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ORIGINAL ARTICLE

Molecular Identification of Giardia Duodenalis Isolates from Children Stool in Diyala Province, Iraq

MOHYMIN SABAH AWAD AWAD[1], TAREQ RIFAAHT MINNAT[2], ANAS A. HUMADI[3]
[1]*MSc student, Zoonosis , Department of Internal and Preventive Medicine, Faculty of Veterinary Medicine, University of Diyala, Iraq*
[2]*Department of Internal and Preventive Medicine, Faculty of Veterinary Medicine, University of Diyala, Iraq*
[3]*Department of Pathology, Faculty of Veterinary Medicine, University of Diyala, Iraq*
Correspondence to: Mohymin Sabah Awad Awad, Email: Mohymin1990@gmail.com

ABSTRACT

One of the most prevalent human intestine protozoan parasites in the world, Giardia duodenalis is indigenous to every continent and infects a broad variety of animal hosts. The goal of the current study was to determine the prevalence of infection among Giardia duodenalis isolates from regional hospitals and private clinics in the Iraqi province of Diyala that would be the subject of additional genetic analysis. From the beginning of October 2021 to the end of January 2022, 100 children's stools were collected from hospitals and health centers in Diyala, Iraq. Additionally, Giardia duodenalis cysts and trophozoites were examined under the microscope, and DNA was extracted using Quick-DNA Fecal/Soil Microbe Kits. One hundred of the stools taken from youngsters show symptoms of diarrhoea, nausea, and abdominal pains Based on microscopic examination and PCR analysis, a 7 percent infection rate was discovered. In addition, genotyping was carried out utilizing G. duodenalis Tpi sequence analysis. The highest infection rate was 10 % in up 4-5 year category and the lowest infection rate 4.65% recorded in up one year category. While, the infection rate was highest in male samples 8.16% than female samples 5.88%. Regarding the giardiasis according to the house system, most people who were infected were living in the city centre at ratio 9.25%, followed by 4.34 % in persons living in the country side. On the other hand, the infection rate of Giardia infection was 6.89%,4.16% and 8.51% to the tap water, Reverse Osmosis(RO) water and filter water respectively. The findings of this study emphasize that area of study was G. duodenalis infection rate with source of infection route and water basis in children in this province. The genetic analysis of the region Beta-giardin and RH11 and TPIA in this study showed that this diagnosis of Giardia duodenalis was made after effective amplification of these regions.

Keywords: Molecular, Infection rate, Risk factors, Giardia duodenalis.

INTRODUCTION

Giardia spp. is an intestinal flagellate protozoan that may be seen in two stages: active trophozoites and latent cysts, which are infective[1,2]. The three species that have been identified are Giardia lamblia, Giardia muris, and Giardia agilis. Giardia lamblia is the only species known to infect humans. The feco-oral route is the primary method of cyst transmission[3]. It is a parasite that is present across the world and has a 20–30% prevalence rate in developing nations. More infections are noted in the late summer. Those who visit places in Africa, Asia, and Latin America where access to clean water is scarce are more likely to get the protozoa[4].

Although some healthy people do not become ill as a result of Giardia lamblia, they can still infect others. Because It is a parasite that is present across the world and has a 20–30% prevalence rate in developing nations. More infections are noted in the late summer. Those who visit places in Africa, Asia, and Latin America where access to clean water is scarce, elderly, and adults with long-term diseases may be more susceptible to developing the protozoa[5]. Giardia is spread mostly through contaminated water and food. Inadequate living circumstances, overcrowding, socioeconomically disadvantaged status, unsafe water supply, unhygienic personal behaviors, and inadequate environmental sanitation status are all contributors[6]. Around 200 million individuals in underdeveloped nations 500,000 cases reported of clinical giardia are reported each year[7].

The illness is especially prevalent in places with poor sanitation and hygiene[8]. Tropical and subtropical locations, as well as metropolitan areas, have a higher incidence than rural areas, where affects up to 30% of the population[9].

