Multilocus sequence typing for understanding the genetic diversity of *Cryptosporidium* spp. if combined with *H. Pylori* in diarrheic immunocompetent Egyptian children

Original Article

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ABSTRACT

Background: Genotypic variation of *H. pylori* is associated with the existence of virulence factors, while different genotypes and subtypes of *Cryptosporidium* spp. are responsible for human cryptosporidiosis.
Objective: To investigate usefulness of multilocus sequence typing (MLST) to analyze the genetic diversity of *H. pylori* and *Cryptosporidium* spp. co-infections in diarrheic immunocompetent Egyptian children. The secondary objective is to determine the detection rate of each pathogen, and co-infection rate, as well as its associated factors.
Subjects and Methods: This cross-sectional study included 305 immunocompetent diarrheic children. Faecal samples were collected and processed using molecular screening techniques to detect and differentiate *Cryptosporidium* spp. and *H. pylori*. *Cryptosporidium* spp. were genotyped by amplifying the 18S rRNA gene, and the gene encoding *Cryptosporidium* oocyst wall protein (COWP) using nested PCR followed by restriction fragment length polymorphism (RFLP). Whereas *H. pylori* strains were identified using PCR to detect genes encoding UreA and CagA. The study also analyzed sociodemographic and clinical parameters to determine associated factors with *Cryptosporidium-H. pylori* co-infection.
Results: Out of the whole studied population, 12.1% had *Cryptosporidium* spp., predominantly *C. hominis* (81.1%); while *H. pylori* DNA was found in 41.0%, with predominance of the CagA$^+$ strain (40.8%). Among the 37 *Cryptosporidium*-positive cases, 27 (73%) had a co-infection with *H. pylori*, and 14 (51.9%) were identified with CagA$^+$ strain. Significant associations were recorded between cryptosporidiosis and factors such as age, gender, source of water and milk, and abdominal pain, while *H. pylori* infection correlated significantly with age, vomiting, and abdominal pain. Co-infections were associated with vomiting and fever, particularly noting that CagA$^+$ *H. pylori* strain significantly correlated with more severe symptoms, indicating its higher pathogenic potential.
Conclusion: This study accepted the complex interplay between *H. pylori* and *Cryptosporidium* spp. in immunocompetent children in Egypt, emphasizing the role of genetic diversity and strain-specific virulence in disease manifestation.

Keywords: 18S rRNA; CagA; co-infection; COWP; *Cryptosporidium* spp.; Egypt; genetic diversity; *H. pylori*; immunocompetent, ureA.

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INTRODUCTION

Diarrheal diseases continue to be a significant contributing factor of mortality for children under five. They are considered an important cause of mortality resulting in 525,000 deaths annually[1]. Additionally, globally, each year, 1.7 billion children with diarrheal diseases are documented[2]. Several pathogens, such as bacteria, viruses, and parasites, can cause diarrhea, but the prevalent siginficant causes in children under five years old include enterotoxigenic *Escherichia coli*, *Cryptosporidium*, *Rotavirus*, and *Shigella*[3].

Cryptosporidium spp. infect a broad spectrum of vertebrates, including humans. On a global scale, the average detection rate of *Cryptosporidium* is estimated to be 7.6%. It is ~4.3% in developed countries, and rises to approximately 10.4% in developing nations. In certain countries, such as Nigeria and Iran, the detection rate can escalate dramatically, reaching rates as high as 69.6%[4]. Acute symptoms such as diarrhea and weight loss in immunocompetent children and adults are suggestive of cryptosporidiosis, while in individuals with weakened immune systems, the infection may be potentially fatal[5]. The report

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of 59% detection rate of cryptosporidiosis among immunocompetent and immunocompromised Egyptian children by nested PCR, highlighted its public health significance and the need for targeted interventions across varied immune statuses[6]. So far, researchers have identified 44 different species and more than 120 genotypes of *Cryptosporidium*. The most commonly encountered species in humans are *C. hominis* and *C. parvum*[7].

In fact, MLST becomes an essential successful protocol to determine the genetic diversity of pathogens. Multiple loci provides a deeper understanding of the population structure than studies that focus on a single locus that provide limited information. It is particularly useful to identify *C. hominis* and, to a lesser degree, *C. parvum*[8].

