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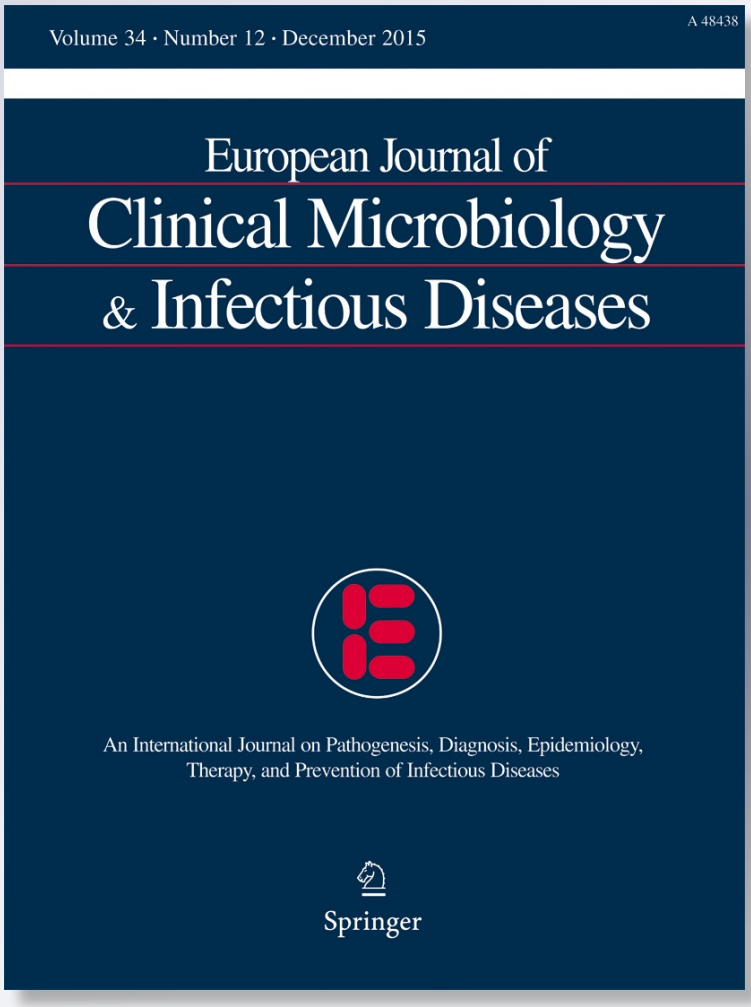

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ORIGINAL ARTICLE

Molecular seasonal, age and gender distributions of *Cryptosporidium* in diarrhoeic Egyptians: distinct endemicity

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Abstract Cryptosporidiosis is a worldwide gastrointestinal disease caused by the protozoan *Cryptosporidium* parasite. It has a broad range of seasonal and age-related prevalence. We aimed to study the molecular prevalence and seasonality of *Cryptosporidium* over a period of 1 year in a cohort of Egyptian diarrhoeic patients. Stool samples were collected from 865 diarrhoeic patients attending outpatient clinics of Cairo University hospitals, from all age groups over a 12-month period, examined microscopically for faecal *Cryptosporidium* oocysts by the acid-fast staining method and for copro-DNA detection using nested polymerase chain reaction (nPCR) assays. PCR-positive samples were characterised molecularly by nPCR-restriction fragment length polymorphism (RFLP) to determine *Cryptosporidium* genotypes. *Cryptosporidium* copro-DNA was detected in 19.5 % of the collected samples throughout the year, with a major peak in summer (August) and a small rise in spring (April). Infection was mainly *C. hominis* (95.8 %) followed by *C. parvum* (3.0 %), affecting all age groups, with predominance in the pre-school age group, and decrease with age. There were statistically significant associations between the detection of *Cryptosporidium* and season, diarrhoea, patient age and drinking water, while gender, contact with animals and presence of mucus in stool showed no association. *Cryptosporidium* in diarrhoeic Egyptians was of distinct endemicity, with the bi-model mostly influenced by population dynamics, with a clear high prevalence in pre-school children and predominating anthroponotic (*C. hominis*) transmission throughout the year. The obtained results highlight *Cryptosporidium* as a water contaminant and an important cause of health problems in Egypt, necessitating further studies of the risk factors.

