

Genotyping and phylogenetic relationship of giardia isolates from Sudanese suspected patients using (TPI) triose phosphate isomerase gene.

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Abstract

Giardia lamblia is one of the most common intestinal pathogens in both humans and animals throughout the world, globally, there are greater than 700,000 deaths per year associated with diarrheal disease, and understanding the organism requires more in-depth knowledge of its biology. The current study aimed to establish a research platform for the molecular characterization of Giardia isolates in Sudan, focusing on the biology of giardia, through, molecular characterization attempts to genotyping. A laboratory-based experimental study was designated. A total of 30 fecal samples of giardia positive from suspected patients after informed consent were used in this study from three states. Molecular characterization of giardia isolates which included DNA extraction by Guanidine HCL after liquid nitrogen treatment. PCR detection and sequencing were obtained. PCR results showed that only 5 samples were positive out of 30 that represent 17% total of cyst samples when using tpi gene 605 bp, in the Sequencing results only two samples were successfully sequenced (one from Khartoum and one from Sinnar states) while bioinformatics results showed similarity between these isolates through alignment and multiple alignments to giardia in Africa. After sequencing of tpi gene, the Sudanese isolates from Khartoum state were found to be identical to genotype (A) with 80%, while samples from Sinnar State were identical to genotype (B) with 90%.

Keywords: Genotyping, Tpi gene, Alignment, Sinnar, Giardia.

Introduction

Giardia was one of the first protozoans to be described. In 1681 van Leeuwenhoek discovered the trophozoites of this genus. The Dutch microscopist made glass lenses and set them into metal frames which he made into simple microscopes. Globally, there are greater than 700,000 deaths per year associated with diarrheal disease. The flagellated intestinal parasite, giardia lamblia is one of the most common intestinal pathogens in both humans and animals throughout the world [1]. *G. intestinalis* has a wide genetic variety and its characterization helps in the understanding of its transmission dynamic [2]. PCR genotypes classification of Giardia in feces depends on quantity and quality of purified DNA and the removal of a great number of inhibitors [3]. Currently, molecular genotyping of *G. intestinalis* is based on analyses of one or several of the following genetic loci: small subunit ribosomal DNA, elongation factor 1- α , histone

2b, and histone 4, β -giardin, glutamate dehydrogenase, and triose phosphate isomerase (ssrDNA, ef, h2b, h4, bg, gdh and *tpi* respectively). The first four (ssrDNA, ef, h2b, h4) are considered conserved genetic markers and the latter three (bg, gdh, *tpi*) are considered more variable [4]. Since this work mainly focuses on *G. intestinalis* infection in humans only genotyping of the two human infecting assemblages A and B is described. Meta-analyses of several molecular typing studies of the human infecting assemblages A and B have shown that assemblage B Giardia is more commonly occurring than assemblage A, in humans [5,6]. The currently utilized loci and especially the more variable loci, provide ample discrimination of assemblage B isolates, whereas little discrimination is found among assemblage A isolates [4-6]. The human infecting *G. intestinalis* assemblages (A and B) have been divided into different sub-assemblages based on sequencing results from the bg, gdh and *tpi* loci.

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Assemblage A is grouped into AI, AII, and AIII, where AII can be further subdivided into AII-1 and AII-2 based on two nucleotide substitutions on the *bg* locus. AII is described to typically infect humans, AI has been found in humans but mainly infects animals and AIII has exclusively been found in animals [5-7]. The routinely used markers for sequence based genotyping of assemblage A provide low resolution between different A isolates, and there is a high necessity to find new molecular markers for genotyping of assemblage A *G. intestinalis*. Sequence-based genotyping of assemblage B has been hampered due to a high frequency of mixed base polymorphisms, seen as double peaks at single nucleotide positions in the sequencing chromatograms [6,7]. Although there is a high level of difference in the sequences between different B isolates, there is currently no functional way of categorizing them into different sub-assemblages this is due to the high frequency of mixed base polymorphisms found in the majority of sequences from clinical samples.