While *H. pylori* primary infection is reportedly prevalent in childhood, its acquirement is apparently infrequent in later life[9]. The established virulence factors produced by *H. pylori* include the genes encoding CagA, VacA proteins which, when present in *H. pylori* strains, is a risk factor predisposing to stomach cancer[10]. Commonly, *H. pylori* and intestinal parasites have similar transmission modes and may also share risk and predictive factors, one of which may aid another in colonizing[11,12]. Because of their frequency, ability to spread, and effective outcomes on children's development and health, these infections are of great interest even though they are self-limiting in immunocompetent hosts. Since severe diarrhea in infants may result in malnutrition and dehydration, it is essential to establish effective prevention and management strategies. This can be achieved by understanding the biodynamics of pathogens causing severe diarrhea, *i.e.*, their genetic variety, and factors with impacts on their pathogenicity and transmission[13,14].

Accordingly, the present study investigates the genetic basis of *Cryptosporidium* and *H. pylori* co-infections in diarrheic Egyptian children using MLST to identify susceptibility variables that may aid in improvement of treatment and prevention procedures.

SUBJECTS AND METHODS

This cross-sectional study was conducted at the Molecular Parasitology Unit, Faculty of Medicine, Cairo University from July to December 2023.

Study design: Target population in our study was diarrheic stool specimens from immunocompetent children. Molecular techniques, including nested PCR and RFLP were used to genotype *Cryptosporidium* spp. and *H. pylori*. Additionally, sociodemographic and clinical data were collected to determine factors associated with co-infection.

Sample size: This was calculated according to a recent hospital-based study conducted in Egypt[15], Assuming a 72.7% detection rate for co-infection of *H. pylori* and *Cryptosporidium*, a margin of error of 5%, and a confidence level of 95%, the sample size was 305 children.

Study participants: Diarrhic stool specimens were collected from participants aged a few months to 12 years. Data such as age, gender, and residence were recorded on each stool sample container. The study did not include children diagnosed with bacterial infections (*e.g.*, *Salmonella, Shigella*), viral infections (*e.g.*, *Rotavirus*), those with compromised immune systems, those who had recently received antibiotic or antiparasitic treatments, and those with chronic gastrointestinal conditions, *e.g.*, inflammatory bowel disease (IBD), celiac disease, and chronic liver disease.

Sample processing: One sample was collected from each child with diarrhea using a sterile, leak-proof plastic container. Stool samples were divided into three portions: one for microscopic examination to identify intestinal parasites *via* direct wet mount, and formalin-ethyl acetate techniques, another for acid-fast (AF) staining to detect *Cryptosporidium* oocysts[16]. The final part was preserved at -20°C for molecular analysis. Additionally, a data collection sheet was used to record each patient's demographic and clinical information.

Extraction of DNA: Following the manufacturer's guidelines[17], the extraction process involved utilizing 200 mg of each stool sample with the QIAamp DNA Stool Mini Kit (catalog number 51504). Following extraction, the DNA was preserved at -20°C until PCR analysis.

Identification of *Cryptosporidium* spp. and subtyping: Following the protocol by Ghaffari and Kalantari[17], a nested PCR was carried out to amplify the 830 bp of the 18S rRNA gene and the 553 bp of the *Cryptosporidium* oocysts wall protein (COWP), We utilized RFLP to identify the *Cryptosporidium* genotypes/species. The restriction enzymes RsaI and SspI digested the amplified PCR products in a 31-µl (total volume) reaction at 37°C for two hours. Following ethidium bromide staining, the digestion products were separated into 3% Metaphor agarose (Table 1).

Molecular identification and detection of H. pylori isolates: Using specific primers aimed at the gene encoding UreA, the recovered DNA was verified to be *H. pylori*, and PCR using a particular primer allowed for the detection of the gene encoding CagA in *H. pylori* strains (Table 1). The PCR products of 200 and 550 bp, respectively, were examined in agarose gel under a UV lamp and in agarose gel stained with ethidium bromide.

Statistical analysis: A version of IBM SPSS (Version 28.0) was used. The data, encompassing both

Table 1. Reaction conditions, targets, restriction enzymes and primers for identification of *Cryptosporidium* spp. subtypes and *H. pylori* genotypes.