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Introduction

Cryptosporidium is a worldwide enteric zoonotic protozoan parasite infecting a wide range of hosts, including mammals, birds, reptiles and fish [1]. Cryptosporidiosis was identified as a worldwide health problem and included in the World Health Organization (WHO) Neglected Diseases Initiative in 2004 [2]. It is a chief cause of diarrhoeal diseases in both developing and developed countries [3]. In Egypt, it is reported as a virulent agent of diarrhoea, especially in childhood, with varied prevalence [4]. Routine diagnosis is by coproscopy and copro-immunoassay is limited, as some *Cryptosporidium* infections escaped detection and species identification. Polymerase chain reaction (PCR)-based methods, beside having high diagnostic performance, have been used for the identification of species and genotypes. PCR followed by restriction fragment length polymorphism (RFLP) analysis or sequencing were required to understand the epidemiology and study outbreaks [5, 6]. Several *Cryptosporidium* species cause human infection, with zoonotic *C. parvum* and anthroponotic *C. hominis* being the main species, contributing to the complexity of cryptosporidiosis epidemiology [7].

Seasonality is a character of many infectious enteric diseases, including cryptosporidiosis. Environmental influences and population socio-demographic and behavioural characteristics are the main drivers of enteric disease seasonality. They affect parasite transmission and spread [8]. Some researchers have reported seasonal variation of cryptosporidiosis in their

studies in developing countries, including Egypt; however, they didn't include all months of the year or they didn't study the molecular identification of *Cryptosporidium* species or studies were done on small sample sizes, which are considered limitations to health studies and statistical power.

This study aimed to determine the molecular prevalence of *Cryptosporidium* in diarrhoeic Egyptians over a 12-month period to assess its true seasonal pattern. Also. patients' age and gender distributions were determined.

Materials and methods

Study population and ethical considerations

This was a cross-sectional study. Stool samples submitted for parasite examination from 862 diarrhoeic patients attending outpatient clinics of Cairo University hospitals, from all age groups over a 12-month period from March 2013 to March 2014 were collected. Their related data were recorded.

The study was ethically approved by the ethical committee of Faculty of Medicine, Cairo University and informed consent was obtained from patients or their relatives and parents of young children, and they responded to questionnaires.

Sample collection and processing

A single faecal sample was obtained from each case. Collected stool samples were examined microscopically for faecal *Cryptosporidium* oocyst by the acid-fast staining method prior to and after concentration. The remaining part of the specimen was stored at −20 °C for molecular studies. Genomic DNA was extracted from the remaining part of fresh frozen faecal samples using the FavorPrep Stool DNA Isolation Mini Kit (Favorgen Biotech Corporation, Ping-Tung 908, Taiwan), according to the manufacturer's instructions after thermal shock of samples (five cycles of deep freezing and boiling in a water bath, each for 5 min), with prolongation of incubation for 1 h at 95 °C after 56 °C at 10 min. Extracted copro-DNA was amplified by nested PCR (nPCR) targeting the COWP gene, using two sets of primers: external primers, BCOWPF (5′-ACCGCTTCTCAACAACCATCTTGTCCTC-3′) and BCOWPR (5′-CGCACCTGTTCCCACTC AATGTAAACCC-3′), which amplify a 796-bp fragment [9], and nested primers, cry-15 (5′-GTAGATAATGGAAGAGATTGTG-3′) and cry-9 (5′-GGACTGAAATACAGGCATTATCTTG-3′), which amplify a 553-bp fragment [10]. The reaction mixture and conditions were done in a total volume of 25 μL, according to Spano et al. [10]. The amplified products were visualised with 1.5 % agarose gel electrophoresis after ethidium bromide staining. PCR products were digested by *Rsa*I (Fermentas UAB, V.Graiciuno 8, LT-02241 Vilnius, Lithuania). Digestion of N-COWP fragments was resolved by electrophoresis in 3.2 % typing-grade agarose gels containing ethidium bromide. The fragments were visualised by UV light to determine the *Cryptosporidium* genotype.

