An Investigation of *Giardia duodenalis* Subgenotype AI in Human and Cattle Isolates in Baghdad

Fadia AL-Kayat[1], M.A.AL-Zuhairy[2], Saad M. Nada[3], Najwa Sh. Ahmed[3]

[1]Dep. Basic Sciences, Dentis College, Baghdad University,
[2]Dep. Public Health, Vet .Med. College, Baghdad University,
[3] Biotechnology Research Center, Al-Nahrain University, Baghdad

ABSTRACT — ***The aims of this study was designed for detection and determined the genotype of Giardia duodenalis isolates from human and cattle. To achieve this goal, thirty stool samples (5g) from patient were collected in sterile plastic cups, another 17 fecal samples from cattle were collected, all samples were examined microscopically by Saline wet-mount method for Giardia detection, Giardia duodenalis cysts were purified by using zinc flotation method, genomic DNA was successfully extracted from all samples by using AccuPrep® Stool DNA extraction kit, the gdh gene (432 bp fragment) were amplified by using specific primers of this gene, and the restriction enzyme 2U of NIaVI was used for amplified gdh gene and found the sequence of this region for 9 human and 17 cattle sample. The results indicated that the restriction pattern showed 9samples in human isolates refer to genotype A (The digested subgenotype AI yields a fragment of 150, 120, 90bp), and 21 samples refer to genotype B (Subgenotype B allele yields an intact 432 bp fragment), while all the 17 cattle samples refer to genotype A, than result of sequencing of gdh gene and alignment showed that the 99% similarity of sample with wild type of the gdh gene of Giardia intestinalis from the Gene Bank, there are one transition at position +92 T/C single nucleotide polymorphism that cause a phenylalnine (F) to phenylalnine substitution. Conclusion: The subgenotype AI of G.duodenalis was found in human and cattle isolate.***

Keywords— *Giardia duodenalis***,** restriction fragment length polymorphism (RFLP), Glutamate dehydrogenase gene (*gdh*) **,** Genotype

1. INTRODUCTION

Giardia duodenalis is a flagellated unicellular microorganism which can cause gastrointestinal infection ranging from mild to severe as well as chronic disease in human while, in domestic animal its show clinical importance and economic significant losses (Nash and Patel, 2010). Most infections result from fecal-oral transmission, ingestion of contaminated water/food and person to person is also common (Mahdy, *et al*., 2009). *Giardia duodenalis* is a complex made up of morphologically indistinguishable isolates that are classified into 7 assemblages. (Kareem, *et al*.,2011). Molecular tools have recently been used in assessment of the zoonotic transmission of giardiasis because of their highly discriminatory power, Polymerase chain reaction (PCR) combination with techniques ,such as restriction fragment length polymorphism (RFLP) or sequencing have been used for detection and genotyping and subgenotyping. *G.duodenalis* assemblage A and B associated with human and domestic animals infection (Breathnach *et al*, 2010). The glutamate dehydrogenase gene (gdh) has been successfully used for genotyping *Giardia* isolates (Leonhard, *et al*., 2007). The aims of this study were designed for detection and determined the genotype of *Giardia duodenalis* isolates from human and cattle.

2. MATERIAL AND METHODS

Thirty stool samples (5g) from patient aged 2-40 years old were collected in sterile plastic cups, another 17 fecal samples from cattle also aged 6 month to 2 years old were collected during September and October / 2012, all samples were examined microscopically by Saline wet-mount method for *Giardia* detection, *Giardia duodenalis* cysts were purified by using zinc flotation method (Zajac, *et al*., 2002), genomic DNA was successfully extracted from all samples by using AccuPrep® Stool DNA extraction kit provided by BioNeer/Korea. The determination of isolates genotype was done by using polymerase chain reaction (PCR), One set of primers for detection of *G.duodenalis* assemblages A and B was used against the coding region of the Glutamate dehydrogenase gene (*gdh*) in a reaction called GDH-PCR, a 432-bp fragment was amplified using the forward primer (GDHiF) 5' CAGTACAACTCT GCTCTCGC3' and the reverse primer (GDHiR) 5' GTTGTCCTTGCACAT CTCC3' (Red, *et al*., 2004). Purification of all the PCR product is essential in order to eliminate other by products. The PCR amplification was performed in a total volume of 25µl containing 5µl DNA, 12.5 µl Go Taq green master mix 2X (Promega corporation, USA), 1µl of each primer (50 pmol), the volume completed with 25µl with nucleases free water. The thermal cycling was done as follows: Denaturation at 94 °C for 10 min, followed by 50 cycles of 94 °C for 35s, 61°C for 35sec, and 72 °C for 50sec, with final incubation at 72 °C for

7min, using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem). The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after ethidium bromide staining. The DNA fragment from the agarose gel was excised with ascalpel and purified with gel/PCR DNA fragments extraction kit Geneaid/ Tywan. In order to determined the subgenotype AI of *Giardia duodenalis* isolate, the restriction enzyme 2U of *NIaVI* was used for amplified *gdh* gene in the restriction fragment length polymorphism assay for 3 hours at 37 C overnight, digested products were electrophoresis on 3.0% agarose gel (Fallah, *et al*., 2008).