Primer	Sequence (5'-3')	Gene	Reaction conditions	Enz.	Ref.
BcowpF	ACCGCTTCTCAACAACCATCTTGTCCT C	*cowp*	35 cycles of 94°C for 60 s, 63°C for 60 s, and 72°C for 60 s 35 cycles of 94°C for 60 s, 54°C for 30 s, and 72°C for 60 s	*RasI*	[18.19]
BcowpR	CGCACCTGTTCCCACTCAATGTAAACC C				
Cry-15	GTAGATAATGGAAGAGATTGTG				
Cry-9	GGACTGAAATACAGGCATTATCTTG				
rRNA F	TTCTAGAGCTAATACATGCG	*18s rRNA*	35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 60 s 35 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 30 s	*SspI*	[20]
rRNA R	CCCATTTCCTTCGAAACAGGA				
rRNA F2	GGAAGGGTTGTATTTATTAGATAAAG				
rRNA R2	CTCATAAGGTGCTGAAGGAGTA				
2F2	ATATTATGGAAGAAGCGAGAGC	*ureA*	35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min 30 s. 35 cycles of t 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, 30 s	--	[21]
2R2	ATGGAAGTGTGAGCCGATTTG				
2F3	CATGAAGTGGGTATTGAAGC				
2R3	AAGTGTTGAGCCGATTTGAACCG				
CagA F1	GGAACCCTAGTCAGTAATGGGTT	*cag*A	35 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 30 s 35 cycles at 98°C for 10 s 63°C for 30 s and 72°C for 30 s	--	[22]
CagA R1	GCTTTAGCTTCTGATACCGCTTGA				
CagA F2	CCAATAACAATAATAATGGACTCAA				
CagA R2	AATTCTTGTTCCCTTGAAAGCCC				

Enz.: Restriction enzymes; **Ref.:** Reference.

qualitative and quantitative types were displayed. Chi-square and Fisher's exact test were used for group comparisons. Univariate analysis was applied to determine the characteristics of potential patients, clinical manifestations, and the relationship between infection and various *Cryptosporidium* spp. and *H. pylori*. This association was quantified using odds ratios, with a *P* value ≤0.05.

Ethical considerations: The study adhered to the ethical guidelines outlined in the Declaration of Helsinki by the World Medical Association. The research ethics committee of Suez University approved this study (Approval number 56023) on June 4, 2023. All children' parents or guardians gave written consent before recrument in the study.

RESULTS

Participant demographics: The study group was divided into 170 (55.7%) boys and 135 (44.3%) girls. The participants' ages ranged from a few months to 12 years, with a mean of 5.5±4.5 years. We categorized the study population into three age groups: infants, preschool, and school-aged children. The preschool group was the largest, comprising 43.3% of the participants, followed by the school-aged group (38.3%). Regarding residential settings, 54.4% of participants were from urban areas, while 45.6% came from rural areas.

Molecular characterization: *Cryptosporidium* spp. was found in 37 samples (12.1%) by amplifying the *18S rRNA* gene using nested PCR. Using RFLP with *SspI* of the amplified product revealed that *C. hominis* was the predominant isolate (30/37; 81.1%). Besides, *cowp* gene's PCR-RFLP study confirmed the species' designation. On the other hand, using PCR-RFLP analysis of the genes encoding 18S rRNA and COWP, seven isolates (7/30; 18.9%) were shown to be *C. parvum* (Figs. 1, 2).

Association between sociodemographic and clinical parameters, and cryptosporidiosis: The overall infection with *Cryptosporidium* was (37/305, 12.1%). This varied significantly by age (*P*=0.002), with a higher mean age of infected patients than that of non-infected (OR= 2.4, 95% CI 0.843-3.957). Significant variation in the detection rate was also observed among different age groups (*P*=0.05). The detection rate varied by sex (*P*=0.02), being higher among males (26/ 37; 70.0%) compared to females (11/37; 30.0%). A statistically significant association was found with the water source (*P*= 0.016), where those using tap water group showed a higher prevalence than those using filtered water. When studying the detection rate of *Cryptosporidium* according to milk source (pasteurized milk versus breastfeeding), it proved significant (*P*=0.033). Among clinical symptoms, abdominal pain was significantly associated with infection (*P*=0.03) (Table 2). Vomiting was more common in patients with *C. hominis* infection (14/30) in comparison to *C. parvum* infection (1/7). Fever, duration of diarrhoea, and abdominal pain were the same in those infected with both species.

Sociodemographic and clinical parameters and *H. pylori* infection: Overall, *H. pylori* tested positive by PCR in 125/305 (41.0%). Age differences recorded in the prevalence of *H. pylori* were strongly significant.

Infected children were of older mean age (OR= 3.0, 95% CI 2.01-3.99). However, the variation in *H. pylori* prevalence was statistically insignificant compared to other sociodemographic variables. Among the clinical symptoms, vomiting and abdominal pain were statistically significantly (P<0.0001, and =0.031, respectively) (Table 3).