Materials and Methods

Sources of DNA

A total of 30 (10 Khartoum, 10 Sinnar, 10 Gezira) fecal samples were used for DNA extraction, sample size was calculated by the formula suggested by [8] $N \geq 50 + 8p$ where p is number of predictors if we predictor (p) = 1 in addition to other samples from culture used as control positive, fecal samples were purified and washed in phosphate buffer saline PBS then confirmed microscopically and counted before used in DNA extraction and preserved -20°C . Ethical approval for the current study was obtained from ethical committee of Tropical Medicine Research Institute (TMRI) Sudan, Khartoum. Standard equipment used in a microbiology and molecular biology laboratory was used.

DNA extraction method

DNA extraction was conducted according to a protocol designated by [9-12] with slightly modification to optimize disruption of the cysts prior to DNA extraction. Cyst samples were treated by boiling in 100°C for 2 min and put in liquid nitrogen for 2 min these steps were repeated 3 times before DNA extracting a guanidine hydrochloride method was used as the following.

1 ml of sample added to 1 ml lyses buffer 2% SDS, 1 ml guanidine hydrochloride, 300 μl ammonium acetate, and 10 μl proteinase K and incubated at 37°C overnight, DNA was harvested, then confirmed and evaluated by scanning Nano drop system for reading DNA and protein concentration, and kept at -20°C as stock for further PCR experiments.

PCR performed according to [13] with slightly modification. Using Triose phosphate isomerase gene *TPI* fragment 605 bp was amplified by using primers AL3543 [5-AAATATGCCTGCTCGTCG-3] forward and AL3546 [5-CAAACCTTITCCGCAAACC-3] reverse as target gene sequence. The reactions were performed by using ready prepared primex (MiQ COMPANY) 20 μl commercial product contain (200 μM dNTP, 1X PCR Buffer, 3mM MgCl_2 , 5U of Tag polymerase). In the reaction we add 1.5 μl forward primer

and 1.5 μl reverse primer, 10 μl DNA template and complete the volume to 20 μl with sterile D.W. and the reaction was conducted at 94°C for 5 min an initial temperature 94°C for 30 sec, 55°C for 45 sec, 72°C for 2 min, 72°C for 10 min to complete 30 cycle a PCR product was confirmed by run in 1.5% agarose with ethidium bromide stain 1% read by Gel Documentation System for target visible bands then record the result. PCR from feces depend on the quality and quantity of purified DNA and removal of great variety of inhibitors such as bilirubin salts, bilirubin, and 10% of formalin can inhibit PCR [3]. This test used in order to control PCR inhibitors substances which may found in faecal materials. The test conducted by added 10 μl DNA template which give positive PCR to that give negative and run it in thermo cycler with same PCR program and the results were records.

Bioinformatics, sequencing and phylogeny analysis

Sequencing was done by www.Bioneer.com Korea to four PCR positive samples. Alignment for sequences was obtained by Basic Local Alignment Search Tool (BLAST) www.ncbi.com which allows rapid sequence comparison of a query sequence against a database in order to compare the similarity between the sequences and other reference sequences on DATA base, DNA sequences are homologous if they are at least 70% identical, and there are three steps to run BLAST. (1) Choose the sequence (query) (2) Select the BLAST program (3) Choose the database to search. Then click BLAST. A phylogenetic tree and multiple alignment was conducted by (clusterW and cluster Omega) software programs in order to study the relationships between different isolates and to compare with other sequences from Data base.

Results

The variant in similarity sequences were subjected to multiple alignment, using ClusterW and cluster Omega software, matching with *tpi* nucleotide sequences of *Giardia intestinalis* assemblage A and B isolate deposited from gene bank under ID (KY444791.1), (gi|1255657882|gb|KY444788.1), (gi|1255657879|gb|KY444787.1), (gi|340885174|gb|HQ836660.1) (LN626349), (LN626350.1).

Result in (Figures 1-4) pair alignment between two sequences sample1 and sample2 shows identity loci (marked with stars * and gabs marked -----) there was identical between both samples. (Figure 5) Multiple alignment sequences between different *Giardia* isolates and sample1,2 showing identity and gabs (Figure 6) Multiple alignment sequences between different *Giardia* isolates and sample1,2 showing identity and gabs. The result show that sample1 identity 80% to genotype (A) or assemblage A, while sample2 identity 90% to genotype (B) assemblage B.

