is a major source of infection, different types of drinking water were recorded of giardiasis cases (filtrated, bottled, tap and boiled), as showed in table (1) high incidence in persons who drunk tap water 55/220(25%) compared to other sources of drinking water, there was highly significant relation between the sources of drinking water and the incidence of giardiasis ($p \leq 0.01$), low incidence was noticed in patients using the boiling water 3/58 (5.17%).

This finding is coincide with the results of Jaeffer (2011) that tap water consumers may be more likely than others to be infect by *G.lamblia*, as the percentage of giardiasis cases 13, 78% for them .The results of Al-Saqur *et al.* (2015) concerning presence of *G.lamblia* in tap water at percentage 3.47% was confirmed the results in current study, that the tap water is probably the source of infection.

Drinking water outbreaks with giardiasis have been reported from Canada, New zealand, Sweden and Germany (Wicki *et al*., 2009). Study in UK found an association between drinking tap water and giardiasis in persons who had not traveled outside the UK and this result consistent with present finding (Stuart *et al*., 2003)

There are different causes which may explain the high incidence among consumers of tap water:

Giardia lamblia cysts relatively resistant to chlorination, particularly if the water is cold, and the amount of chlorine used routinely in drinking water is not sufficient to kill *G. lamblia* (CFSPH, 2012).

Giardia spp. is widely spread in both humans and other mammalian species, permitting for frequent contamination of surface water supplies (Al-Taie and Ali, 2009).

Giardia lamblia cysts are widely distributed in the environment, cysts can penetrate physical barriers in water treatment processes and it disinfectant resistant (Jaffer, 2011).

There are several factors play important roles in the effectiveness of sterilization of water like: temperature, pH and turbidity of the water, as well as contact time with the chemical materials (CFSPH, 2012).

Giardia lamblia has a low infectious dose for humans (Jaffer, 2011).

The fecal contamination of water and sewer overflows which happened during storm events and discharge raw sewage from sewer pipes into waterways, are common contributors to high fecal pollution levels in urban areas (Hadi and Faraj, 2008).

In Iraq, the amount of tap water which delivered from the native supplies is not enough. So, people are use the water pumps to receive more of water amounts to their homes; this process may be a principal cause of contamination of drinking water with sewage which contain various types of pathogens and may overlap with pollutants and sewage materials that permeate by pumping power to old corroded water pipes (Al-Saqur *et al*., 2015).

Detection of *G.lamblia* in Clinical Samples by Real Time PCR (RT-PCR)

Recent establishment of based quantitative PCR has been advanced in *Giardia* diagnostics with an increasing success rate in detecting the disease (Ankarklev, 2012). RT-PCR combines the amplification and detection into a single step thereby eliminating the need for any post amplification processing of the sample (Singh *et al*., 2014). In the present study, RT-PCR was applied to the diagnosis of *G.lamblia* in clinical specimens, figure (1) clarified the 68 specimens showed positive result for *G.lamblia* by three detection methods. 63 samples analyzed by RT-PCR. Five samples not being examined by the RT-PCR because the amount of DNA were not enough to accomplish, the amplification of *tpi* gene succeed in 59/63(93.65%) specimens,

These results agree with Al-kayat (2013) who succeeded in identifying (90.8%) samples positive for *G.lamblia* by RT-PCR assay, other study conducted in Canada by Guy in 2004 detected by RT-PCR all specimens that were given positive result for *Giardia* in microscope 15/15(100%), and no false negatives were recorded. But current result was much higher compared with results in Egypt by Helmy *et al.* (2009) who obtained 42.3% *tpi* gene amplification rate by same approach. The failure of amplification and given false negative results for some samples may be due to the low quantity of DNA samples, low concentration of DNA, degrading in time, presence of some of PCR inhibitors such as lipid, hemoglobin, cellulose, bile salts, polysaccharides from mucus, bacteria, food degradation product, handling error (Al-Kayat 2013), and may be reasons related to the primers such as sensitivity of primers (Nantavisai *et al*., 2007).

Table.1 The Percentage of Infection According the Source of Drinking Water

Source of water	No. of Infection	No. of non infection	Total cases	Percentage %
Tap water	55	165	220	**25
Boiling water	3	55	58	5.17
Filtered water	5	27	32	15.62
Bottle water	5	60	65	7.69
Total	68	307	375	18.13

Chi-square = 18.4597. p-value is .000354 **P≤ 0.01

Table.2 RT-PCR Assays Detection and Genotyping *G.lamblia* Isolates

PCR assay	No. of samples with assemblage A and percent %	No. of samples with assemblage B and percent%	No. of mixed samples (A+B) assemblage and percent%	Total and percent%	False negative samples and percent%
RT-PCR for 63 positive samples	-	15 (25.42)	44 (74.57)	59 (93.65)	4 (6.34)