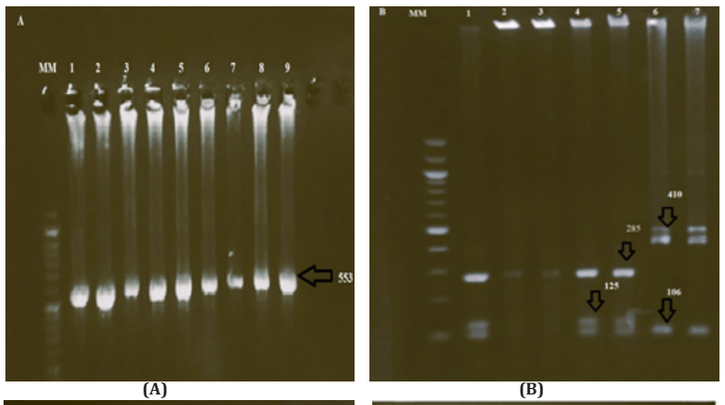

Fig. 1. Agarose gel electrophoresis **(A):** Gene encoding COWP of *Cryptosporidium* spp. at 553 bp. **MM:** 100 bp DNA molecular weight marker "ladder". **Lane 1:** Positive control at 553 bp. **Lanes 2-9:** Positive samples; **(B):** After digestion by *RsaI*, **MM:** 100 bp DNA molecular weight marker "ladder". **Lanes 1–5:** Positive *C. hominis* samples (285,125, 106). **Lanes 6,7:** Positive *C. parvum* samples (410 and106).

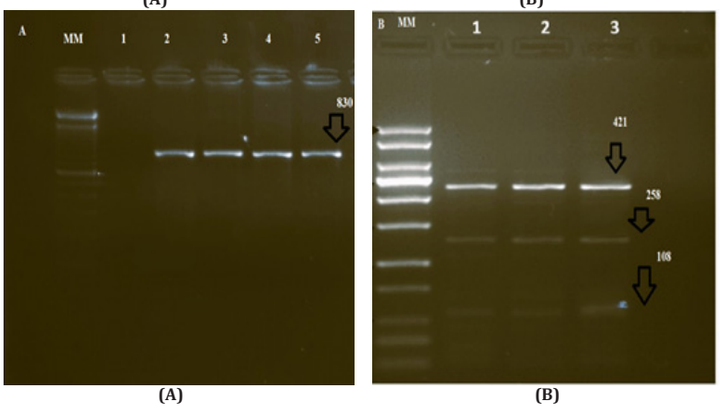

Fig. 2. Agarose gel electrophoresis **(A):** 18SrRNA of *Cryptosporidium* spp. at 830 bp. **MM:** 100 bp DNA molecular weight marker "ladder". **Lane 1:** Negative control. **Lanes 2-5:** Positive samples; **(B)** After digestion by *SspI*, **MM:** 100 bp DNA molecular weight marker "ladder". **Lanes 1–3:** Positive *C. hominis*/*C. parvum* samples (421, 258, 108).

Table 2. Sociodemographic, clinical data, and *Cryptosporidium* infection.

Variables			*Cryptosporidium* infection			Statistical analysis	
			Total No. (%)	Infected No. (%)	Not infected No. (%)	OR (CI 95%)	Significance (P value)
Age			5.5 ± 4.5	7.6 ± 4.6	5.2 ± 4.5	2.4 (0.843-3.957)	0.002*
Age groups	**Infants (<1)** **Preschool child (>1-5)** **School child (>5-12)**		56 (18.4%) 132 (43.3%) 117 (38.3%)	2 (5.4%) 17 (45.9%) 18 (48.7%)	54 (20.1%) 115 (43%) 99 (36.9%)	NA	0.05*
Sex	**Male** **Female**		170 (55.7%) 135 (44.3%)	26 (70.0%) 11 (30.0%)	144 (53.7%) 124 (46.3%)	1.99 (1.11-3.55)	0.02*
Residence	**Urban** **Rural**		166 (54.4%) 139 (45.6%)	22 (59.5%) 15 (40.5%)	144 (53.7%) 124 (46.3%)	1.28 (0.73-2.24)	0.39
Water source	**Tap water** **Filtered water**		291 (95.4%) 14 (4.6%)	32 (86.5%) 5 (13.5%)	259 (96.6%) 9 (3.4%)	0.207 (0.057-0.751)	0.016*
Milk source	**Raw (cow milk)** **Pasteurized milk** **Breastfeeding**		134 (43.9%) 92 (30.2%) 79 (25.9%)	15 (40.5%) 15 (40.5%) 7 (19.0%)	119 (44.4%) 77 (28.7%) 72 (26.9%)	NA	0.033*
Clinical Symptoms	**Vomiting**	Yes	105 (34.4%)	15 (40.5%)	90 (33.6%)	1.35 (0.76 to 2.41)	0.305
		No	200 (65.6%)	22 (59.4%)	178 (66.4%)		
	Fever	Yes	57 (18.7%)	9 (24.3%)	48 (17.9%)	1.44 (0.724-2.857)	0.299
		No	248 (81.3%)	28 (75.7%)	220 (82.1%)		
	Abdominal pain	Yes	277 (90.8%)	37 (100.0%)	240 (89.6%)	23.32 (1.347-403.67)	0.03*
		No	28 (9.2%)	0 (0.0%)	28 (10.4%)		
Total No. (%)			305 (100%)	37 (12.1%)	268 (87.9%)		