Sequencing and Sequence Alignment

Sequencing of Glutamate dehydrogenase gene (***gdh***) gene (A1) 26 sample (9 human and 17 cattle) was performed by Macro gen company, USA. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program. The results were compared with data obtained from Gene Bank published ExPASY program which is available at the NCBI online.

3. RESULTS

The genomic DNA from 30 samples were extracted using wizard genomic DNA promega, *gdh* gene from genomic DNA were amplified by using specific PCR primers, results shown in figure (1) indicated that a yield of a single band of the desired product with a molecular weight about 432 bp for *gdh* gene was obtained.

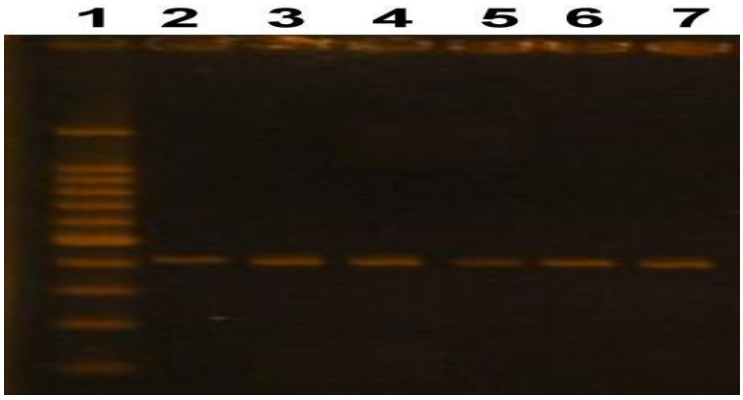

Figure (1) : Agarose electrophoresis of PCR amplification for *gdh* gene (432bp). Lane 1 represents DNA ladder (100bp), Lane 2,3,4 represent human samples, 5,6,7 represent cattle samples. Fragments were resolved on 1.5% agarose gel and visualized by Ethidum bromide staining.

The results indicated that the restriction pattern showed 9/30 (30%) samples in human isolates referred to genotype A (PCR products with 2U of *NIaIV* enzyme, the digested subgenotype AI yields a fragment of 150, 120, 90bp), and 21/30 (70%) samples refer to genotype B (Subgenotype B allele yields an intact 432 bp fragment), while all the 17 cattle samples refer to genotype A. Subgenotype determination by RFLP analysis recorded that all the 9 genotype A samples in human belong to A1, and all the 21 genotype B samples were belong to BIV. In cattle results showed that all the positive samples belong to subgenotype AI (Figure 2 and 3). According to sex, most of subgenotype A1 samples 7/9 (77.77%) were belong to males and 2/9 (22.22%) were belong to females, while for the subgenotype B1V the results showed 10/21 (47.6%) samples were belong to male and 11/21 (52.38%) samples recorded in females. Subgenotype A1 was seen in 4/18 (22.22%) samples aged under or equal to 10 years old, and 14/18 (77.77%) samples recorded as B1V within the same age category,the remaining samples 12 belong to person aged more than 10 years old showed 5/12 (41.6%) samples refer to A1 and 7/12 (58.33%) samples refer to subtype B1V.

