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Molecular Study of Co-Infection between *Giardia Lamblia* And *Helicobacter Pylori* In Non- Symptomatic Children In Thi-Qar Province,Iraq

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Abstract:
The current study was carried out in Thi-Qar Province which included a collection of stool samples from non-symptoms children. The aim of the study was to detect of co-infection of *Giardia. lamblia* with *Helicobacter pylori* in non-symptoms children. Atotal 96 stool samples taken children were under five years of both sex and examined by PCR for detection of *G. lamblia*. The results showed that the percentage of positive samples was 32 positive and 64 negative. The most infection was in females more than males. The highest infection with *G. lamblia* according to PCR was in rural more than in urban population. The highest infection rate with *G. lamblia* in the age group (1-12 months) , Patients and the lowest age group (37-48 months) .A total of 96 samples were examined by PCR for diagnosis of *G. lamblia* also to detection of *H. pylori* . The results showed 32 positive samples with *G. lamblia* and only 14 positive samples with *H. pylori*. The number of samples that have co-infection between *G. lamblia* with *H. pylori* is 13. The BLAST results for DNA sequencing showed that isolation for *G. lamblia* different with the isolates recorded at the National Center for Biotechnology (NCBI); therefore, these isolates were registered in NCBI as new isolates under the GenBank accession numbers, that including two isolates per microorganism. *G. lamblia*, isolate IQ No.1 (MN096738) and *G. lamblia* co-infection with *H. pylori* isolate IQ No.2 (MN096739). The accessions number for isolates of *H. pylori* are, seq1(MN115555) and *H. pylori* co-infection with *G. lamblia* isolates seq2 (MN115556).

***Keywords**: Giardia lamblia, Helicobacter pylori, co-infection, PCR.*

Introduction:

Giardiasis (disease caused by *Giardia lamblia*) occurs worldwide and may infect up to a third of the population in developing countries. The disease is reported from other mammals also, which serves to make it difficult to eradicate (1). Approximately about 200 million of people in the world are with clinically manifested giardiasis, with 500,000 new cases per year (2). *Giardia lamblia*, also known as *Giardia intestinalis*, is a flagellated parasitic microorganism, that colonizes and reproduces in the small

intestine, causing giardiasis, the parasite attaches to the epithelium by a ventral adhesive disc or sucker, and reproduces via binary fission (3). *G. lamblia* does not spread via the bloodstream, nor does it spread to other parts of the gastrointestinal tract, but remains confined to the lumen of the small intestine (4). *Giardia* trophozoites absorb their nutrients from the lumen of the small intestine.

Helicobacter pylori (*H. pylori*) is a type of bacteria. These germs can enter human and live digestive tract. After many years, they can cause sores, called ulcers, in the lining of human stomach or the upper part of small intestine , for some people, an infection can lead to stomach cancer. Infection with *H. pylori* is common, about two-thirds of the world's population has it in their bodies. For most people, it doesn't cause ulcers or any other symptoms (5). *H. pylori* is small scale aerophilic winding molded exceptionally motile with four to six lophotrichous flagella Gram-negative microscopic organisms that colonize the stomach of human and cause gastrointestinal disease (6). *H. pylori* is contagious, although the exact route of transmission is not *known*, but Person-person transmission by either the oral-oral or fecal-oral route is most likely (7). Gastrointestinal infections are major causes of morbidity and mortality throughout the world, and particularly in developing countries. Causes of gastrointestinal disease include a wide variety of bacteria, viruses and parasites (8). In low-income countries co-infections involving several different pathogens commonly occur. Several recent cross-sectional studies from different locations, have reported a potential association between *G. lamblia* and *H. pylori*. Both organisms colonize the gastrointestinal tract in their human hosts and both organisms are known to infect children at a high rate (9).