Data displayed as number (No.) and percentage (%); **NA:** Not applicable; ***:** Significant (P<0.05).

Table 3. Sociodemographic, clinical data, and *H. pylori* infection.

Variables			*H. pylori* infection			Statistical analysis	
			Total No. (%)	Infected No. (%)	Not infected No. (%)	OR (CI 95%)	Significance (*P* value)
Age			5.5 ± 4.5	7.6 ± 4.6	5.2 ± 4.5	2.4 (0.843-3.957)	0.002*
Age groups	**Infants (<1)** **Preschool child (>1-5)** **School child (>5-12)**		56 (18.4%) 132 (43.3%) 117 (38.3%)	2 (5.4%) 17 (45.9%) 18 (48.7%)	54 (20.1%) 115 (43%) 99 (36.9%)	NA	0.05*
Sex	**Male** **Female**		170 (55.7%) 135 (44.3%)	26 (70.0%) 11 (30.0%)	144 (53.7%) 124 (46.3%)	1.99 (1.11-3.55)	0.02*
Residence	**Urban** **Rural**		166 (54.4%) 139 (45.6%)	22 (59.5%) 15 (40.5%)	144 (53.7%) 124 (46.3%)	1.28 (0.73-2.24)	0.39
Water source	**Tap water** **Filtered water**		291 (95.4%) 14 (4.6%)	32 (86.5%) 5 (13.5%)	259 (96.6%) 9 (3.4%)	0.207 (0.057-0.751)	0.016*
Milk source	**Raw (cow milk)** **Pasteurized milk** **Breastfeeding**		134 (43.9%) 92 (30.2%) 79 (25.9%)	15 (40.5%) 15 (40.5%) 7 (19.0%)	119 (44.4%) 77 (28.7%) 72 (26.9%)	NA	0.033*
Clinical Symptoms	**Vomiting**	Yes	105 (34.4%)	15 (40.5%)	90 (33.6%)	1.35 (0.76 to 2.41)	0.305
		No	200 (65.6%)	22 (59.4%)	178 (66.4%)		
	Fever	Yes	57 (18.7%)	9 (24.3%)	48 (17.9%)	1.44 (0.724-2.857)	0.299
		No	248 (81.3%)	28 (75.7%)	220 (82.1%)		
	Abdominal pain	Yes	277 (90.8%)	37 (100.0%)	240 (89.6%)	23.32 (1.347-403.67)	
		No	28 (9.2%)	0 (0.0%)	28 (10.4%)		
Total No. (%)			305 (100%)	37 (12.1%)	268 (87.9%)		

Data displayed as number (No.) and percentage (%); **NA:** Not applicable; ***:** Significant (*P*<0.05).

Association between sociodemographic and clinical parameters, and *H. pylori* CagA⁺ status: In patients infected with *H. pylori*, CagA⁺ strains were identified in 40.8% of them. Detection rate of CagA⁺ *H. pylori* strains varied with water source (*P*= 0.046), being higher in the tap water group than in the group consuming filtered water. The variation in the detection rate of CagA⁺ *H. pylori* strains was statistically insignificant compared to other variables. Among the clinical symptoms, vomiting and fever were associated with CagA⁺ *H. pylori* (*P*= 0.01, and 0.004 respectively) (Table 4).

Table 4. Sociodemographic, clinical parameters and *H. pylori* CagA status.