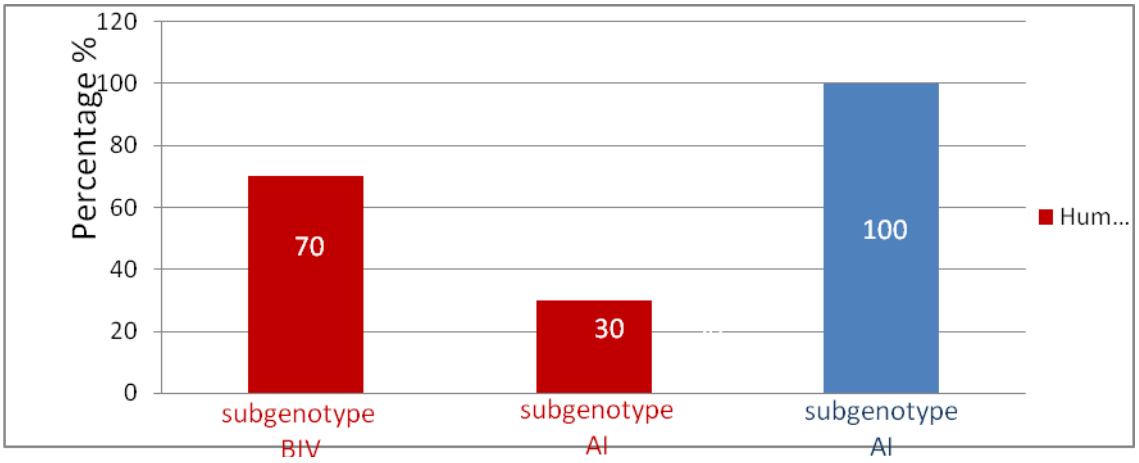

Figure (2): Percentage of *G.duodenalis* subgenotype by using RFLP analysis.

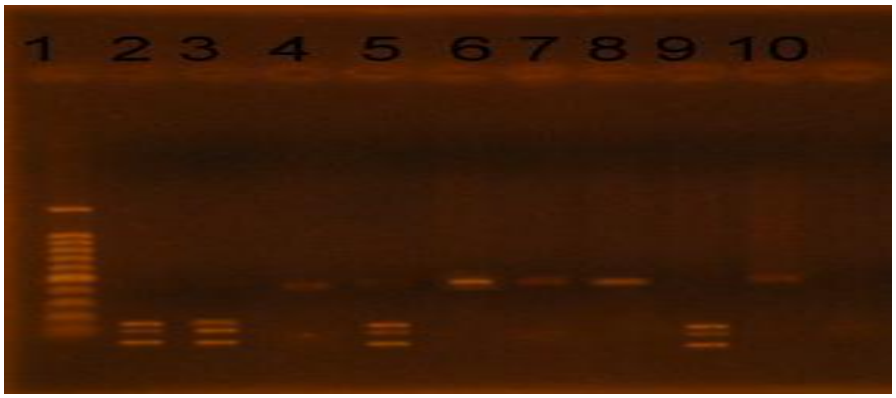

Figure (3): RFLP analysis using *NIaIV* restriction enzyme. Lane 1 represent DNA ladder (100bp); Lane 2,3 represent subgenotype AI (150, 120, 90bp) in human; Lane 5,9 represent subgenotype AI in cattle. Lane 4,6,7,8,10 represent genotype B (430bp) in human. Fragment was resolved on 3% agarose gel and visualized by Ethidum bromide staining.

Sequencing of this gene was performed to detect of genotype of *Giardia duodenalis* isolate from 9 human and 17 cattle. Sequences alignment using BLAST and BioEdit showed that the 99% similarity or homology of sample with wild type of the *gdh* gene of *Giardia intestinalis* from the Gene Bank (Figure 4), there are one transition at position +92 T/C single nucleotide polymorphism that cause a phenylalnine (F) to phenylalnine substitution in codon 92, Table (1) shows the type of single nucleotide polymorphism and the effect of these mutations and Table (2) shows the translation of *gdh* gene of sample (Human and Cattle) to a protein sequence. The results refer to *Giardia duodenalis* genotype A1 isolate from human and cattle.

Giardia intestinalis isolate A1 glutamate dehydrogenase (GDH) gene, partial cds

Score	Expect	Identities	Gaps	Strand	Frame
641 bits(347)	2e-180()	349/350(99%)	0/350(0%)	Plus/Plus	

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Query  55   GGCTTTGAGCAGATCCTGAAGAACTCCCTTACCACGCTCCCGATGGGCGGTGGTAAGGGC  114
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  108  GGCTTTGAGCAGATCCTGAAGAACTCCCTTACCACGCTCCCGATGGGCGGTGGTAAGGGC  167

Query  115  GGCTCCGACTTCGATCCTAAGGGCAAGTCGGACAACGAGGTCATGCGCTTCTGCCAGTCC  174
           |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
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Sbjct  168  GGCTCCGACTTCGATCCTAAGGGCAAGTCGGACAACGAGGTCATGCGCTTTTGCCAGTCC  227

Query  175  TTTATGACCGAGCTCCAGAGGCACGTCGGGGCTGACACCGACGTTCCTGCTGGCGATATT  234
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  228  TTTATGACCGAGCTCCAGAGGCACGTCGGGGCTGACACCGACGTTCCTGCTGGCGATATT  287

Query  235  GGCGTCGGCGGTCGCGAGATCGGTTATCTGTTTGGACAGTACAAGCGCCTCAGGAACGAG  294
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  288  GGCGTCGGCGGTCGCGAGATCGGTTATCTGTTTGGACAGTACAAGCGCCTCAGGAACGAG  347

Query  295  TTCACGGGCGTCCTCACGGGCAAGAACATCAAGTGGGGCGGGTCTCTCATCAGGCCAGAG  354
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  348  TTCACGGGCGTCCTCACGGGCAAGAACATCAAGTGGGGCGGGTCTCTCATCAGGCCAGAG  407

Query  355  GCCACAGGGTATGGAGCTGTCTACTTCCTGGAGGAGATGTGCAAGGACAA  404
            ||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  408  GCCACAGGGTATGGAGCTGTCTACTTCCTGGAGGAGATGTGCAAGGACAA  457
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Fig. (4): Sequencing of sense flanking the partial ***gdh*** **gene** for 9 Human and 17 cattle as compared with wild type ***gdh*** obtained from Gene Bank.