Aim of study:

Molecular diagnosis of *G. lamblia* and *H. pylori* by using conventional PCR, detection co-infection rate between *G. lamblia* and *H. pylori*, study of some factor affecting the spread such as age, gender, geographer and sequencing analysis for genes of *G. lamblia* and *H. pylori*

Methods:

Polymerase chain reaction (PCR)

The PCR technique was performed for detection *G lamblia* and *H. pylori* for stool samples. This method was carried out according to method described by (10,11) as following steps:

Genomic DNA Extraction and genomic DNA estimation The extracted genomic DNA from stool samples was measued by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm) , and PCR master mixed was prepared by using (**Maxime PCR PreMix Kit**), that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler.

Table (1): The PCR primers for detection *Giardia lamblia* and *H. pylori*

Primer	Sequence 5'-3'		amplicon
Giardia lamblia	F	GGGCTAGAAGGCGATCAGAC	543 bp
	R	GGCGCCTACAAGACATTCCT	
H. pylori	F	AAGCTTTTAGGGGTGTTAGGGGTTT	297 bp
	R	AAGCTTACTTTCTAACACTAACGC	

PCR Thermocycler Conditions

PCR thermocycler conditions using convential PCR thermocycler system as following table (2)

Table (2): PCR Thermocycler Conditions

PCR step	Temp.	Time	No. of Cycle
Initial Denaturation	95ºC	5min.	1
Denaturation	95 ºC	30sec.	30 cycle
Annealing	58 ºC	30sec	
Extension	72 ºC	1min.	
Final extension	72 ºC	5min.	1
Hold	4 ºC	Forever	-

PCR product analysis

The PCR products of was analyzed by agarose gel electrophoresis following steps:

1- Agarose gel (1%) was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µl of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volts and 80 AM for 1hour.

5- PCR products were visualized by using UV Transilluminator.

Results:

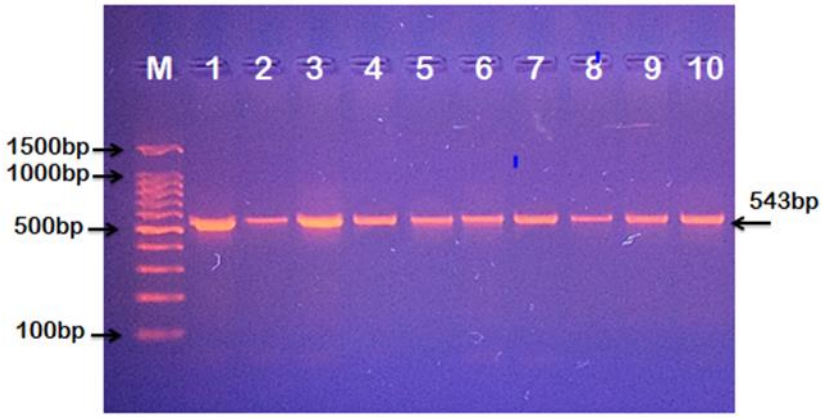
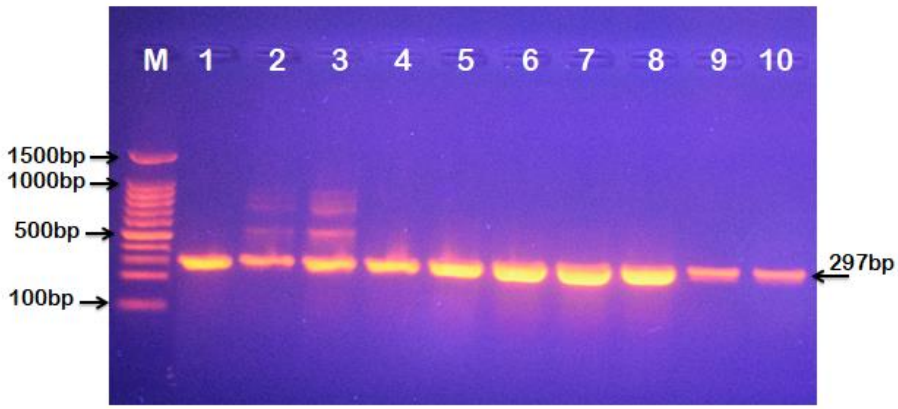


Figure (1): Agarose gel electrophoresis showed that PCR product analysis for 18S ribosomal RNA gene in Giardia lamblia positive isolates. M (Marker ladder 1500-



(100bp). Lane (1-10) some positive Giardia lamblia stool samples at 543bp product size.
Figure (2): Agarose gel electrophoresis showed that PCR product analysis for CagA gene in Helicobacter pylori positive isolates. M (Marker ladder 1500-100bp). Lane (1-10) some positive Helicobacter pylori stool samples at 297bp product size.