Molecular genetics Innovative and efficient tools to describe Giardia isolates and G. duodenalis-specific have been successfully created using approaches. Small subunit ribosomal RNA (SSU-rRNA), -giardin (bg), glutamate aminotransferase (gdh), elongation factor 1-alpha (ef-1), triose phosphate isomerise (tpi), and variant surface protein (vsp) gene analyses have been the mainstays of biochemical investigations of G. intestinalis[10]. These instruments are often used to distinguish between Giardia at the species/assemblage and genotype levels as well as to identify G. duodenalis genotypes in clinical specimens. The SSU-rRNA, gdh, tpi, and bg genes addressed are typically what decide the usefulness of molecular diagnostic techniques. It is helpful to study population genetics, epidemiology, and taxonomy of giardiasis in people and domestic animals by using these loci for genotyping or subtyping of G. duodenalisand or sequencing features[11].

Giardiasis is a significant cause of diarrhea, hence it was chosen to look at the infection incidence and risk factors among diarrheic infants and kids in the Diyala region of Iraq.

RESOURCES AND PROCEDURES

Area: This study was conducted from first October 2021 to last January 2022 on children living in different area of Diyala Province

Samples and Laboratory methods: 100 stool samples were collected from both healthy children and those with probable diarrhoea. Every aspect of their water and housing systems supply information, as well as their age group and gender, were recorded. Cysts are obtained from the children's stool (Cheesbrough, 1998). The samples were collected in little containers (50 ml) containing about 20-30 gram of faces in each container. After that, a volume of 20-25 ml of normal saline was added to the sample to dilute it, and then the material was separated into two layers using a 40 S 9*9 Mesh. The filtrate was placed in a centrifuge tube and spun at 1500 rpm for 10 minutes. Then toss out the supernatant. To re-suspend the sediment, a 33 % zinc sulphate solution is mixed with the same amount of sediment then centrifuged for 15 minutes at 1200 g. Transferring the supernatant to a centrifuge tube and diluting it with four times the volume of water, and centrifuged for another ten minutes at 1500 g[12].

Morphological identification and cyst counting: The cyst samples are placed on slides and covered with 20 mm square coverslips, as stated before. Every slide should be thoroughly examined. Under a 40 and 100 optical microscope (with oil immersion), all slides are inspected. The densities of cysts were measured using a haemocytometer[13]. Counting white blood cells was used for counting cysts to measure the dosage of infection using iodine solution as a dilution in this operation. The counting chamber was covered with a cover slip, and 10/platform was poured onto the platforms. as a consequence of the cyst count determined by haemocytometer technique. Each platform's four corner squares (1mm2) were counted, and cyst density was computed as follows[13].

$$\frac{cyst\ no.}{4\ mm^2}\ x\ \frac{10}{1\ mm}\ x\ \frac{1\ mm^3}{ml} = cysts/ml$$

Cyst no. = number of cyst in 4 chambers
4 mm² = total area
1 mm³ = total volume.

DNA extraction and molecular analysis: Having followed the owner's manual, the DNA was extracted from sediment samples using a Quick-DNA Fecal/Soil Microbe Kits Isolation Kit (ZYMO RESEARCH). Human samples were detected independently for each sample. Giardia species' beta-giardin (bg) and triose phosphate isomerase (tpi) genes were amplified and sequenced using PCR. Table provides information about primers and cycle conditions. (1,2,3,4)

Table 1: The specific primer of gene Beta-giardin

Primer	Sequence	Tm (ºC)	GC (%)	Product size
Forward	5′-GCGAGGAGGTCAAGAAGTC-3′	56.70	57	500bp base pair
Reverse	5'-GAGCGTGTTGACGATCTTGT -3′	54.78	60	

