UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Ciencias Biológicas y Ambientales

Genotyping of *Giardia intestinalis* in Clinical Samples

Articulo Académico

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Licenciatura en Biología

Trabajo de titulación presentado como requisito

para la obtención del título de

Licenciado en Biología

Quito, 21 de mayo de 2019

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

COLEGIO DE CIENCIAS BIOLOGICAS Y AMBIENTALES

HOJA DE CALIFICACIÓN
DE TRABAJO DE TITULACIÓN

Genotyping of *Giardia intestinalis* in Clinical Samples

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Quito, 21 de mayo de 2019

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RESUMEN

Giardia intestinalis es un parásito intestinal distribuido en todo el mundo y afecta principalmente a países en vías de desarrollo. Para evaluar el potencial zoonótico de este parásito se realizó la caracterización genética de dos genes: Beta-giardina (bg) y la deshidrogenasa de glutamato (gdh) loci a partir de aislados de 11 muestras de heces humanas para generar una perspectiva de los ensamblajes y subensamblajes circulantes en una población rural. Para bg se utilizó un protocolo de PCR anidado para amplificar un fragmento de 511 pb aproximadamente dentro del gen y para gdh se utilizó un protocolo semi anidado de PCR para amplificar un fragmento de 432 pb aproximadamente dentro del gen. Los fragmentos obtenidos de los dos genes fueron secuenciados. Los resultados obtenidos evidencian la presencia de los ensamblajes A y B los cuales pueden infectar tanto a humanos como a animales transmitiéndose mediante el consumo de agua o alimentos con presencie de quistes o por el contacto con heces de organismos infectados.

Palabras clave: *Giardia intestinalis*, ensamblajes, beta-giardina, glutamato deshidrogenasa.

ABSTRACT

Giardia intestinalis is an enteric parasite with a worldwide distribution, presenting higher rates of prevalence in developing countries. Giardiasis is considered zoonoses and some authors have proposed a sub-classification of *G. intestinalis* in eight genetic assemblages (A–H). To assess the zoonotic potential of this parasite, molecular analyses of the beta-giardin (bg) and the glutamate dehydrogenase (gdh) genes were performed from 11 human stool samples isolates of rural communities near Quito. A nested PCR protocol was necessary to amplify a fragment (511 bp) of the bg gene and another fragment (432 bp) of the gdh gene, then were sequenced. Results showed the presence of sub-assemblages AII, BI and BII belong to assemblages A and B respectively. These assemblages have been found in humans as well as in animals supporting the zoonotic potential of the waterborne transmission of the cysts in these rural communities near to Quito.

Key words: *Giardia intestinalis*, assemblages, beta-giardin, glutamate dehydrogenase.

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Introduction

Giardia intestinalis (syn. Giardia lamblia and Giardia duodenalis) is one of the most common parasites encountered in most mammals (Feng & Xiao, 2011). This specie is the only one within the genus that can be isolated from human as well as livestock samples (Beck et al., 2011). The parasite is distributed all around the globe causing symptomatic infections in developed countries and a larger number of asymptomatic infections in developing countries (Hellard, 2000). Being the cause of Giardiasis, this parasite represents a major concern in the public health of developed and developing countries (Sprong, 2009). The similarity among several gastroenteritis diseases represents an issue for the diagnosis of the disease and its proper follow up by the health entities (Thompson, 2008). A large number of asymptomatic patients infected or carriers present another concern for the correct assessment of the prevalence of the parasite and the infection around the world (Eisenberg, 2002).

G. intestinalis causes giardiasis in various groups within mammals as well as in humans so it is considered a zoonotic disease (Feng & Xiao, 2011). Based on genetic and molecular analyses using the tpi, bg, gdh, SSU rRNA and ef1α genes*, G. intestinalis* can be represented as a complex composed by 8 members called assemblages classified according to their host range (Feng & Xiao, 2011). Assemblages A and B are the ones that have a wider range than all the other assemblages described, being found in humans, domestic animals, and cattle (Ryan & Cacciò, 2013). Assemblages C and D have a very similar host range between them, being found in domestic animals such as cats and dogs, having more present in canines (Palmer et al. 2008).