Variables			*H. pylori* CagA status			Statistical analysis	
			Total No. (%)	Infected No. (%)	Not infected No. (%)	OR (CI 95%)	Significance (*P* value)
Age			7.3 ± 4.6	7.5 ± 4.8	7.2 ± 4.4	0.3 (-1.952-1.35)	0.719
Age groups	**Infants (<1)** **Preschool child (>1-5)** **School child (>5-12)**		17 (13.6%) 54 (43.2%) 54 (43.2%)	10 (19.6%) 18 (35.3%) 23 (45.1%)	7 (9.5%) 36 (48.6%) 31 (41.9%)	NA	0.241
Sex	**Male** **Female**		74 (59.2%) 51 (40.8%)	32 (62.7%) 19 (37.3%)	42 (56.8%) 32 (43.2%)	1.28 (0.729-2.265)	0.387
Residence	**Urban** **Rural**		73 (58.4%) 52 (41.6%)	28 (54.9%) 23 (45.1%)	45 (60.8%) 29 (39.2%)	0.815 (0.465-1.429)	0.475
Water source	**Tap water** **Filtered water**		116 (92.8%) 9 (7.2%)	45 (88.2%) 6 (11.8%)	71 (96.0%) 3 (4.0%)	0.306 (0.095-0.983)	0.046*
Milk source	**Raw (cow milk)** **Pasteurized milk** **Breastfeeding**		66 (52.8%) 42 (33.6%) 17 (13.6%)	29 (56.9%) 14 (27.4%) 8 (15.7%)	37 (50.0%) 28 (37.8%) 9 (12.2%)	NA	0.269
Clinical Symptoms	**Vomiting**	Yes	65 (52.0%)	32 (62.7%)	33 (44.6%)	2.08 (1.182-3.66)	0.01*
		No	60 (48.0%)	19 (37.3%)	41 (54.4%)		
	Fever	Yes	20 (16.0%)	13 (25.5%)	7 (9.5%)	3.16 (1.43-6.98)	0.004*
		No	105 (84.0%)	38 (74.5%)	67 (90.5%)		
	Abdominal pain	Yes	120 (96.0%)	50 (98.0%)	70 (94.6%)	2.58 (0.489-13.62)	0.265
		No	5 (4.0%)	1 (2.0%)	4 (5.4%)		
Total No. (%)			125 (100%)	51 (40.8%)	74 (59.2%)		

Data displayed as number (No.) and percentage (%); **NA:** Not applicable; ***:** Significant (*P*<0.05).

Association between sociodemographic and clinical parameters and cryptosporidiosis co-infected with *H. pylori:* Among the 37 *Cryptosporidium*-positive cases, 27 (73%) were co-infected by H.pylori, with 14 (51.9%) identified as CagA⁺ *H. pylori* strain. There were no significant differences among clinical symptoms; but, both fever and vomiting were significantly associated with co-infection by CagA⁺ *H. pylori* strain and *Cryptosporidium* (P<0.0001, and =0.004 respectively) (Table 5).

Table 5. Sociodemographic and clinical data in *Cryptosporidium-H. pylori* co-infections.

Variables			Co-infection with ***H. pylori* CagA**			**Statistical analysis**	
			Total No. (%)	**Infected No. (%)**	**Not infected No. (%)**	**OR (CI 95%)**	**Significance (P value)**
Age			8.0 ± 4.0	8.6 ± 4.1	7.0 ± 4.7	1.6 (1.89-5.09)	0.354
Age groups	**Infants (<1)**		2 (7.5%)	2 (14.2%)	0 (0.0%)	NA	1.0
	Preschool child (>1-5)		13 (48.1%)	6 (42.9%)	7 (53.8%)		
	School child (>5-12)		12 (44.4%)	6 (42.9%)	6 (46.2%)		
Sex	**Male**		18 (66.7%)	10 (71.4%)	8 (61.5%)	1.57 (0.868-2.824)	0.137
	Female		9 (33.3%)	4 (28.6%)	5 (38.5%)		
Residence	**Urban**		16 (59.3%)	9 (64.3%)	7 (53.8%)	1.58 (0.896- 2.794)	0.114
	Rural		11 (40.7%)	5 (35.7%)	6 (46.2%)		
Water source	**Tap water**		24 (88.9%)	12 (85.7%)	12 (92.3%)	0.46 (0.178-1.199)	0.113
	Filtered water		3 (11.1%)	2 (14.3%)	1 (6.7%)		
Milk source	**Raw (cow milk)**		11 (40.7%)	7 (50.0%)	4 (30.8%)	NA	1.0
	Pasteurized milk		13 (48.2%)	5 (35.7%)	8 (61.5%)		
	Breastfeeding		3 (11.1%)	2 (14.3%)	1 (7.7%)		
Clinical Symptoms	**Vomiting**	Yes	11 (40.7%)	7 (50.0%)	4 (30.8%)	2.33 (1.31-4.17)	0.004*
		No	16 (59.3%)	7 (50.0%)	9 (69.2%)		
	Fever	Yes	7 (25.9%)	6 (42.9%)	1 (6.7%)	10.45 (4.41- 24.78)	0.0001*
		No	20 (74.1%)	8 (57.1%)	12 (92.3%)		
	Abdominal pain	Yes	27 (100.%)	14 (100.0%)	13 (100.0%)	1.0 (0.019- 50.894)	1.0
		No	0 (0.0%)	0 (0.0%)	0 (0.0%)		
Total No. (%)			27 (100%)	14 (51.9%)	13 (48.1%)		