Coproscopy was carried out in the Diagnostic & Research Unit of Parasitic Diseases (DRUP) and the copro-nPCR assay was held in the Lab of Molecular Medical Parasitology (LMMP), Department of Medical Parasitology, Faculty of Medicine, Cairo University, Egypt.

Table 1 Diagnostic yield of the used nested polymerase chain reaction (nPCR) assay for the detection and genotyping of *Cryptosporidium* within the study group

nPCR-RFLP		Frequency	%
Negative		694	80.5 %
Positive	*C. hominis*	161	18.7 % (95.8 % within positive group)
	C. parvum	5	0.6 % (3.0 % within positive group)
	Non-typed	2	0.2 % (1.2 % within positive group)
	Total	168	19.5 %
Total		862	100 %

Data are presented as frequency and %

Statistical analysis

Data were tabulated and processed by the Statistical Package for the Social Sciences (SPSS) version 17 (Chicago, IL, USA) for statistical analysis. Positive rates were expressed as percentages. Differences in prevalence rates among groups of the

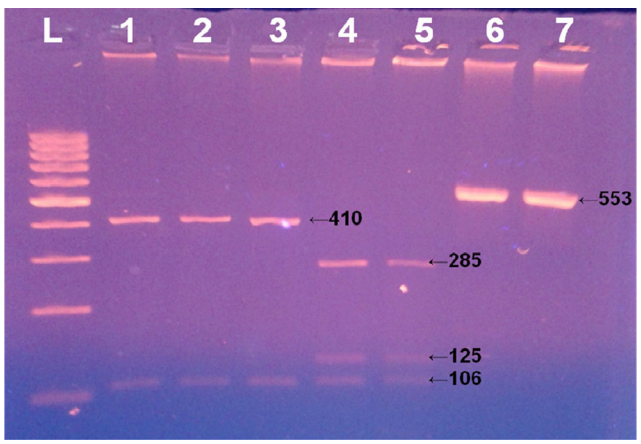

Fig. 1 Agarose gel electrophoresis showing: L: 100-bp DNA molecular weight marker; lanes 1–3: restriction fragment length polymorphism (RFLP) products after digestion with *Rsa*I endonuclease with *Cryptosporidium parvum* genotype 2 digestion products at 34, 106 and 410 bp (the 34 band is very small, faint and difficult to see); lanes 4 and 5: RFLP products after digestion with *Rsa*I endonuclease with *C. hominis* digestion products at 34, 106 and 285 bp (the 34 band is very small, faint and difficult to see); lanes 6–7: products of the nested polymerase chain reaction (nPCR) targeting the COWP gene of *Cryptosporidium* at 553 bp

studied variables were compared by the Chi-square test. Data were considered significant for a p-value<0.05.

Results

Out of 862 examined stool samples with nPCR, copro-DNA was detected in 168 (19.5 %) stool samples of diarrhoeic patients. Among them, infection was mainly *C. hominis* in 161 (95.8 %) samples, followed by *C. parvum* in 5 (3.0 %) samples; the remaining two cases were non-typed. The diagnostic yield of the used nPCR assay for the detection and genotyping of *Cryptosporidium* within the study group is represented in Table 1. *Cryptosporidium* oocysts were detected in 64 (7.4 %) samples using MZN-stained stool smears.