Table 2: The optimum condition of detection for Beta-giardin

No.	Phase	Tm (ºC)	Time	No. of cycle
1-	Initial Denaturation	94ºC	5 min.	1 cycle
2-	Denaturation -2	94ºC	30 sec.	35 cycle
3-	Annealing	58ºC	30 sec.	
4-	Extension-1	63ºC	30 sec.	
5-	Extension -2	72ºC	10 min.	1 cycle

Table 3: Specific primer TPiA of gene tpi

Primer	Sequence	Tm (ºC)	GC (%)	Product size
Forward	5'-CGCCGTACACCTGTC - 3'	52.6	66.7	332 base pair
Reverse	5'-AGCAATGACAACCTCCTTCC - 3'	56.8	50.0	

Table 4: The optimum condition of detection for TPiA of gene tpi

No.	Phase	Tm (ºC)	Time	No. of cycle
1-	Initial Denaturation	94ºC	5 min.	1 cycle
2-	Denaturation -2	94ºC	30 sec.	35 cycle
3-	Annealing	58ºC	30 sec.	
4-	Extension-1	63ºC	30 sec.	
5-	Extension -2	72ºC	10 min.	1 cycle

Following ethidium bromide or Red Stain staining, the PCR products were separated on a 2 percent agarose gel electrophoresis and observed by exposure to ultraviolet light (302 nm). MacroGen Korea did the gene sequencing, and the Basic Local Alignment Search Tool (BLAST) and BioEdit programs were used for the homology search. BLAST is a tool that may be found online at http://www.ncbi.nlm.nih.gov. PCR products were purified and sequenced using an ABI 3130 Genetic Analyzer and the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

In order to identify the sample, the sequence was examined in the nucleotide databases using NCBI's Basic Local Alignment Search Tool Bio ID software and uploaded to Ncbi (ID). By using Bio ID software, related alleles from the sample or s were collected from the NCBI's rna database (www.ncbi.nlm.gov/nucleotide) (Tamura et al, 2011).

Statistic evaluation: Using SPSS version 23.00, data were arranged, recorded, and statistically examined. P values have been computed. The frequency data were compared using the Chisquare test (2). Significant (S) values are those with a P value 0.05.

RESULTS

The infection rate of Giardia according to the age group categories: The overall infection rate of Giardiasis were (7%) in all different age categories. The highest infection rate 10 % up 5 year category, while the lowest infection rate 4.65% recorded in up to one year category. Where a significantly at p<0.05 as (Table.1)

Table 1: The infection rate of Giardia according to the age groups categories.

Age group	Up to 1 year	Up to 2 years	Up to 3 years	Up to 4 years	Up to 5 years	Total
Examined	43	14	11	12	20	100
Positive	2	1	1	1	2	7
Infection rate%	4.65	7.14	9.09	8.33	10	
X^2= 0.748	p-value =0.945					

The infection rate of according to the gender: The infection rate of Giardia duodenalis infection was highest in male samples 8.16%, while female stool samples had a lower prevalence of 5.88%. Where a significantly at p<0.05 (Table 2).

Table 2: Prevalence and distribution of Giardia according to the gender

	Male	Female	Total
Examined	49	51	100
Positive	4	3	7
Infection rate%	8.16	5.88	
X^2= 73.97	p-value =0.0		

Distribution of Giardia according to the house system: Regarding the Giardia duodenalis according to the house system, most people who were infected were living in the city center at ratio 9.5% , followed by 4.34 % Giardia infection in persons living in the country side there is no significant difference, at p<0.05 (Table 3).

Table 3: Infection rate and distribution of Giardia spp. according to the house system.

	Type of house system		
	Country side	City centre	Total
Examined	46	54	100
Positive	2	5	7
Infection rate%	4.34	9.5	
X^2= 0.92	p-value =0.92		

Infection rate and distribution of Giardia according to source of water: The infection rate of Giardia duodenalis was 8.51%, 6.89% and 4.16% to the filter water, tap water and RO water respectively (Table 4).