The remaining members of the complex have usually a more restricted host range described, assemblage E is present in domestic animals such as cattle, assemblage F is present in the feline group, and assemblage G can be found in rodents (Cacciò & Ryan, 2008). The newest assemblage reported and described is the one infecting and present in seals, assemblage H (Lasek-Nesselquist et al. 2009). Using more specific criteria in phylogeny studies assemblages A and B can be subdivided into 3 groups for assemblage A and 4 groups for the assemblage B (Monis, 1999).

Assemblages A and B having such an open host range present an interesting subject of research with the purpose of understanding the epidemiology of the infection in humans and for tracing the sources of the infection (Sprong, 2009). Further study on these two assemblages may also help to unveil and understand the zoonotic potential of this parasite, which can cause the infection to humans coming from animals and to animals from humans (Isaac-Renton, 1994). Studies on the genetic characterization at the triosephosphate isomerase (tpi), beta-giardin (bg) and glutamate dehydrogenase gene (gdh) loci show that the genetic variability among the 8 accepted assemblages

may be greater than it was first conceived (Cacciò & Ryan, 2008). The aim of this study was the genetic characterization of *G. intestinalis* isolates from human stool samples positives by microscopy to identify the assemblages that may be present in rural communities near Quito.

Material and Methods

Sample collection

The samples used in this study were obtained from the Clinical Laboratory that belongs to the Instituto de Microbiología of the Universidad San Francisco de Quito. The samples belong to the PRISA research project of the Institute for the study of different parasites in human samples. The presence of the parasite within the samples was confirmed using a microscope and the Crypto/Giardia Duo-Strip (CorisBioconcept) according to the instructions given for the manufacturer.

DNA extraction

The DNA was extracted from aliquots of each positive sample using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to the directions provided with the kit. Purified DNA samples obtained after the extraction was diluted to half the original concentration in several aliquots and stored at -20 °C for downstream molecular analyses.

Primers used

For the amplification of the gdh gene fragment the GDHeF (TCAACGTYAAYCGYGGYTTCCGT), GDHiR (GTTRTCCTTGCACATCTCC), and the GDHiF (CAGTACAACTCYGCTCTCGG) primers were used (Read et al., 2004). For the amplification of the bg gene fragment the G7eF (AAGCCCGACGACCTCACCCGCAGTGC), G759eR (GAGGCCGCCCTGGATCTTCGAGACGAC), G99iF (GAACGAACGAGATCGAGGTCCG), and the G609iR (CTCGACGAGCTTCGTGTT) primers were used (Gil et al., 2017).

Molecular analysis

Aliquots resulted from the extraction process were used for semi-nested and nested PCR protocols at the glutamate dehydrogenase (gdh) and beta-giardin (bg) loci respectively. The semi-nested-PCR protocol applied in the study by Read et al. (2004) with a few modifications was used to amplify a fragment of nearly 432 bp within the gdh gene (2004). PCR reaction mixtures (10 µL) were composed of 1 µL of sample DNA, 0.5 µM of each primer (GDHeF/GDHiR in the first part of the protocol and GDHiF/GDHiR in the second part), and 0.5 units of *Taq* DNA Polymerase recombinant (Invitrogen, Thermo Fisher Scientific), 1 µL of Buffer 10X (Invitrogen) with 0.2 mM dNTPs and 1.5 mM of MgCl2 (Invitrogen). The two protocols were composed of a denaturation step at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C for

30 sec and 72 °C for 1 min, with a final extension of 72 °C for 10 min (Read et al., 2004).