Phylogeny relationships obtained by using ClusterW and cluster Omega software in order to study the relationships between different isolates. (Figure 7) a phylogram which show that sample 2 significantly related to assemblage B (KY444791.1) both were branched from one root assemblage B. (Figure 8) a phylogram which show that sample 1 significantly related to assemblage A (gi|1255657879|gb|KY444787.1) both were branched from one root assemblage A. (Figure 9). A phylogram which show that both samples 1,2 were related to

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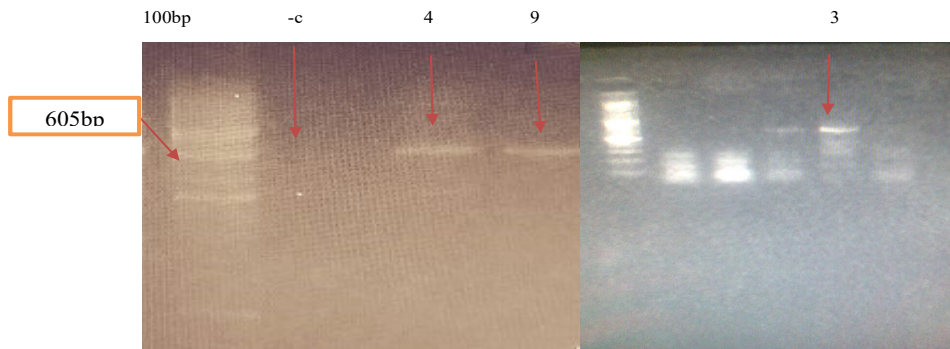


Figure 1. Ethidium bromide-stained 1.5% agarose gel electrophoresis of PCR products for *tpi* gene 100-base pair DNA marker; -C negative control (distilled water) 4, 9 and 3 positive samples.

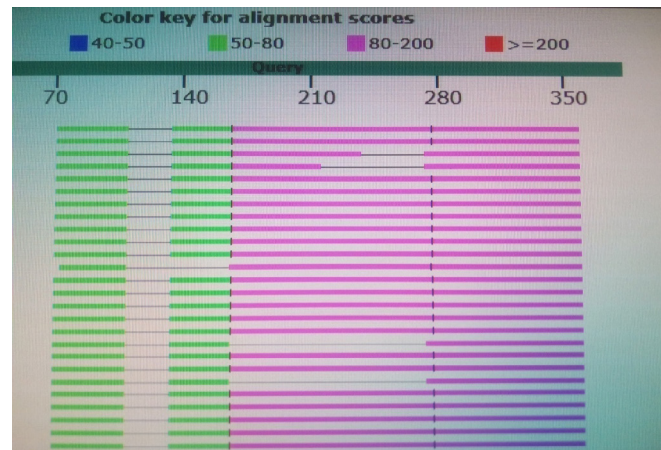


Figure 2. Result of similarity sequence of sample 4 pink color indicate to 80% of similarity.

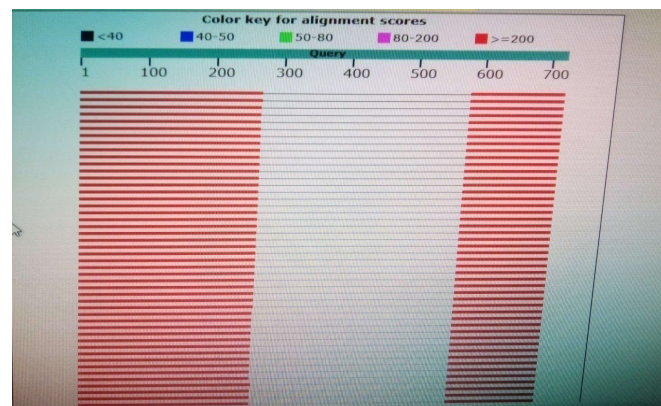


Figure 3. Result of similarity sequence of sample 9 red color shows 90 to 100% of similarity.

