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Original Article

Evaluation of a New Primer In Comparison With Microscopy for the Detection of *Giardia lamblia* Infection in Stool Samples

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Abstract

Background: Among the most important parasitic disease, causing diarrhea, *Giardia lamblia* is noteworthy. Nowadays detection methods for these parasites include parasitological methods such as microscopic examination. The sensitivity of these methods relies on the expertise and experience of examiners. In contrast, molecular methods such as PCR are less dependent on the expertise of the examiner. Here we developed a PCR for the detection of *G. lamblia* genome in stool samples in comparison with microscopy, which is the gold standard.

Methods: For the evaluation of primers, 22 positive samples and 47 negative samples were used. QIAamp DNA Stool Mini Kit (QIAGEN, Germany) was used for DNA extraction from feces. Primers for PCR were designed using Primer-BLAST which uses Primer 3 to designing specific primers (NCBI/ Primer-BLAST).

Results: Sensitivity of the PCR was done with 100% (95%CI: 84.56-100) for the detection of *G. lamblia* DNA isolated from patients stool samples which were positive for *G. lamblia* cysts and/or trophozoites using microscopy as gold standard. In comparison with microscopy, PCR had showed the specificity of 97.87% (95%CI: 88.71-99.95).

Conclusion: We designed new primers for the *Giardia,* and PCR method for the rapid and accurate identification of *Giardia* parasites established. With consideration to the routine diagnosis techniques in medical parasitology and their limitations such as time consuming, laborious, less sensitivity etc. This *G. lamblia* PCR is a sensitive and specific application for the diagnosis of *G. lamblia* and provides us a reliable method in the routine intestinal parasitic infection laboratory diagnosis.

Introduction

Among non-viral diarrhea, causing agents *Giardia lamblia* Infection is very prevalent (1). Microscopy is the usual method has been used for diagnosis of *G. lamblia* infections by using stool samples; closely rely upon times of sampling, application of concentration methods and how much expertise and experience has the technician performing the test. Consequently, microscopic test for the diagnosis of *G. lamblia* in stool samples is behind hand and uneconomical (2), due to expensiveness of hand working in comparison to machine work in the developed countries. Over light microscopy, there are some immunological methods for the diagnosis of *G. lamblia* cysts or trophozoites in biological samples. One is the detection of *Giardia* antigens in the stool of patient (3). Application of enzyme immunoassay for antigen detection does not eliminate the need to analyze multiple stool specimens for accurate detection of parasite (4).

In general, conventional methods using microscopy, biochemistry and immunological approaches have serious restrictions such as essential need to a high degree of expertise and experience in the diagnosis of *Giardia* (5). Therefore, molecular techniques with high sensitivity, specify and workable there have been in demand. In recent for the detection of diarrhea causing agents such as viruses and bacteria many PCR based methods have been published but for the diagnosis of parasites it seems to be less addressed and, application of these methods for the diagnosis of parasitic diseases in routine laboratories is still very limited (2). DNA isolation from stool samples is very complicated and grinding. Nowadays, there are some methods for nucleic acids isolation from biological samples, which could remove PCR inhibitors (6).

Combination of simple and quick DNA isolation methods with easy genome amplification and detection techniques lets us to use these techniques in routine laboratory platforms for the detection of parasites. In this regard, we developed a PCR for the detection of *G. lamblia* genome in stool samples in comparison with microscopy, which is the gold standard.

Material and Methods

More than 500 stool samples in the years 2012 to 2013 were examined, among which 22 positive samples and 47 negative samples were used. The samples from different parts of Iran such as Tehran and Bandar Abbas were collected. A standard diagnostic method in this study (Golden Standard) was parasitological method, which is describing as follow. Microscopic examination was performed for all samples. For loose, soft and formed specimens formal-ether concentration was performed, then, wet mount prepared slides stained with temporary iodine stain and investigate for cyst and/or trophozoite of *Giardia* using 400 × magnifications. For watery and dysenteric samples, iodine stained wet mount preparation had done also without a formal-ether concentration for the detection of active trophozoites.

We used QIAamp DNA Stool Mini Kit (QIAGEN, Germany) for DNA extraction from feces, 180 to 220 mg feces suspended in 1.4 ml of ASL buffer provided in kit and following the other commands recommended by manufacturer, DNA was isolated with QIAamp DNA Stool Mini Kit spin columns.

Primers for PCR were chosen using Primer-BLAST. It uses Primer3 to design PCR primers and then uses BLAST and global alignment algorithm to design specific primers (NCBI/ Primer-BLAST) (7), based on the *G. lamblia* Cathepsin L-like protease gene sequence for *G. lamblia* (GenBank accession no. XM_001706220.1) that *G. lamblia* DNA should be detected specifically.

The *G. lamblia* specific primers consisted of forward primer GlF (5 ′- AATCTGTT-GACTTAAGGGAGTA-3 ′; positions185–206), reverse primer GlR (5 ′-ATTGAGTCATT-ATAGGGATTGT-3 ′; positions 647–626), product length: 463 base pairs (Fig. 1).

The specificity of the PCR was checked out by a range of DNA extracted of various microorganisms includeing *E. histolytica*, *E. dispar*, *Entamoeba coli*, *Blastocystishominis, Cryptosporidum* spp. and from *Escherichia coli*, *Vibrio cholerae*, and *C. albicans*.