To amplify the fragment of 511 bp within the bg gene the nested PCR protocol proposed by Gil et al. with some modification was used (2017). PCR reaction mixtures (10 µL) consisted of 1 µL of sample DNA, 0.5 µM of each primer (G7eF/G759eR in the primary reaction and G99iF/G609iR in the secondary reaction), 1 unit of *Taq* DNA Polymerase recombinant (Invitrogen, Thermo Fisher Scientific), and 1 µL of Buffer 10X (Invitrogen) with 0.2 mM dNTPs and 1.5 mM of MgCl2 (Invitrogen). The first PCR reaction was conducted with a denaturation step of 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 40 sec with a final step of 72 °C for 7 min. The conditions for the second PCR protocol were the same as the ones of the first protocol except that the annealing temperature was 55 °C (Gil et al., 2017).

All PCR protocols were conducted with positive and negative samples confirmed in the laboratory used as controls and were carried out on a T100™ Thermal Cycler of Bio-Rad Laboratories, Inc.

The PCR amplicons obtained from both protocols were visualized in a 1.5% agarose gels (Invitrogen, Thermo Fischer Scientific) stained with ethidium bromide to confirm the presence of 511 bp and 432 bp fragments respectively. The amplicons were sent to Functional Biosciences for sequencing.

The sequences resulting were edited using the Pregap and Gap programs from the Staden Package software (Bonfield & Staden, 1996). Sequence multiple alignments were performed using the ClustalW software and inspected by eye (Thompson et al. 1994). Neighbor-joining analysis was selected for the phylogeny analyses. Neighbor-joining method was performed using MEGA 10.0 software (Kumar et al., 2004). Bootstrap of 500 replications was carried out to support the nodes.

Results

The sequences from bg amplicons (511 bp) showed 36 variable sites, 3 of which were informative. Gdh sequences revealed 51 variable sites, 4 of them informative. For gdh the nucleotide composition was 21% T, 31% C, 19% A and 29% G and 12% T, 31% C, 28% A and 29% G for bg. All sample isolates corresponded to either assemblage A or B. The bg gene shows the presence of sub-assemblages AII and BI that gather all samples analyzed (Figure 1). Three sub-assemblages were found using the gdh gene; AII, BI and BII (Figure 2), the only representative of the last one being sample 145 (Figure 3).