Table 4: Prevalence and distribution of Giardia according to source of water

	Source of water			
	Tap water	RO	Filter water	Total
	29	24	47	100
Positive	2	1	4	7
Infection rate %	6.89	4.16	8.51	
X^2= 0.461	p-value =0.794			

Severity infection of Giardia: To determine the severity of infection according to the cysts count of Giardia duodenalis, the formula used to count the cysts and found that 3 sample from 7 positive samples were contain 10 cysts in ml and two of the samples contain 7 cysts in ml and the other were 3 and 5 cysts in ml (Table 5).

Table 5: Severity of Giardia infection according to the cyst counting.

Severity of infection according to the cyst count	
Samples	Cysts in ml
1	10
2	7
3	10
4	3
5	5
6	10
7	7

Giardia Morphological identification: Giardia species have cysts of ovoid. The cysts are ovoid, measuring 10–14 µm (wide-long). Each cyst contains four nuclei. The axonemes and median bodies are prominent (Fig. 1).

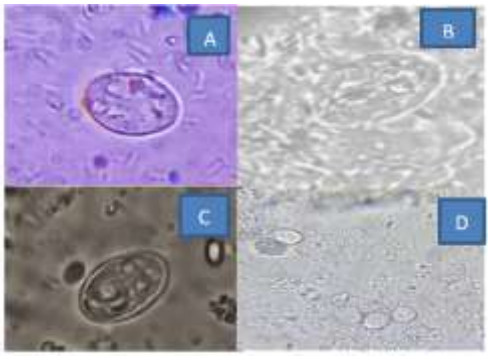

Figure 1: A: Giardia duodenalis stained new methylene blue stain show the cyst morphology, B, cyst of Giardia without any stain :C : cyst of Giardia stained with Iodine. D: different stages of Giardia cyst. The images are captured by a digital camera.

Molecular identification: Detection of samples using conventional PCR: Seven fecal samples of children were collected. Extracted samples from respective cases of each case were screened by polymerase chain reaction. Two pairs publishing primers designed in the region of Beta-protein gene as previously described with the aim to generate a clear band of copy DNA with size of 500 base Paris . These samples were screened by conventional PCR to amplify the Beta gene.. Oligonucleotide primer sequence that represented the partial amplification in the Beta gene region to detect Giardia genotyping was chooses. The entire amplified DNA showed identical mobility on 2% agarose gel. Out of 100 samples (human) 7 samples (7%) were positive. Positive samples generated a specific DNA band of 500bp figure (2)

Molecular identification Giardia by PCR from children: Using Beta-giardin primers(500 bp) for common Giardia spp and TPIA primers (332bp) for Giardia duodenals There were seven positive samples of stool from children were diagnosed by PCR to identify of Giardia for each primers (Seven positive for Beta-giardin 500 bp& TPIA 332bp) . All these samples were given positive results for Giardia by isolation. The total infection rate detected by PCR was 7%. With infection rate 8.16 and 5.88 for male and female respectively Table (6) Figure (2&3).

Table 6: Show the primers that used in this study and the positive and negative samples from human.

No of samples	Beta-giardin 500 bp	TPIA 332bp
1	-	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	-	
9	+	

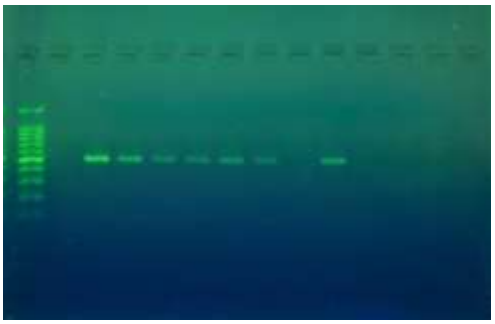

Figure 2: The PCR result's 500 bp band size. Electrophoresis on 2 percent agarose at 5 volts per square centimeter was the procedure employed. TBE buffer for an hour at 1x. N: Seven human samples were used with a DNA ladder (100). Beta-giardin primers of 500 bp.