Data displayed as number (No.) and percentage (%); **NA:** Not applicable; ***:** Significant (P<0.05).

DISCUSSION

Diarrhea remains a significant health issue in children, frequently attributed to pathogens as *Cryptosporidium* and *H. pylori*. *Cryptosporidium* is a notable cause due to its high detection rate and potential for severe outcomes. Although *H. pylori* is primarily associated with stomach infections, it can also contribute to diarrheal cases. Co-infections by these pathogens can make diagnosis and treatment more difficult, which emphasizes the necessity for thorough microbiological analysis in children with diarrhea to enhance management techniques and understand their epidemiological impact in countries like Egypt[15].

Our present study investigated the genetic diversity of *Cryptosporidium* and *H. pylori* using MLST. This approach is validated by Robinson *et al*.[23], for its effectiveness in differentiating between closely related strains, offering insights into their transmission patterns and evolutionary backgrounds. It was possible to identify a predominance of certain strains of *C. hominis* and CagA⁺ *H. pylori*, recognized for their increased virulence; this observation aligns with Uran-Velasquez *et al*.[8]. Species of *Cryptosporidium* were identified in 12.1% (37/305) of the study population through COWP and 18s rRNA gene analysis. This highlights the significance of the parasite in children with diarrhea. The predominance of *C. hominis*, found in 81.1% of our cases, is consistent with previous findings[24], emphasizing its high detection rate in children. These results indicate the need for effective measures to control spread of cryptosporidiosis, particularly in regions like Egypt, where such infections are endemic. However, the detection of *C. parvum* (18.9%) also raises concerns over potential zoonotic transmission routes, highlighting the importance of considering the presence of multiple species in surveillance and control efforts[14,15]. These study results indicate a dominant anthroponotic transmission route for *C. hominis* identified in low-income countries The findings of Yang *et al*.[25] pointed to a major anthroponotic transmission route for *C. hominis* found in low-income countries. Regarding the association of demographics with cryptosporidiosis, our results indicated that in contrast to the lower infection rates in infants, older children had a higher detection rate , especially in the school- and preschool-age groups. This finding contradicts other previous studies[4,15,26], who reported higher cryptosporidiosis rates among infants. Conversely, Zhao *et al*.[27], documented similar results suggesting

the occurrence of variations in *Cryptosporidium* epidemiology across different populations and regions. The observed gender disparity in cryptosporidiosis rates, with males showing a higher incidence than females with a significant association (P=0.002), suggests a potential gender-based difference in exposure or susceptibility. This supports other researches suggesting that gender-specific behaviors might influence infection risks. In contrast, Bitilinyu-Bangoh *et al*.[28] found no evidence of significant correlation between gender and cryptosporidiosis .

Our findings revealed that cryptosporidiosis was higher among urban residents (59.5%) than their rural counterparts (40.5%), though this difference did not attain statistical significance. Contrastingly, according to Leder *et al*.[29], a higher detection rate of cryptosporidiosis is found in rural areas compared to urban areas. It may be justified by the differing environmental and sanitation conditions prevalent in rural versus urban environments. Moreover, in our analysis, tap water and raw milk consumption were significantly associated with cryptosporidiosis (P=0.016, and 0.033, respectively). This association highlights potential risk factors for transmission within the study population and underscores the importance of addressing these practices in public health strategies to mitigate infection risk. To reduce the risk of infection, public health interventions should prioritize addressing these practices, as this association suggests possible risk factors for transmission within the study population. Similar results were recorded by Khan *et al*.[30] who registered drinking water as the primary route of *Cryptosporidium* exposure and infection.