Tab (1): Types of mutations detected in partial ***gdh*** gene.

No.	Location of gene bank	Nucleotide change	No. of sample	Amino acid change	Predicted effect	Type of mutation
1	T/C 27	TTT/TTC	26 (9 Human +17Cattle	(F) / (F)	Silent	Transition

Tab (2): Amino acid sequences of partial *gdh* gene for 9 Human and 17 cattle as compared with wild type *gdh* obtained from Gene Bank.

	Sequencing of amino acid	Sample
1	GFEQILKNSLTTLPMGGGKGGSDFDPKGKSDNEVMRFCQSFMTELQRHVGADTDVPAG	Wild type
2	GFEQILKNSLTTLPMGGGKGGSDFDPKGKSDNEVMRFCQSFMTELQRHVGADTDVPAG	26

Abbreviations: K: lysine; V: Valine; S: Serine; L: Lysine; T: Threoine; F: Phenylalanine; A: Alanine; E: Glutamic acid; D: Asparagine; R: Arginine; Q: Glutamine; G: Glysine; P: Proline.

4. DISCUSSION

The Molecular characterization of this study revealed that only genotype A and B were found to be associated with human infection (Breathnach, *et al.*, 2010). Thirty human *G.duodenalis* isolates were studied by RFLP analysis of PCR product of *gdh* locus, subgenotype AI was detected in all genotype A (30%) and all genotype B were identified as subgenotype BIV (70%). Subgenotype AI was observed in all cattle fecal isolates. The differences in social and environmental condition might have contributed to the variations in the distribution of *G. duodenalis* assemblages in human, asymptomatic carriers, length of diarrhea, type of epidemiological study and use of diagnosis test of difference sensitivity and specificity, in general the prevalence was strongly associated with a variety of risk factors including host, sociodemograph environmental and zoonoticn (Maria, *et al*., 2007). The reason for the wide occurrences of subgenotype AI may due to contamination of public water with raw sewage from animals and humans sources (Berrilli, *et al*., 2006). The finding of this study was agreement with a study done in Egypt which showed that 41 isolate of *G. duodenalis* were detected by PCR-RFLP, 24 (58.5%) were containing AI and 7 (17.1%) were containing AII also 8 (19.5%) were containing B and 2 (5%), samples had a mixture of AII and B (Moshira, *et al*., 2009). The result of this study show agreement with Parima, *et al*. (2010) who reported that BIV was the majority (46%) subgenotype among 66 of human *G. duodenalis* isolates and AI recorded 21%, while AII and BIII recorded 30%, 4% respectively. Another study revealed all human isolates were recorded as AI, while B was not found (Aline, *et al*., 2011). This finding also corresponds to a study showed 38/54 samples (70.4%) were found to be AI and the remaining 16/54 (29.6%) were found to be subgenotype B (Cuneyt, *et al*., 2012). Two studies were also proved the occurrence of subgenotype of AI in human isolates (Lalle, *et al*., 2005 and Volotao, *et al*., 2007). In cattle, The similar results were detected in a study that described the presence of subgenotype AI in 15% with varying levels of 8 to 45% concluding that calves might be a source of *G. duodenalis* infection in human (Trout, *et al*., 2004). Also, Feng, *et al*. (2008) showed the existence of AI in 4/48 samples. Another agreement was shown by a study in Europe in which 113 samples was tested, subgenotype AI was detected in 70 sample and 39 belong to AII, while 4 belongedio AIII (Sprong, *et al*., 2009). The results observed was influence of multifactors system like host, parasitic agents, management, hygiene, environmental effect, and transmission process in which multiple transmission pathway is known, but transmission via water may be particular significance in areas with large numbers of domestic cattle. This is because cattle are typically maintained at high densities and produce large quantities of fecal waste. Further, the cysts excreted in their feces are environmentally resistant, immediately infectious to susceptible hosts and are prone to moving passively across land escapes and into water ways via runoff (Appelbee, *et al*., 2003). Conclusion: The subgenotype AI of *G. duodenalis* was found in human which indicate that the participation such hosts in zoonotic cycle of giardiasis is possible.

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