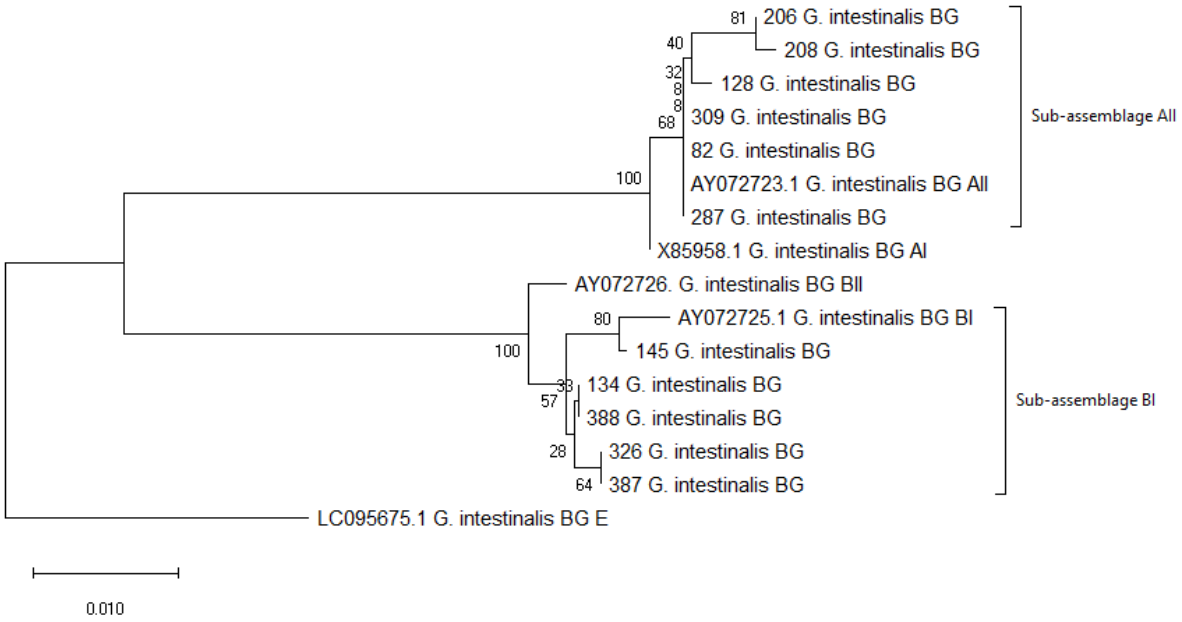

Figure 1 Neighbor-joining tree obtained from nucleotide analyses of Beta-giardin gene sequences of 11 samples Reference sequences of sub-assemblages are shown with each accession number from GenBank, Assemblage E was used as root of the tree

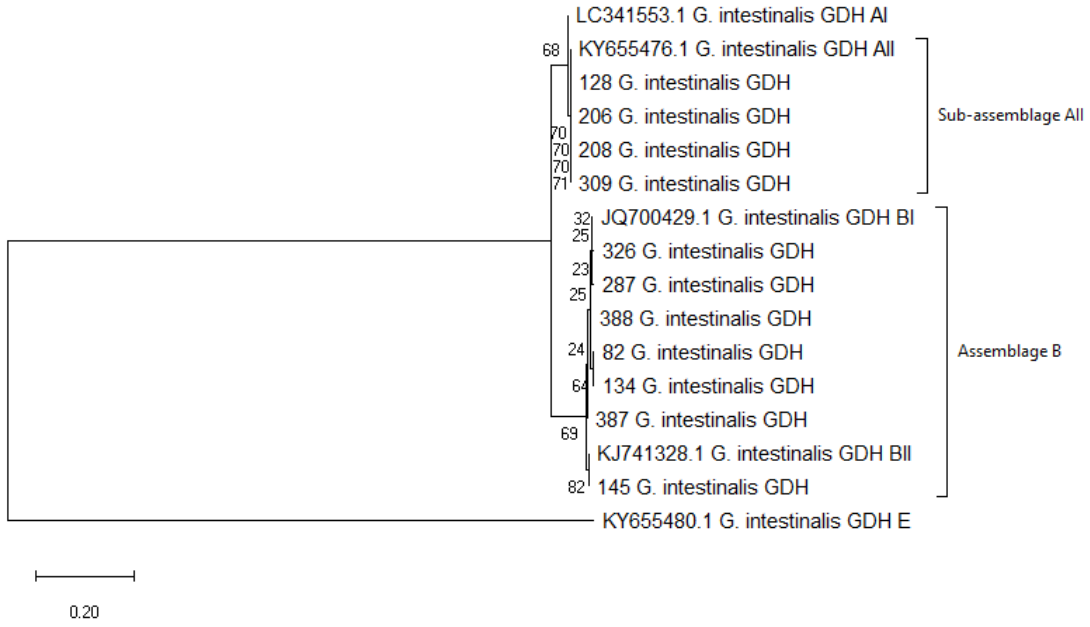

Figure 2 Neighbor-joining tree obtained from nucleotide analyses of Glutamate dehydrogenase gene sequences of 11 samples Reference sequences of sub-assemblages are shown with each accession number from GenBank, Assemblage E was used as root of the tree