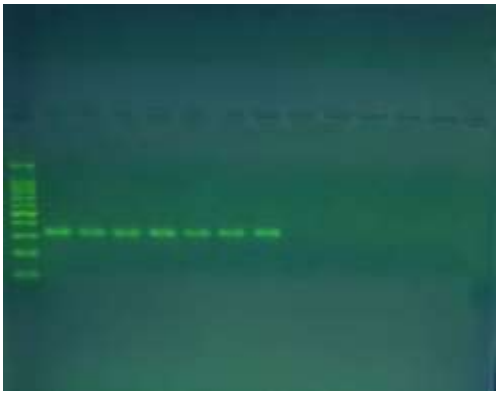

Figure 3: 332 bp is the band size of the PCR product. The process used 1.5 percent agarose and a 5 volt/cm2 voltage. 1:30 hours of 1x TBE buffer. N: DNA stairway (100). utilizing a particular TPIA gene primer

Results of sequences: The Macrogen lab in South Korea provided high-quality sequencing of the tpi gene, and the findings of the sequences indicated that the samples were Giardia dudenalis, for the sent three samples with a PCR product size of approximate 332 bp, and these sequence were BLAST and the sequences were more than 99 % resampled to Giardia dudenalis ID: MG924457.1.

Submission of local Iraq isolate in NCBI: From three PCR samples that were selected for positive sequencing by forward and reverse primers, ten samples of PCR products (human) were extracted. The sequences were used in the No. 1 NCBI gene bank database (MG924457.1). To identify the converging sequences stored in the gene bank, these sequences were examined using the BLAST-NCBI tool, available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequences (1-3) had 99.4% similarity to the USA isolate of Giardia duodnalis (MG924457.1) and were registered at the NCBI under the accession number ID: MG924457.1.

Figure 4: 332 bp is the band size of the PCR product. The process used 1.5 percent agarose and a 5 volt/cm2 voltage. 1:30 hours of 1x TBE buffer. N: DNA stairway (100).

Table 7: Include the total samples (human) and their identity percentage with other genotype of Giardia duodnalis that were collected in this study

Source: Giardia duodnalis (Giardia lamblia)			
No.	Sequence ID with compare	Source	Identities
1	ID: MG924457.1	Giardia duodnalis isolate 105N triosephosphate isomerase (TPI) gene	100%
1	ID: MG924457.1	Giardia duodnalis isolate 105N triosephosphate isomerase (TPI) gene	100%
1	ID: MG924457.1	Giardia duodnalis isolate 105N triosephosphate isomerase (TPI) gene	100%

Sample 1 C01_2_HAF.ab1 317
Giardia duodnalis isolate 105N triosephosphate isomerase (TPI) gene, partial cds
Sequence ID: MG924457.1 Length 480 Number of Matches: 1,
Range 1: 118 to 434

Score	Expect	Identities	Gaps	Strand
586 bits(317)	4e-172	317/317(100%)	0/317(0%)	Plus/Plus

QUERY 1
AGTTGAGGATAGCAGCGCAGAATGTGTACCTAGAGGGGAACGGGGCGT
GGACTGGCGAGA 60
SBJCT 118
AGTTGAGGATAGCAGCGCAGAATGTGTACCTAGAGGGGAACGGGGCGT
GGACTGGCGAGA 177
QUERY 61
CAAGTGTTGAGATGCTTCAGGACATGGGTTTGAAGCATGTGATAGTAGG
GCACTCTGAAA 120
SBJCT 178
CAAGTGTTGAGATGCTTCAGGACATGGGTTTGAAGCATGTGATAGTAGG
GCACTCTGAAA 237
QUERY 121
GACGCAGAATCATGGGGGAGACCGACGAGCAAAGCGCCAAGAAGGCT
AAGCGTGCCCTGG 180
SBJCT 238
GACGCAGAATCATGGGGGAGACCGACGAGCAAAGCGCCAAGAAGGCT
AAGCGTGCCCTGG 297
QUERY 181
AAAAGGGGATGACGGTCATCTTCTGCGTCGGAGAGACCTTGGATGAGC
GCAAGGCCAACC 240
SBJCT 298
AAAAGGGGATGACGGTCATCTTCTGCGTCGGAGAGACCTTGGATGAGC
GCAAGGCCAACC 357
QUERY 241
GCACCATGGAGGTGAACATCGCCCAGCTTGAGGCGCTTGGCAAGGAG
CTCGGAGAGTCCA 300
SBJCT 358
GCACCATGGAGGTGAACATCGCCCAGCTTGAGGCGCTTGGCAAGGAG
CTCGGAGAGTCCA 417
QUERY 301 AGATGCTCTGGAAGGAG 317
SBJCT 418 AGATGCTCTGGAAGGAG 434