Cryptosporidium was detected in the collected study samples throughout the year, with a large increase in June to September, peaking in August and a very small rise from February

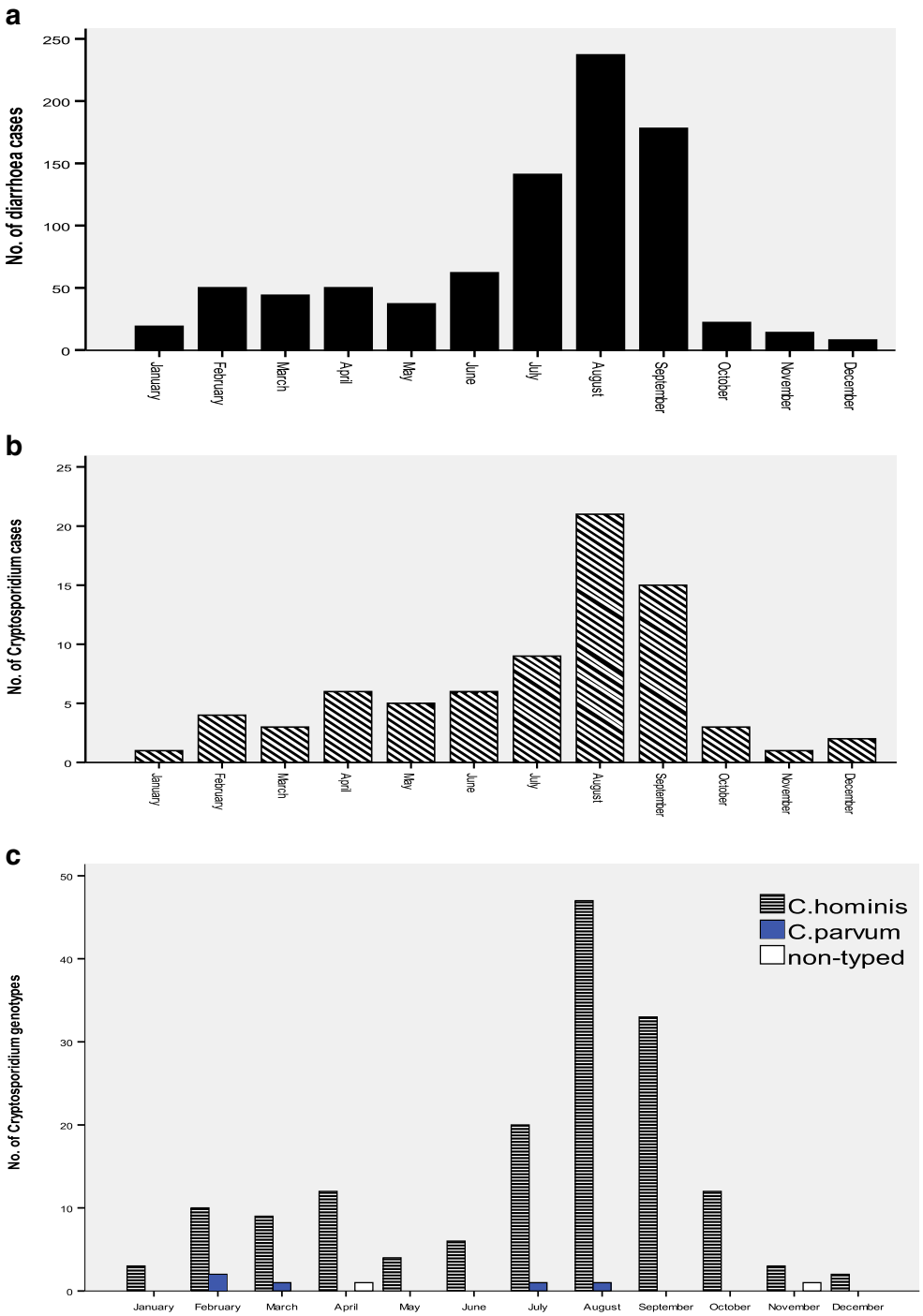

Fig. 2 Seasonal distribution of cases of diarrhoea (**a**), *Cryptosporidium* (**b**) and its species (**c**) among diarrhoeic patients positive by nPCR (p-values for the seasonal distribution of diarrhoea [**a**] was 0.0001 and for *Cryptosporidium*-positive cases [**b**] was 0.003)

