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Detection of Giardia Lamblia Virus (GLV) by real-time PCR assay from diarrheic patients in Najaf/Iraq.

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Abstract

Background: ***Giardia lamblia*** **is one of the most public intestinal protozoa in a large number of vertebrates. This parasite causes giardiasis infection in affected people. So far, several scattered reports have shown the existence of a double-stranded RNA (dsRNA) virus called** *Giardia Lamblia* **Virus (GLV) in different** *Giardia* **species using molecular methods. The current study aimed to detect of GLV in** *G. lamblia* **species diagnosed in fecal samples of Iraqi patients.**

Materials and Methods: In this study that was done from December 2018 to July 2019, 750 specimens of stool were collected from patients who attended to hospitals in the Najaf Al-Ashraf province, Iraq. The *G. lamblia* **occurrence was examined by direct smear with Lugol's iodine, concentration by formalin ethyl acetate and PCR. The quantitative reverse transcription polymerase chain reaction (RT-qPCR) used for GLVscreening.**

Results: The Lugol's iodine smear revealed that 13.3% (n=100/750) of samples had positive results for *G. lamblia***. Also, the formalin ethyl acetate concentration technique showed similar results. The** *G. lamblia* **was detected in 80% (n=80/100) of fecal samples using PCR. The results of RT-qPCR showed that 12 samples (15%) had positive results for GLV while 68 specimens (85%) had negative results.**

Conclusion: This study exposed the presence of GLV in 15% of *G. lamblia* **isolates collected from Iraqi patients. It is recommended to perform the sequencing analysis of these viruses in future project and compare them with the existing Gene bank sequences.**

Keywords: Giardia *lamblia* virus, Giardia duodenalis, Giardia duodenalis, Giardiasis.

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Introduction

Giardia lamblia (syn. Giardia intestinalis, Giardia duodenalis) is one of the most common intestinal protozoa in a huge number of vertebrates, as well as domestic and wild animals and humans [1]. *Giardia lamblia* has two morphological stages including cyst and trophozoite, the cyst with two or four nuclei and trophozoite that was flagellated and live in the upper portion of the small intestine and is responsible for creating clinical signs of giardiasis in affected people. The cyst form that excreted with the host feces and remain in the environment for weeks is responsible for transmitting the parasite through fecal-oral route to others [3,4]. This parasite includes 8 main assigned genotypes A to H [1,2]. Although the infectivity of genotypes A and B has been established in humans, evidence of genotype E infection has also been reported in human [5].

Currently, giardiasis is one of the major individual and social health problems in different countries of the world known as a zoonotic disease [1,2]. About 280 million people worldwide were affected with giardiasis annually [6]. This infection has a wide range of clinical symptoms from asymptomatic form to acute and chronic diarrhea, abdominal pain, anorexia, nausea, vomiting, and general weakness [3,4]. Several studies have reported a various prevalence rates of *G. lamblia* ranging from 1.7 % to 72% in different regions of Iraq [7,8].

So far, some scattered reports have shown the existence of a double-stranded RNA (dsRNA) virus called *Giardia Lamblia Virus* (GLV) in different *Giardia* species in variety of animals using molecular methods [9,10]. Introduced in 1980s, this virus belongs to the genus *Giardia virus*, family *Totiviridae*. The dsRNA viruses have also been identified in other protozoa such as *Trichomonas*, *Cryptosporidium*, and *Leishmania* [10]. The presence of these viruses may affect the pathogenicity of the mentioned parasites in their hosts [4,10]. To the best of our knowledge, there is currently no published study inspecting the presence of the GLV in *G. lamblia* species collected from patients with giardiasis in Iraq. So, this study aimed to investigate the occurrence of GLV in fecal samples collected from Iraqi patients suffered from giardiasis using quantitative reverse transcription polymerase chain reaction (RT-qPCR).