Sample 2 C01_2_HAF.ab1 317
Giardia duodnalis isolate 105N triosephosphate isomerase (TPI) gene, partial cds
Sequence ID: MG924457.1 Length 480 Number of Matches: 1,
Range 1: 118 to 434

Score	Expect	Identities	Gaps	Strand
586 bits(317)	4e-172	317/317(100%)	0/317(0%)	Plus/Plus

Query 1
AGTTGAGGATAGCAGCGCAGAATGTGTACCTAGAGGGGAACGGGGCGT
GGACTGGCGAGA 60
Sbjct 118
AGTTGAGGATAGCAGCGCAGAATGTGTACCTAGAGGGGAACGGGGCGT
GGACTGGCGAGA 177
Query 61
CAAGTGTTGAGATGCTTCAGGACATGGGTTTGAAGCATGTGATAGTAGG
GCACTCTGAAA 120
Sbjct 178
CAAGTGTTGAGATGCTTCAGGACATGGGTTTGAAGCATGTGATAGTAGG
GCACTCTGAAA 237
Query 121
GACGCAGAATCATGGGGGAGACCGACGAGCAAAGCGCCAAGAAGGCT
AAGCGTGCCCTGG 180
Sbjct 238
GACGCAGAATCATGGGGGAGACCGACGAGCAAAGCGCCAAGAAGGCT
AAGCGTGCCCTGG 297
Query 181
AAAAGGGGATGACGGTCATCTTCTGCGTCGGAGAGACCTTGGATGAGC
GCAAGGCCAACC 240
Sbjct 298
AAAAGGGGATGACGGTCATCTTCTGCGTCGGAGAGACCTTGGATGAGC
GCAAGGCCAACC 357
Query 241
GCACCATGGAGGTGAACATCGCCCAGCTTGAGGCGCTTGGCAAGGAG
CTCGGAGAGTCCA 300
Sbjct 358
GCACCATGGAGGTGAACATCGCCCAGCTTGAGGCGCTTGGCAAGGAG
CTCGGAGAGTCCA 417
Query 301 AGATGCTCTGGAAGGAG 317
Sbjct 418 AGATGCTCTGAAGGAG 434

Sample 3 C01_2_HAF.ab1***317***
Giardia duodnalis isolate 105N triosephosphate isomerase (TPI) gene, partial cds
Sequence ID: MG924457.1 Length 480 Number of Matches: 1,
Range 1: 118 to 434

Score	Expect	Identities	Gaps	Strand
586 bits(317)	4e-172	317/317(100%)	0/317(0%)	Plus/Plus