Molecular results
percentage of infected and non-infected of stool samples with *Giardia lamblia* and co-infection with *H. Pylori* by using PCR.
The current study includes examination of 96 stool samples from children with non-symptoms examined by using conventional PCR. Table (4-4), showed 32 positive (33.33%) with *G. lamblia* and 14 positive (14.58%) with *H. pylori*, and co-infection were 13 (13. 54%).Table(4-4).

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Table (3): Percentage of infected and non-infected stool samples with *Giardia lamblia* and *co -infection with H. Pylori* by using PCR.

Number of samples examination	*G. lamblia* +		*G. lamblia*-		*H. Pylori*+		*H. Pylori*-		Co-infection	
	No.	%	No.	%	No.	%	No.	%	No.	%
96	32	33.3	64	66.6	14	14.58	82	85.4	13	13.54

Distribution of infection with *G. lamblia* and co-infection with H. *pylori* according to the Gender by using PCR Technique.

Distribution of *G. lamblia* and co-infection with *H. pylori* according the gender of infected children found (18) (56.25%) in female, and (14) (43.75%) in male, infected with *G. lamblia*. and (8) (61.53%) in male, and (5) (38.46%). in female co-infection with *H. pylori*. Figure (3).

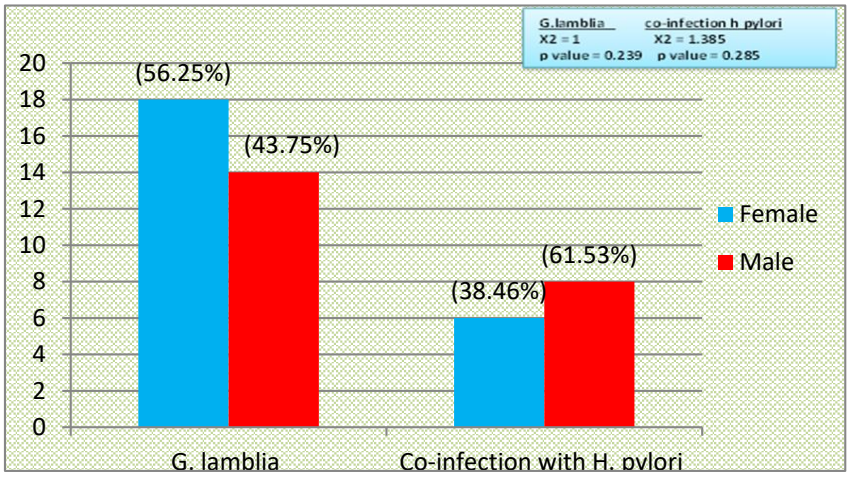

Fig (3): Distribution of infection with *G. lamblia* and co-infection with H. *pylori* according to the Gender by using PCR Technique.

Distribution of infection with *G. lamblia* and co-infection with *H. pylori* according to the Habitation (Rural and Urban) by PCR technique.

The percentage of infected with *G. lamblia* in rural Habitation (18) (56.25%) and in urban (14) (43.75%), and percentage of co-infection with *H. pylori* in rural Habitation (11) (84.61%) and in urban (2) (15.38%). Figure (4).