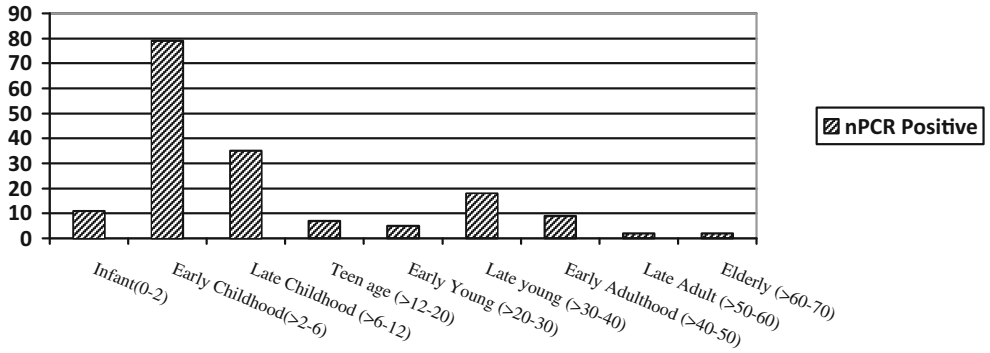

Fig. 3 Age distribution of *Cryptosporidium* among diarrhoeic patients positive for *Cryptosporidium* by nPCR (*p*-value for the age distribution was 0.0001)

to April, peaking in April. It affects both sex and all age groups, with predominance in the pre-school age group (Figs. 1, 2, 3 and 4).

Among the studied variables, there were statistically significant associations between season (month), diarrhoea seasonality, patient age and drinking water and detection of *Cryptosporidium*, while gender, contact with animals and presence of mucus in stool showed no association (Figs. 2, 3 and 4 and Table 2).

Discussion

In our study, *Cryptosporidium* was a prevailing protozoan with distinct endemicity among diarrhoeic Egyptians. *Cryptosporidium* transmission occurred throughout the year and was due to sporadic rather than outbreak-associated infections. The bi-model seasonal pattern was identified with a major seasonal summer peak, preceded by a small spring peak and both peaks showed *Cryptosporidium* human strain (*C. hominis*) predominance. There was a close association between cryptosporidial infection and the occurrence of diarrhoea. The period with an increase in *Cryptosporidium* prevalence was associated with higher prevalence in diarrhoea during the same period.

A wide range of *Cryptosporidium* molecular prevalences (4.6–25 %) was reported in Egypt, and most of the studies reported a high prevalence [6, 11–13]. We reported a prevalence of 7.4 % using MZN-stained stool smear. Coproscopy had specificity; all of them were positive by PCR. However, it was of limited sensitivity, with many cases that escaped diagnosis.

The UK, oceanic countries (Australia and New Zealand), Northern Americas (USA and Canada) and European countries had clear bi-modal peaks, with one major peak in spring (UK, oceanic countries) or late summer and early autumn (Northern Americas) attributed to the human strain and an additional smaller second peak related to the bovine strain with an increase in animal contact; the lowest cases were in winter [14]. In Brazil [15], Ethiopia [16], the Philippines [17] and many tropical countries, transmission was associated with rainy seasons [18, 19].

In Egypt, there is a little winter rainfall; however, cryptosporidiosis was more prevalent in summer, as also reported in some tropical countries with little rainfall, such as Peru, which also shows the prevalence of cryptosporidiosis in warm