Materials and Methods

Ethics

The study protocol was approved by ethical committee in college of medicine/university of Kufa according to the Declaration of Helsinki.

Study area and patients

In this cross-sectional study that was performed from December 2018 to July 2019, the stool samples were collected from patients suffering from gastrointestinal disorders who referred to hospitals in the Najaf Al-Ashraf province, Iraq. The patients selection was depended on the clinical symptoms that approved by a gastro enterologist. The epidemiological and clinical information were recorded in a questionnaire.

Sample collection and detection of giardia lamblia

All samples used in the current study was those collected from our previous investigation and put in disposable plastic container and kept in cool box and transferred immediately to the laboratory for phenotypic analysis [11]. The Lugol's iodine smear was done for *G. lamblia* detection in stool samples by the microscopic examination. Finally, the formalin ethyl acetate concentration technique was performed to enhance the detection chance according to previous described protocol [12]. The positive specimens for cyst or trophozoite of *G. lamblia* were stored at -20 °C for further PCR and RT-qPCR analysis.

DNA extraction

The DNA extraction was carried out using AccuPrep® stool DNA extraction kit (Bioneer, Korea) according to the manufacturer's technical manual. Briefly, 200 mg of the stool sample was added to a tube containing 20 µl of proteinase K, then 400 µl of SL buffer was added to the sample and vortexed. The tube was incubated for 10 min at 60 ℃. The tubes were centrifuged at 13000 rpm for 5 min and the supernatant was transferred to a new tube. In next steps, 400 µl of SB buffer (10 minutes incubation at 60 ℃) and 100 µl of isopropanol were added. This liquid was transferred to the binding column tube and centrifuge at 8000 rpm for 1 min. The solution was discarded from the collection tube and 500 µl of WA1 buffer was added to the column and centrifuged at 8000 rpm for 1 min. Again, the solution was discarded and 500 µl of W2 buffer was added and centrifuged for 1 min at 8000 rpm. Same as previous step, the solution was discarded from the collection tube. The collection tube was spined down once more at 13000 rpm for 1 minute to remove the remaining ethanol. Then, the binding column was transferred to a 1.5 ml tube and 50-200 µl of EA buffer was added and remained for 1 minute to permeate the column. Finally, the column was centrifuged at 8000 rpm for 1 min to yield DNA.

PCR detection of Giardia lamblia

The extracted DNA was measured by PCR using a pair of specific primers for small subunit ribosomal RNA (ssrRNA) gene (530 bp) of *G. lamblia*. The used primers were as follows: forward primer (5'-GCGATCAGACACCACCGTAT-3') and reverse primer (5-CGCCTACAAGACATTCCTGGT-3') [11]. The PCR reaction was accomplished in a final volume of 20 µl containing 1.5 µl of distilled water, 0.5 µl of each forward and reverse primer (10 µM), 12.5 µl of master mix 2X, and 5 µl of extracted DNA. The PCR assay was done in the Biometra thermocycler (Biometra GmbH, Germany) with following program: an initial denaturation at 95 ºC (5 min), 30 cycles of denaturation at 95 ºC (30 sec), annealing at 58 ºC (30 sec) and extension at 72 ºC (1 min), with a final extension at 72 ºC (5 min). Finally, the PCR products were separated by 1% agarose gel electrophoresis and visualized by a UV illuminator system.

Detection of GLV by RT-qPCR (RNA extraction)

From 100 µl of each stool sample that give positive results for ssrRNA gene of *G. lamblia* in PCR step by Bioneer RNA extraction Kit (Korea) following manufacturer's protocol, the RNA was extracted and checked with Nanodrop spectrophotometer at 260/280 nm (Thermofisher Scientific, USA).