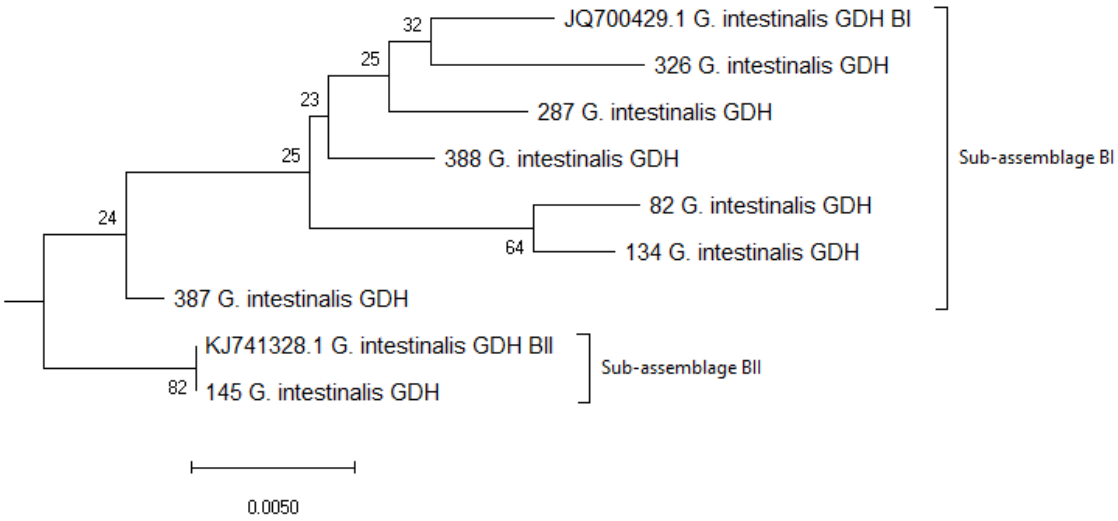

Figure 3 Assemblage B branch of the Glutamate dehydrogenase gene sequences Neighbor- joining tree obtained zoomed in

Discussion

The results obtained in this study show the presence of the two assemblages that are well known for their wide host range capability (Monis et al., 1999). These two assemblages show high genetic polymorphism within the genes that were used (Cacciò et al., 2008). Regarding the genes used, previous studies show that the mutations in the bg gene are mostly synonymous, whereas in the gdh gene the mutations have higher rates of non-synonymous substitutions revealing different selection pressures that act upon these genes as a possibility (Cacciò et al., 2008).

Sub-assemblages AII and AI can be found in humans as well as in animals, AI is more commonly found in pets and also in livestock, whereas sub-assemblage AII is mostly found in humans with some cases in livestock (Sprong et al., 2009). Sub-assemblage AII can also be found in cultured as well as in wild fish, either marine or fresh water, from isolates, using the gdh gene (Yang et al., 2010). These findings support the zoonotic potential of assemblage A and unveil the need to increase the research of this zoonotic disease as well as the full host-parasite interaction that is yet to be understood (Sprong et al., 2009). Assemblage B is predominantly found in human isolates with a low prevalence in livestock, can also be found in non-human primates living in captivity, but can be explained with the close interactions with humans (Sprong et al., 2009). The heterogeneity rate of this assemblage has shown to be much higher than the one found in assemblage B (Cacciò et al., 2008). The members of this assemblage present

large genetic differences from each other which can provide evidence of host-specific sub-assemblages within this group (Monis et al., 1999).

The zoonotic potential of the disease is clear giving that the same assemblages can be found in humans and animals, but the trigger mechanisms have yet to be studied (Feng & Xiao, 2011). Using the existing database of different isolates from humans and various animals groups as well as by the characterization of zoonotic genotypes using several loci could help to unveil the real amount of zoonotic diseases in humans (Feng & Xiao, 2011).

Conclusion

This pilot study suggests the potential zoonotic transmission of *Giardia intestinalis* however, it is necessary the analysis of isolates from animals that have close interactions with humans such as cattle or pets to correlate these findings. The inclusion of the tpi gene in the analysis could help in the correct positioning of the isolates among the assemblages and sub-assemblages. This study showcases the need for research regarding the parasite and its genomic characterization.

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