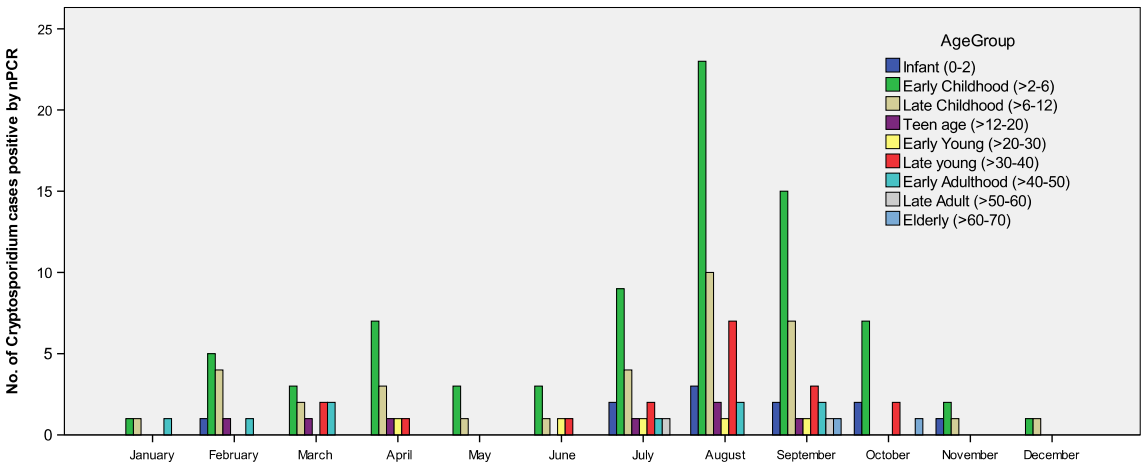

Fig. 4 Pattern of seasonal age group distribution of *Cryptosporidium* species among diarrhoeic patients positive by nPCR (*p*-value for the age group distribution was 0.0001)

Table 2 nPCR-positive cases of cryptosporidiosis in association with the different studied variables other than season and age group

		nPCR			
		Negative	Positive	Total	*p*-Value*
Child/adult	Child	401 (46.5 %)	125 (14.5 %)	526 (61.0 %)	0.0001
	Adult	293 (34.0 %)	43 (5.0 %)	336 (39.0 %)	
Gender	Male	368 (42.7 %)	87 (10.1 %)	455 (52.8 %)	0.773
	Female	326 (37.8 %)	81 (9.4 %)	407 (47.2 %)	
Type of water	Tape	674 (78.2 %)	150 (17.4 %)	824 (95.6 %)	0.0001
	Filter	12 (1.4 %)	12 (1.4 %)	24 (2.8 %)	
	Mineral	8 (0.9 %)	6 (0.7 %)	14 (1.6 %)	
Animal contact	Yes	222 (25.8 %)	54 (6.3 %)	276 (32.0 %)	0.969
	No	472 (54.8 %)	114 (13.2 %)	586 (68.0 %)	
Mucus	Yes	604 (70.1 %)	152 (17.6 %)	756 (87.7 %)	0.223
	No	90 (10.4 %)	16 (1.9 %)	106 (12.3 %)	
Total		694 (80.5 %)	168 (19.5 %)	862 (100.0 %)	

Data are presented as frequency and %

*p-Value<0.05 is significant

seasons [20], while in Kuwait, it occurred during the cool season [3].

The difference in *Cryptosporidium* genotypes distribution was attributed to differences in the influences of infection sources. The bovine strain *Cryptosporidium* peak was attributed to the land use pattern with contamination of the water supply from young livestock (main reservoir) by hydrological phenomena in areas with rainfall/flood events and agricultural practices related to calving, while human strain predominance was related to water contamination from human activities with person-to-person transmission [8, 21].

This global *Cryptosporidium* species shift seems not to be applied in our study in Cairo, Egypt, with both peaks being attributed to the predominating human strain, with distinct endemicity

Similar to our results, Abd El Kader et al. [11] showed an outcome of 80 % for *C. hominis* and 40 % for *C. parvum*, with *C. parvum* predominating throughout the year and *C. hominis* mainly in August. Helmy et al. [22] found that *C. hominis* was 1.6 times more prevalent than *C. parvum* in children in Ismailia, Egypt. Contradictory to our results, Eida et al. [23] reported *C. parvum* predominance in Egypt. El-Shazly et al. [24] and Abd El Kader et al. [11] reported that the highest prevalence was in summer but the latter recorded, besides the major summer peak, another smaller one in spring in Cairo and attributed it to bovine strains.