Gopfert *et al*.[31] drew attention to the connection between unpasteurized milk intake and outbreaks of cryptosporidiosis, with many confirmed cases previously experiencing gastrointestinal disturbances and diarrhea. Notably, among our patients abdominal pain emerged as a significant symptom associated with the infection (P=0.03). In our study, infections by *C. hominis* were associated with a higher incidence of frequent vomiting than *C. parvum*, implying that *C. hominis* infections may exhibit greater severity. This finding is consistent with the allegation that *C. hominis* generally causes more severe symptoms than *C. parvum*[32].

A higher age detection rate correlated with positive *H. pylori* infection indicating that age could play a role in influencing infection rates. This observation is supported by two studies that noted a linear increase in *H. pylori* infection rates with advancing age, suggesting that a similar age-related trend may exist[14,33]. In the present study, children from almost all age groups were infected with *H. pylori*, which indicates that the infection is more common in children of school age and preschool age, but this difference was statistically insignificant. Thus, implementing a health awareness program in schools to improve kids' behavior and personal hygiene may help lower the risk of illness. According to our data, male infection rates were insignificantly higher than female infection rates (OR=1.23, P=0.476). According to Abbas *et al*.[34], acquirement of *H. pylori* infection was linked to male gender. Our results align with those of Ibrahim *et al*.[15] who found no statistically significant correlation between illness and place of residence, milk source, or drinking water. While *H. pylori* infection was rpeportedly high in childhood non-ulcer dyspepsis yet it was not associated with specific gastrointestinal symptoms[35]. On the other hand our study found an association between *H. pylori* infection and symptoms such as vomiting (P<0.0001) and abdominal pain (P=0.031), underscoring the impact of the infection on gastrointestinal health. Our study also observed CagA$^+$ *H. pylori* strains in 40.8% of cases, associated with vomiting (P=0.01) and fever (P=0.004), and suggesting that individuals with CagA$^+$ strains may experience more severe symptoms. The link between tap water and CagA positivity (P=0.046) suggests a potential route of transmission or risk factor associated with water quality. Studies supporting these findings might focus on the virulence of CagA$^+$ strains and their impact on clinical outcomes, as previously reported[36,37]. Converse arguments may highlight the multifactorial nature of *H. pylori* infection symptoms, suggesting that other bacterial factors or host responses play a significant role.

We recorded an association of some sociodemographic and clinical parameters with co-infection by *Cryptosporidium* spp. and *H. pylori*, including CagA$^+$ and negative strain, contributing to the ongoing research in this area. According to Ibrahim *et al*.[15], immunocompromised children who frequently experience diarrhea are often co-infected by *Cryptosporidium* spp. and *H. pylori*, with a significant detection rate of both pathogens. Significant associations have been noted between co-infection with *H. pylori* CagA$^+$ strains and clinical symptoms, including fever (P<0.0001) and vomiting (P=0.004), indicating that these more pathogenic strains may cause more severe systemic and gastrointestinal symptoms. Additionally, the presence of abdominal pain among all co-infected individuals indicates that it is a common symptom of co-infection, unaffected by The *H. pylori* strain's pathogenicity. This indicates that CagA+ *H. pylori* strains may have an aggravating effect on co-infection symptoms.

In conclusion, this study revealed significant findings with implications for public health. Identifying *C. hominis* as the predominant species of *Cryptosporidium* emphasizes the need for targeted interventions in Egypt; *i.e.*, the co-infection dynamics between *H. pylori* and *Cryptosporidium* spp. highlights how difficult it is to diagnose and treat diarrheal illnesses in this population. Future research should focus on pathogenic genetic diversity to enhance diagnostic accuracy and

treatment efficacy. Additionally, our MLST analysis suggests critical insights into the epidemiology and pathogenicity of these infections, providing a basis for improved diagnostic, prevention, and treatment strategies.

Our study has some limitations. The sample size provided initial insights into the genetic variability of *Cryptosporidium* and *H. pylori* based on MLST in Egypt; a broader study encompassing a more diverse population across various regions of Egypt would enhance our findings. Additionally, our genetic analysis focused on specific loci; exploring a more comprehensive array of genetic markers and virulence factors for *H. pylori* could yield a more detailed understanding of the epidemiology and pathogenicity of these infections.

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