Table.3 Number and Percentage of *G.lamblia* Assemblage Isolated from Environmental Samples

RT-PCR assay	No. of samples with assemblage A and percent %	No. of samples with assemblage B and percent%	No. of mixed samples (A+B) assemblage and percent%	Total and percent%	No. of non amplified samples percent%
RT-PCR for 7 positive samples	1 (25)	2 (50)	1 (25)	4 (57.14)	3 (42.85)

Figure.1 Amplification Curve of Some *G.lamblia* Isolates from Fecal Samples of Human Detected by RT-PCR, Horizontal Line: Threshold Line

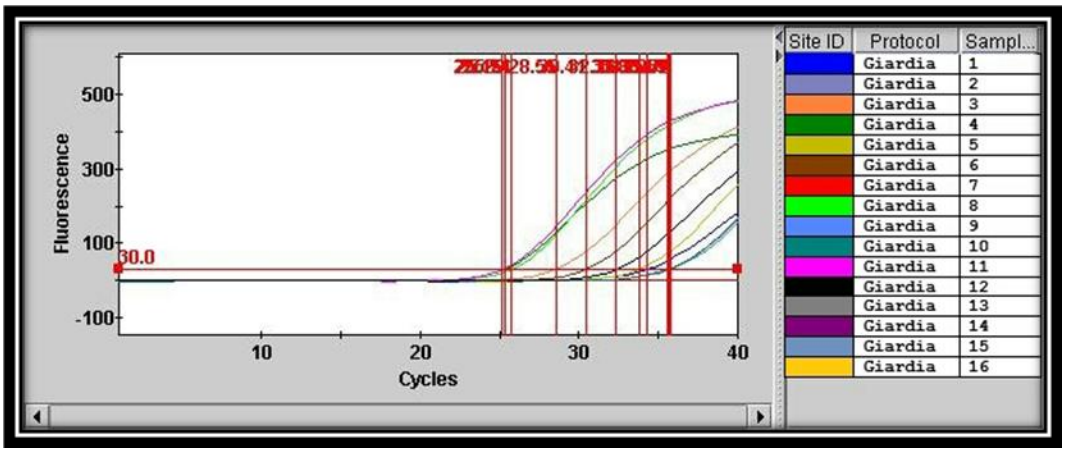
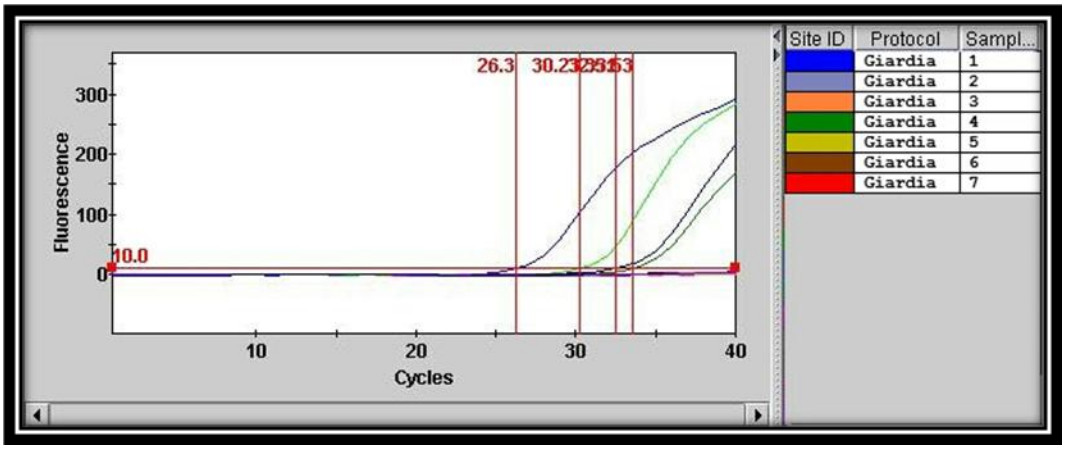


Figure.2 Amplification Curve of 4 *G.lamblia* Isolates from Environmental Samples Detected by RT-PCR, Horizontal Line: Threshold Line



In the RT-PCR assay obtained result demonstrated that the mix infection were predominant where the infection rate of assemblage(A+B) 44/59(74.57%), while the assemblage B frequented alone in fifteen cases (25.42%), it didn't recorded the cases with assemblage A only, table(2).

The occurrence of mixed infection as shown by the RT-PCR assay in current study may be due to recombination between assemblages A and B which recently suggested, or presence of both types of cysts in the fecal sample (i.e., a mixed infection) and the high sensitivity of RT-PCR enabled to detect the low number of cysts in clinical samples (Al-Meida *et al*., 2010).