C. hominis predominance in this report is similar to studies conducted in Uganda [19], Malawi, Kenya [25], South Africa [26], Australia, Canada, Japan, USA and developing countries [27].

Contradictory to our results, *C. parvum* was more often detected in humans than *C. hominis* in studies in Middle Eastern countries [3, 4, 28–31] and European countries, such as Portugal [32], UK [33] and Ireland [34]. Spain and Wales showed a relatively balanced relation of *C. hominis*/*C. parvum* [35].

Having anthroponotic *Cryptosporidium* strain predominance with no association between *Cryptosporidium* prevalence and animal contact in our study population reveals that population dynamics influence the transmission pattern of *Cryptosporidium* in Cairo. Water contamination may be the key determinant of distinct seasonality in Egypt and may be coupled with population dynamics that increase person-to-person transmission in hot months and spring due to outdoor activities, including recreational water use [36]. In addition, attendees of Cairo university hospitals come from urban and peri-urban areas of higher population densities and compromised infrastructures that favour person-to-person transmission and had low socio-economic class populations, depending mainly on chlorinated water, in which *Cryptosporidium* can survive. All cases were endemic, as none of the study population had history of travel. The lack of proper sanitation and infrastructure may be the origin of water contamination by faecal materials or they may become contaminated by storage in dirty containers [37].

Socio-demographic and behavioural differences between our study populations and those in other studies may explain the differences in the prevalence of the most frequent *Cryptosporidium* species. Also, these studies were products of reduced sample sizes and sample collection was not included in all months of the year.

Our study deduced statistically significant differences for *Cryptosporidium* between children and adults. Cryptosporidiosis affects all age groups, with the highest prevalence level among pre-school children aged 2–6 years (32.5 %), followed by older children aged 6–

12 years (20.8 %), and it decreases with age. Similarly, Abdel-Messih et al. [38], in Egypt, confirmed that 61.9 % of infected cases were related to this age group. The high prevalence of cryptosporidiosis in children has been reported in many countries, including Canada, USA, New Zealand, Ireland, England and France [14, 20, 34, 39, 40]. This high incidence of the disease in children may be related to the lack of pre-existing immunity, as older people may get exposed to *Cryptosporidium* infection in their lifetime. Moreover, children were more exposed to water during playing, increasing the chance of getting infected, and there was a more frequent attendance by physicians of diarrhoeic children than adults [11, 40].

In this study, *Cryptosporidium* was detected in males (n=455) more than females (n=407); however, the difference in sex distribution was statistically insignificant. This is in accordance with the results of many studies [11, 34, 39, 40], suggesting significant male infection predominance. In contrast, Yoder et al. [14] found that most of the reported cases in 2005 occurred among females. However, they stated that their data on race and ethnicity were incomplete.

Conclusion

There was a distinct endemicity of *Cryptosporidium* seasonality in diarrhoeic Egyptians, with a clear high true prevalence and predominance of anthroponotic (*C. hominis*) transmission throughout the year, and cases were due to sporadic rather than outbreak-associated infections. Differences in sex distribution for *Cryptosporidium* was not significant but was significant for age distribution, and pre-school children showed the highest level. Molecular tools are a must in *Cryptosporidium* prevalence studies to identify species and sub-genotypes.

The obtained results are important for the development of public health strategies, and improvement of disease prediction, prevention and control in the absence of treatment or reliable vaccine. The results also highlight *Cryptosporidium* as a water contaminant and an important cause of health problems in Egypt necessitating further studies of the risk factors.

Contribution of each author All manuscript authors contributed to every activity of it; idea of paper, study design, collection of materials, methodology, writing the paper and revising it.

Conflict of interest The authors declare that they have no competing interests

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