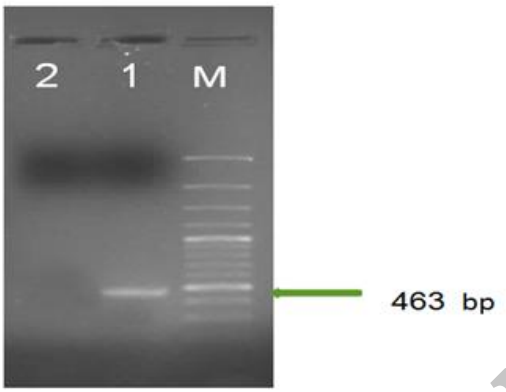

Fig. 1: 1% Agarosegel stained by ethidiumbromid shows PCR product by Gl F&Gl R primers
M: size marker, Lane 1: G. Lamblia DNA, Lane 2: Negative control

In DNase and RNase free PCR tubes Amplification reactions were performed in a volume of 50 ml with 10x PCR buffer, 1mM MgCl2, 5 µl of the DNA template from the stool samples, 1 unit/µl Taq DNA Polymerase and 20 pmol/µl of each specific primer. PCR was carried out by using a peqSTAR thermocycler (Peqlab, Germany) under the following conditions: 5 min at 94°C followed by 35 cycles of 60 s at 94 °C, 90 s at 55 °C, and 2 min at 72 °C followed by 10 min at 72 °C.

Results

DNA of 47 stool samples, which were negative for parasitic infections in microscopy, had tested. Sequencing had done on the amplicon produced from *Giardia* samples achieved of culture for analyzing, and verified to present image of the accurate sequence of the targeted DNA.

Sensitivity of the PCR was done with 100% (95%CI: 84.56-100) for the detection of *G. lamblia* DNA isolated from patients stool samples which were positive for *G. lamblia* cysts and/or trophozoites using microscopy as gold standard. In comparison with microscopy, PCR showed 1 false positive of 47 samples which were negative by microscopy in which the specificity of the test calculated to be 97.87% (95%CI: 88.71-99.95). Thus, positive predictive value (PPV) of the PCR was achieved of 95.65% (95%CI: 78.05-99.89), and the assay negative predictive value (NPV) was 100% (95%CI: 92.29-100). The accuracy of the test was 98.5%.

Discussion

Prevalence of *G. lamblia* in developed countries is 2 to 7% and in developing countries is about 20 to 30% (11, 12). Molecular methods have high sensitivity against conventional methods (13), among these approaches, PCR have been used in studies for molecular epidemiology assessments and understanding about zoonotic transmission possibility of *Giardia* (14). Potentially in developed settings it could be used for diagnostic proposes.

In our study, we used well-marked positive and negative stool samples as controls. In comparison with microscopy as the gold standard designed PCR results showed a sensitivity of 100% (95%CI: 84.56-100), the *G. lamblia* PCR achieved specificity of 97.87% (95%CI: 88.71-99.95) for *G. lamblia*. PCR showed the positive predictive value (PPV), and the negative predictive value (NPV) of 95.65% (95%CI: 78.05-99.89) and 100% (95%CI: 92.29-100) respectively.

It is very challenging to elect the 'gold standard' in the studies like our study (2), but scien-

tists stated that, microscopy is still the gold standard for the detection of many protozoan parasites (8). In this way, we used the microscopy as gold standard and compared our PCR with it. Therefore, PCR showed high sensitivity and specificity in comparison with microscopy for the diagnoses of *G. lamblia* infections. These findings are alike with the findings of Verweij et al. (2) who used a real-time PCR for the diagnoses of *G. lamblia* infections. One false positive result was observed by the PCR, occurrence of false positive results can be due to many various causes such as contamination which could happen accidentally during the procedure or can occurs due to usage of previously contaminated reagents, even it could happen because of aerosol contamination. However, contamination is the Achilles' heel in the open tube system tests like PCR in contrast to those with closed tube systems such as real-time PCR.

There were no false negative results achieved by the PCR, the main reason for this as the case may be the existence of appropriate DNA template in the reaction which we had used. As it proved there were many PCR inhibition factors in the stool samples which in this study using the QIAamp DNA Stool Mini Kit PCR inhibitors removed as manufacturer described.

In the clinical laboratory, *Giardia* diagnosis may face various problems, in some cases where *Giardia* is not seen in feces but clinical symptoms are suspecting, in such cases duodenal sampling by a string test for microscopic examination is recommended (9) , but it is unpleasant for patient and slightly could be an invasive procedure. In situations like these a test which is potent to track parasites' footprint like PCR will have a higher value.

Immunological tests like direct fluorescent antibody (DFA) test are commercially available but limitation is requirement of laboratories access to a fluorescence microscope (10).

Anyway, with consideration to the routine diagnosis techniques in medical parasitology and their limitations such as; time consuming, laborious, less sensitivity etc., this *G. lamblia* PCR is a sensitive and specific application for the diagnosis of *G. lamblia* and provide us a reliable method in the routine intestinal parasitic infection laboratory diagnosis.

As well as possible, PCR proffer multiplex characterization of various objects in the same sample. Therefore, in subsequent steps authors will try to develop a multiplex PCR with using this *G. lamblia* PCR in combination with PCR assays for other pathogens like *E. histolytica* and *C. parvum*.

Conclusion

This PCR showed reliable sensitivity and specificity for the detection of *G. lamblia* in stool samples and has capability to perform in the routine diagnostic laboratories.

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The authors declare that there is no conflict of interests.

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