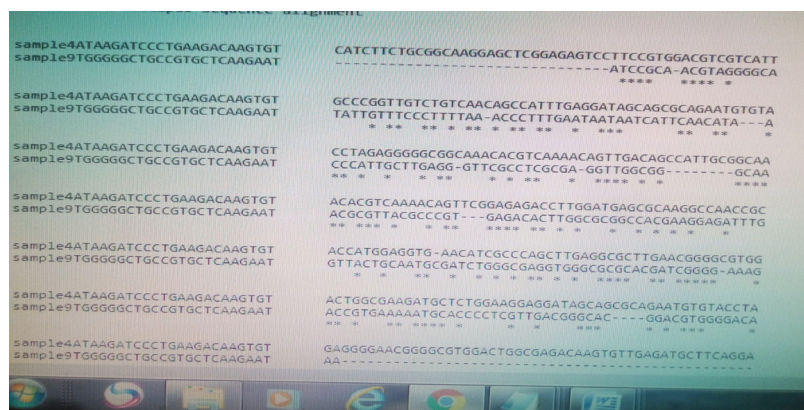


Figure 4. Pair alignment between two sequences sample4 and sample9 showing identity and gaps.

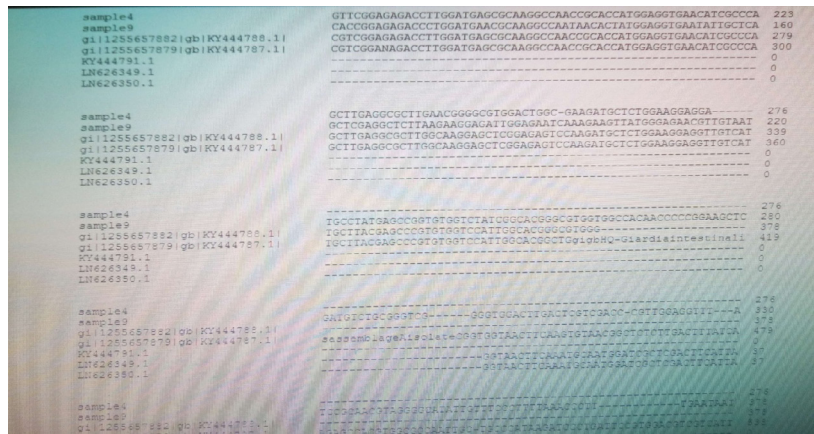


Figure 5. Multiple alignment sequences between different Giardia isolates and sample 4,9.

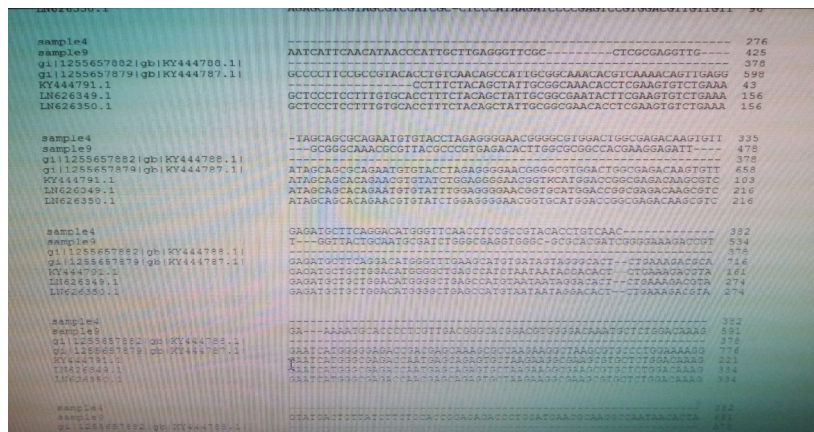


Figure 6. Multiple alignment sequences between different Giardia isolates and sample 4,9.

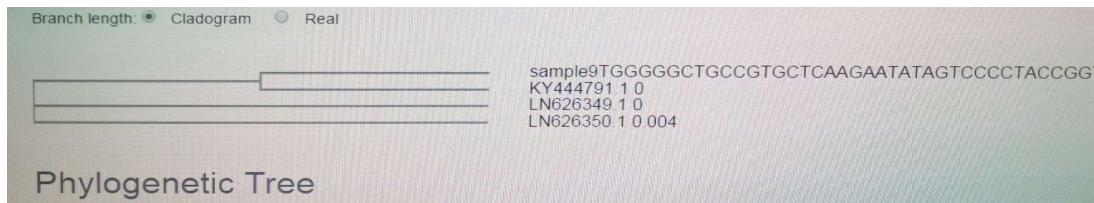


Figure 7. Phylogenetic tree showing position of sample 9 and its relationships among different isolates of *G.intestinalis* assemblage B.