Query 1
AGTTGAGGATAGCAGCGCAGAATGTGTACCTAGAGGGGAACGGGGCGT
GGACTGGCGAGA 60
Sbjct 118
AGTTGAGGATAGCAGCGCAGAATGTGTACCTAGAGGGGAACGGGGCGT
GGACTGGCGAGA 177
Query 61
CAAGTGTTGAGATGCTTCAGGACATGGGTTTGAAGCATGTGATAGTAGG
GCACTCTGAAA 120
Sbjct 178
CAAGTGTTGAGATGCTTCAGGACATGGGTTTGAAGCATGTGATAGTAGG
GCACTCTGAAA 237
Query 121
GACGCAGAATCATGGGGGAGACCGACGAGCAAAGCGCCAAGAAGGCT
AAGCGTGCCCTGG 180
Sbjct 238
GACGCAGAATCATGGGGGAGACCGACGAGCAAAGCGCCAAGAAGGCT
AAGCGTGCCCTGG 297
Query 181
AAAAGGGGATGACGGTCATCTTCTGCGTCGGAGAGACCTTGGATGAGC
GCAAGGCCAACC 240
Sbjct 298
AAAAGGGGATGACGGTCATCTTCTGCGTCGGAGAGACCTTGGATGAGC
GCAAGGCCAACC 357
Query 241
GCACCATGGAGGTGAACATCGCCCAGCTTGAGGCGCTTGGCAAGGAG
CTCGGAGAGTCCA 300
Sbjct 358
GCACCATGGAGGTGAACATCGCCCAGCTTGAGGCGCTTGGCAAGGAG
CTCGGAGAGTCCA 417
Query 301 AGATGCTCTGGAAGGAG 317
Sbjct 418 AGATGCTCTGAAGGAG 434

DISCUSSION

Giardia duodenalis infected people at all ages, while it is more common in children. The increased incidence in children suggests that adults have acquired some level of infection resistance[14,15]. Infections from humans and water have been recorded in all countries[16]. Direct stool sample inspection is still the most common method of diagnosis, however quick copro-antigen detection tests and molecular approaches are becoming more popular[17,18].

Giardiasis was more common in boys than girls among young children. (8.16%) than female children (5.88%) in the current research, which is consistent with earlier studies[19]. This is

most likely owing to male children's more infected of exercise and exposure to the outdoors as as opposed to females. Giardiasis prevalence and incidence varied with age. The age range of 3-5 years was shown to have the highest prevalence of giardiasis in the current study, followed by the 1-3 year age group. This might be explained by these children's increased activity, which includes spending more time outside the house and at playgrounds than younger children, as well as a lack of personal cleanliness compared to older children. This conclusion is consistent with prior researchears[20,21], which found that Early children were found to be at the greatest danger, whereas older children and adults were shown to be at a lower risk.[20,22]

Children aged 1 to 2 years had a decreased frequency of giardiasis, which may be related to the community's tradition of extended breastfeeding.: exclusive breastfeeding was found to have a 5-fold protective effect against giardiasis when compared to no breast-feeding among infants aged 0–18 months[1]. Breast-feeding was found to be protect children up to 6 months of age from Giardia infections in a hospital-based surveillance study[23]. This protection, however, may be independent of the protective antibodies found in mother's milk. Certain components of non-immune milk have been demonstrated to be capable of killing Giardia trophozoites in vitro[24].

This study gives information on the genetic of Giardia spp. among children in the Diyala proviance, despite the fact that the possibility of zoonotic transmission of Giardia infections is still debatable. Our findings show that Giardia was found in many different places. Giardia duodnalis, a frequent genotype found in the region of Diyala, was examined in this study. [25,26], despite the fact that it has also been documented in a number of animal studies and is mostly seen in mammals [26,27]. However, our results in this study imply that Giardia duodenalis may be a common infection distributed by or among youngsters. The current study is the first to describe the genetic characteristics of G. duodenalis isolates from young children with diarrhea who reside in various geographic areas of Diyala. More epidemiological research should be done in areas where people and animals coexist closely and/or where infection is endemic in order to have a better knowledge of how G. duodenalis interacts with its hosts and its pathogen and the extent to which animals can cause Giardiasis in humans.

Children in the Diyala province have a high frequency of giardiasis. Several risk variables, such as water source and resident housing, were linked to Giardia prevalence, emphasizing the importance of parental education and sanitation in children's health. The Giardia infection is a serious problem that requires further research in order to develop effective preventative and treatment methods.

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