These results agreed with Al-Meida *et al*. (2010) who showed in their research at the *tpi* locus of the 10 isolates typed as assemblage A by standard PCR, 9 could be analyzed by RT-PCR, and all were classified as assemblage A plus B similarly, of the 18 isolates typed as assemblage B by standard PCR, analyzed by RT-PCR showed that two

isolates were classified as assemblage B, whereas 16 were (A+B).

Other studies observation were not compatible with the current results, that the majority of clinical samples were assemblage B, such as findings of Amar and colleagues in 2003 by RT-PCR and RFLP analyses of the *tpi* gene for sub genotyping of *G.lamblia* . Guy *et al*.(2004) proved results by RT-PCR Assay for detection and genotype differentiation of *G.lamblia* in stool specimens, where they noted that percentage of assemblage A was 20% while assemblage B was 60% and mixed specimens of assemblage (A+B) were 20%. In Egypt study conducted by Helmy *et al*. (2009) who used nested RT- PCR for targeting the *tpi* gene to detect and genotype *G.lamblia* in human feces, of the total samples, the percentage of assemblage A (75.6%), assemblage B (19.5%) and (5%) were mixed of assemblage (A+B).

The results of predominant of assemblage A, B and mixed infection may be subject to a number of factors; geographic locations of the patients studied, common source of human infection, contamination of public water with raw sewage from animal and human sources (Helmy *et al*., 2009).

The advantages of using RT-PCR are high sensitivity and specificity, it is able to detected DNA from a single trophozoite or cyst, moreover it could differentiate assemblages A and B of *G.lamblia* with a sensitivity of detecting an equivalent of one cyst of *G.lamblia* (Feng and Xiao, 2011). RT-PCR detects reaction progress, speed of analysis and precise measurement of the examined material in the samples (Singh *et al*., 2014), and the risk of contamination of the samples which may be occurred in conventional PCR may be eliminated by applying the RT-PCR (Adamska *et al*., 2010).

Detection and Genotyping *G.lamblia* by RT-PCR Technique in Environmental Samples

The majority of diagnostic methods used in the clinical practice have a limited application regarding the detection of protozoa in water samples, the most important causes restriction is much lower numbers of cysts in water in comparison with material collected from patients (Adamska *et al*., 2010). Treatment of the water samples had been conducted to reduce the contamination and inhibitors which present in environmental samples, because the efficiency of DNA extraction methods is determined by the DNA recovery rate and PCR inhibitor reduction during DNA extraction (Skotarczak, 2009). In current study the DNA extracted from the environmental samples directly without diagnosed by microscope, the process of extracting DNA succeeded only in seven samples from eighteen samples.

As it was mentioned previously the important route of transmission *G.lamblia*, is water either drinking water or recreational water and many of waterborne outbreaks of giardiasis happened in world's. In Baghdad the source of tap water that used from the people is Tigris river after series of treatment, in the same time many cases of giardiasis recorded that the contamination water is the cause, and which supports this findings study conducted by Al-Saqur *et al*. (2015) who scientifically confirmed the presence of *Giardia* in the water that reaches the houses.

In this study *tpi*A and *tpi*B primers that used to identify the *G.lamblia* in fecal samples used again to detect and genotype *G.lamblia* only in 7 water river samples, because the process of extracting DNA failed in the remaining eight samples 4/7 (57.14%) ,

three samples given negative results in RT-PCR although the process of DNA extraction was successful, all the positive samples for *G.lamblia* were collected in the summer and autumn season. one of samples represented assemblage A, two samples represented assemblage B and one sample mixed of assemblage (A+B) 25%,50%,25% respectively, table (3) and figure (2).

The failure of amplification of the *tpi* gene of *G.lamblia* in environmental samples as compared with other studies (regardless the type of target gene) may be due to that samples are rich in PCR inhibitors, such as humic acids, potassium dichromate, formaldehyde which could be co-extracted with DNA during the isolation and purification process and interfere with the PCR amplification (Skotarczak, 2009), so the recent studies used some materials to avoid the effect of PCR inhibitors such as Bovine Serum Albumin BSA (Anceno *et al*., 2007), beside low number of cysts in water samples. In general, the diagnosis of *Giardia* cysts in environmental samples is difficult and requires long time and intensive work and also well professional and experienced personnel (Carmena, 2010)

Current study agree with Guy *et al*. (2003) who found *G.lamblia* in 4 environmental water bodies and detected by RT-PCR with *bg* target gene, they reported in their study the role of some materials in improve the efficiency of PCR and amplified the target gene. In Iran Mahmoudi *et al*. (2011) demonstrated the presence of *Giardia* spp. in all 5 water samples from river in north of Iran by nested- PCR with 435bp fragment of *gdh*. Anceno *et al*. (2007) detected and genotyping *G.lamblia* isolated from surface and waste water in 13 out of 16 samples and all samples tested positive for both assemblages (A+B) of *G. lamblia* and this is not were consistent with current results about the distribution of assemblage.

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