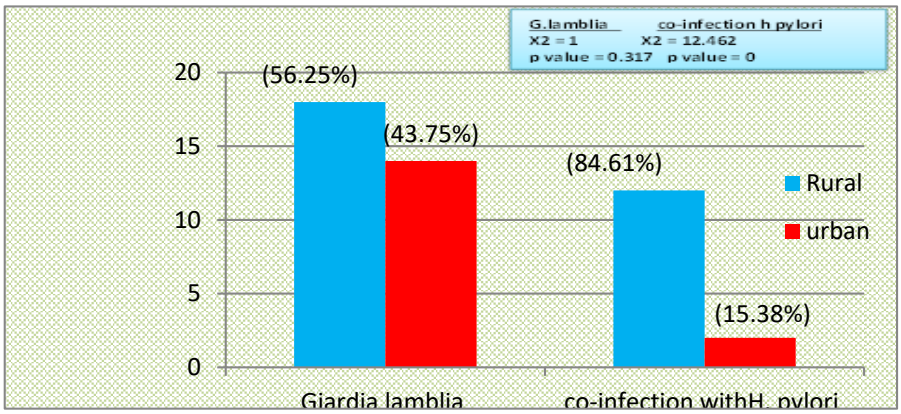

Fig (4): Distribution of infection with *G. lamblia* and co-infection with H. *pylori* according to the Habitation (Rural and Urban) by PCR technique.

Distribution of infection with *G. lamblia* and co-infection with *H. pylori* according to the Age groups by PCR Technique.

Table (4), shows the highest infection in the *G. lamblia* in age groups of 1-12 months (20) (62.5.85%) and the lowest was in the age of 13-24 months (4) (12.5%). And in co-infection with H. *pylori* the highest infection was in age groups 1-12 months (7) (53.84%), and the lowest infection was in age of 37-48 months 4(14.28%).

Table (4): Distribution of infection with *G. lamblia* and co-infection with *H. pylori* according to the Age groups by PCR Technique.

Age groups	*G. lamblia*		co-infection *H. pylori*	
	No.	%	No.	%
1-12	20	62.5	7	53.84
13-24	4	12.5	0	0
25-36	6	18.75	4	30.76
37-48	2	6.25	2	15.38
Total	32	100	13	100
X2	33.333(**)		10.974(*)	
P value	0		0.012	

3. * : significant difference at p < 0.05
4. ** : significant difference at p < 0.01

DNA Sequence results:

G. lamblia

DNA sequencing analysis of *G. lamblia* complete gene showed clear genetic variation between *G. lamblia* isolates from different hosts according to phylogenetic tree analysis that analyzed local *G. lamblia* Human isolates with Standard NCBI-BLAST *G. lamblia* isolates. As show in figure (5).

The local *G. lamblia* Human isolates (No.1 – No2) were show different from other host isolates with closed related to NCBI-Blast *G. lamblia* (**HQ179632.1**). The Homology sequence identity between local

G. lamblia (Human) isolates gene and NCBI BLAST *G. lamblia* isolates. The local *G. lamblia* Human isolates (No.1 – No2) were show (99-100%) homology identity to NCBI-BLAST Human isolate (**HQ179632.1**).

Figure (5): Multiple sequence alignment analysis of 18S ribosomal RNA gene in Giardia intestinalis Iraq No.1 isolates and NCBI-Genbank Giardia intestinalis isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in 18S ribosomal RNA gene.

Figure (6): Multiple sequence alignment analysis of 18S ribosomal RNA gene in Giardia intestinalis Iraq No.2 isolates and NCBI-Genbank Giardia intestinalis isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in 18S ribosomal RNA gene.

Helicobacter. pylori

The DNA sequencing analysis of *H. pylori* CagA gene complete gene was show clear genetic variation between *H. pylori* isolates from different hosts according to phylogenetic tree analysis that analyzed local *H. Pylori* Human isolates with Standard NCBI-BLAST *H. pylori* isolates. As show in figure (7).
The local *H. Pylori* Human CagA gene isolates (No.1 – No2) were show different from other host isolates with closed related to NCBI-Blast *H. pylori* (**CP002073.1**).
The Homology sequence identity between local *H. pylori* (Human) isolates CagA gene and NCBI BLAST *H. pylori* isolates. The local *H. Pylori* Human isolates (No.1 – No2) were show (99-100%) homology identity to NCBI-BLAST Human isolate (**CP002073.1**).