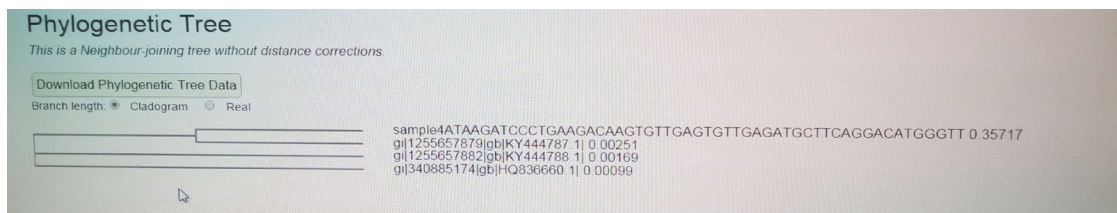


Figure 8. Phylogenetic tree showing position of sample 4 and its relationships among different isolates of *G.intestinalis* assemblage A.

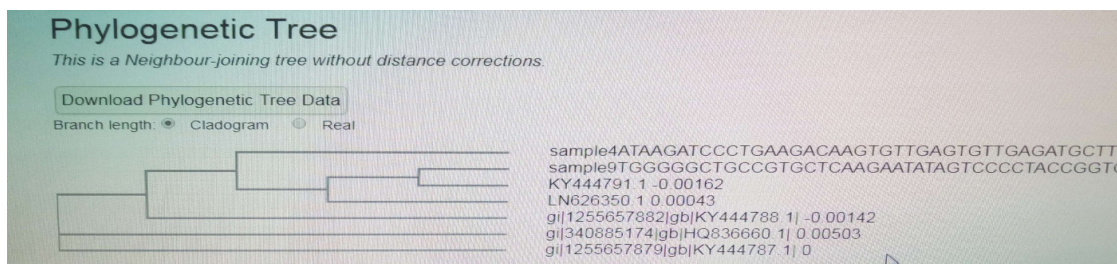


Figure 9. Phylogenetic tree showing relationships between both sample 4,9 and other different isolates of *G.inestenlis*.

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Table 1. PCR results.

Locality	PCR +VE	PCR -VE	TOTAL
Khartoum	2(20%)	8(80%)	10
Sinnar	3(30%)	7(70%)	10
Gezira	0(0%)	10(100%)	10
Total	5(17%)	25(83%)	30

each other. While sample 4 was close related to assemblage A and sample 9 related to B

Discussion

Molecular characterization was studied; DNA extraction was conducted by Guanidine HCL after Liquid nitrogen treatment. PCR result show that only 5 samples were positive out of 30 that represent 17% from total of cyst samples (Table 1) when using *tpi* gene 605bp lower grade of PCR may due to DNA conc, purity and PCR inhibitors, but it considered high sensitive. Sequencing result obtained by bioneer company(Korea) only two samples were successfully sequenced (sample 1 Khartoum and 2 Sinnar), bioinformatics results similarity in alignment and multiple alignment show that sample1 identity 80% to genotype (A) or assemblage A, while sample2 identity 90%to genotype (B) assemblage B. Phylogeny relationships obtain by using ClusterW and cluster Omega software in order to study the relationships between different isolate (Figures 7-9) which show that sample 9 significantly related to assemblage B (KY444791.1) while sample4 related to assemblage A.(gi|1255657879|gb|KY444787.1) and both samples 4,9 are each related to other (Figure 9) Similar result was obtained by [13] in Pakistan [14] confirmed PCR as sensitive technique in compared with ELISA and microscopic technique when used *gdh* gene which obtain that PCR more specific and sensitive than other techniques , In addition, the genetic characterization of the parasite was carried out by amplifying and sequencing the glutamate dehydrogenase (*gdh*) gene in southern Brazil [15], the DNA sequences revealed that 7 (36.8%) out of 19 isolates belonged to assemblage B, while 6 of them (31.6%) belonged to assemblage C, 5 (26.3%) to assemblage A and 1 (5.3%) to assemblage D this is other supportive study to our finding which concluded that the giardia isolates from Sudanese suspected patients was found resemble to geno type B after sequencing of *tpi* gene and bioinformatics analysis.

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