RT-qPCR protocol

The master mix of RT-qPCR was prepared according to the kit instructions. The components of RT-qPCR reaction were added into RT-qPCR tube that contains TaqMan probe premix and Rocket Script reverse transcriptase (8 wells strips tubes). The components were mixed by Exispin centrifuge (Bioneer, Krea) for 3 minutes at 3000 rpm to the resuspension of all components. The following specific primers and probe were used for screening of GLV: forward primer: TGGCGGTTCAGTTTATGCAC, reverse primer: TTGTTGAAACTGCGCTTGCC, probe: FAM-TGCGAGGTCGGAGCTCTGGG-BHQ1. The RT-qPCR was done with ABI device (Thermofisher Scientific, USA) in the following program: 1 cycle of reverse transcription at 50 °C for 15 min, 1 cycle of PCR pre-denaturation at 95 °C for 5 min, 50 amplification cycles each consisting of denaturation at 95 °C-20 sec, and annealing /extension 60 °C- 60 sec. The analysis of RT-qPCR data was calculated by the threshold cycle number (CT value) that obtainable the optimistic amplification of GLV in RT-qPCR cycle number.

Results

Out of the 750 cases that were recorded as a gastrointestinal disturbance, 100 cases (13.3%) were reported as positive for giardiasis by Lugol's iodine smear. Also, the formalin ethyl acetate concentration technique showed similar results in comparison with Lugol's iodine smear. These positive samples were collected from 64 males and 36 females, respectively. Patients in the age group of 10 years and below had the highest frequency of parasite (50%) (Table 1). The *G. lamblia* ssrRNA gene was detected in 80% of (n=80/100) fecal samples using PCR. The results of RT-qPCR showed that 12 samples (15%) had positive results for GLV while 68 specimens (85%) had negative results.

Discussion

In this study, the prevalence of G. lamblia was 13.3% using Lugol's iodine smear and formalin ethyl acetate concentration that was higher than previous reports (7.9%) from same region in Iraq (12). However, the prevalence of this protozoan in Najaf and some other cities has always been higher than other regions of Iraq, according to available reports (12). Another

study by Hasan et al. (13) from Tikrit city, Iraq showed the prevalence of 20.87% and 11.54% in rural and urban areas, respectively. The total infection rate was 14.30% that showed higher prevalence in comparison with the current research. These differences may be due to the type of population being studied, the sample size, the season of sample collection, and the protozoan detection method. Other factors including nutritional, socio-economical, demographical, geographical conditions, environmental, as well as health-related behavior have direct influence on prevalence rates of intestinal parasites (12). The current study revealed that the highest rate sexes were recorded in the ≤10 year age group. This result was corresponding to previous report by Samie et al. (14) from South Africa. They claimed that individuals with the age group of 3-20 years are more prone to the *G. lamblia* infection. Also, the findings of the current study were consistent with another study by Salman et al. (15) from Kirkuk province, Iraq who showed higher incidence rate (5.99%) of giardiasis in stool samples of studied patients aging from 1 to 10 years in comparison with other age groups. Also, the *G. lamblia* was detected in more males that was similar to previous studies (12,13). One of the reasons for this phenomenon may be due to the increased activity of men in polluted environments outside the home and contact with poor hygiene conditions.

The current study was the first research that evaluated the occurrence of GLV in fecal samples collected from Iraqi patients. The occurrence rate of GLV was 15 % using the RT-qPCR. This virus was first observed in the *G. lamblia* Portland 1 strain in 1986 (15). So far, the GLV has been reported in *Giardia* species from animals including dog, cat, beaver, guinea pig, and sheep (9). When searching the previous literature, we couldn't find many similar studies to compare the results of the current experiment with them. Previous studies showed that not all *Giardia* species are susceptible to GLV infection that may due to the lack of appreciate receptors (16,17).

Conclusion

This study revealed the presence of GLV in 15 % of *G. lamblia* isolates collected from Iraqi patients. It is recommended to perform the sequencing analysis of these viruses in future project and compare them with the existing Genebank sequences.

Acknowledgments

None

Conflict of Interest

The authors declare no conflict of interest.

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