Figure (7): Multiple sequence alignment analysis of cagA gene in local Helicobacter pylori No.1 isolates and NCBI-Genbank Helicobacter pylori isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in cagA gene.

Figure (8): Multiple sequence alignment analysis of cagA gene in local Helicobacter pylori No.2 isolates and NCBI-Genbank Helicobacter pylori isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in cagA gene.

Discussion:

The results of PCR showed that the positive samples with *H. pylori* are 14 (14.58%) from 96 stool sample collected from children with non-symptom, while the positive samples that have co-infection between *G. lamblia* with *H. pylori* are (13.54%). These results agreed with a study in Italy carried by (12), where they found that *H pylori* infection in 37 of 41 (90.2%) patients with gastric giardiasis, while the study disagrees with another study carried by (13)
, who revealed that co-infections of *G. lamblia* and *H. pylori* were found in 4 patients out of 130 (3.8%) in Iran, also disagree with (14) in Lebanese, who found comparable to *H. pylori* prevalence rate (21.0%) among asymptomatic children. The high rates of co-infection between *G. lamblia* and *H. pylori* and leads to the hypothesis there may be presence mechanistic or pathological link (15). Co-infection of *G. lamblia* with *H. pylori* is common in all age groups particularly in under fifth years-aged children and caused gastrointestinal problems (16). In most of the cases, antral mucosa colonized with *Giardia* was found to be co-infection with *H. pylori* (17,18).

The results of current study using PCR technique showed distribution of *G. lamblia* and co-infection with *H. pylori* according the gender of infected children same as in direct examination for detection of *G lamblia* alone while different in co-infection, according to PCR results the percentage of infection with *G. lamblia* is (56.25%) in females and (43.75%) in males. These results agree with (19) in Brazil, who recording infection with *G. lamblia* in females more than males. While the rate of co-infection between *G. lamblia* with *H. pylori* according to PCR technique is (61.53%) in males more than (38.46%) in females. The high rate of co-infection in males more than females may be due to males are more active, and more mixing in society than females

While the results of PCR show the distribution of *G. lamblia* according to age groups are highest rate in age groups of 1-12 months (62.85%), while the lowest infection rate was in age group 37-48 months(٦,٢٥ %).

The current study agrees with the study by (20), who recorded high percent of infection with *G. lamblia* in children with 1-year-old. In our result the distribution of co-infection between *G. lamblia* with *H. pylori* according to age groups show the highest infection was in age group 1-12 months (53.84%), and the lowest infection was in age group of 13-٢٤ months (0%).

The results of PCR technique according to habitation were similar as the results of direct examination for both *G. lamblia* and co-infection with *H. pylori*. These results agree with the study by (22) who demonstrated the effect of education on infection with the *G. lamblia* and *H. pylori* in children who living rural area in India, and also agree with (23) their recording gave similar percentage with the present study. This indicates the role of socioeconomic factors in the prevalence of parasitic and bacterial infections**.**

The DNA sequencing analysis of *G. lamblia* complete gene was show clear genetic variation between *G. lamblia* isolates from different Human according to phylogenetic tree analysis that analyzed local *G. lamblia* Human isolates with Standard NCBI-BLAST *G. lamblia* isolates

Our results show that molecular characterization of the gene has genetic variation in *G. lamblia* isolates from stool samples of child host. This finding was agreement with previously (24), who reported more variation in *G. lamblia* genes.

Our results show that molecular characterization of the gene has genetic variation in *H. pylori* isolates from stool samples. This finding was agreement with previously study who reported more variation in many types of samples (25).

Conclusions:

Prevalence of *G. lamblia* and co-infection with *H. pylori* among non-symptomatic children in Thi-Qar province, not significant different between male and female, Rural areas were highest rates of infection than urban areas between with *G. lamblia* and co-infection with *H. pylori*, the PCR technique is more sensitive and efficient than direct smear to determine the *G. lamblia*, age group (1-12 months) was more susceptible to infection by the *G. lamblia*. and co-infection with *H. pylori* and new genetic mutations have been recorded for both *G. lamblia* and *H. pylori* for the first time in Iraq by the present study.

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