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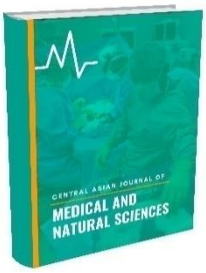

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Molecular Detection of *Cryptosporidium spp.* from Human in Iraq

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Abstract: This investigation was done for molecular detection of Cryptosporidium spp. From human in Iraq. Descriptive cross-sectional research was undertaken, whereby 300 faecal samples were collected from outpatients at a hospital over the period spanning from 2020 to the middle of 2022. Every new specimen was obtained in a sterile and clearly marked container with a screw-top lid. Subsequently, the samples were promptly frozen on a refrigerated pack that was appropriately labelled with the relevant patient information. Prior to sample collection, verbal agreement was obtained from each participant, as well as from the parents or guardians of babies and young children. Demographic data, including age, gender, place of residence, level of education, as well as other relevant information, were obtained by the administration of a specifically constructed questionnaire for the purpose of this research. Microscopic examination was conducted on stool specimens with the modified Ziehl Neelsen method. Macroscopic Examination: The naked eye was used to assess the consistency, odour, as well as the presence of blood, mucus, and pus in each fresh sample. PCR method was used to identify the presence of Cryptosporidium spp. by targeting the 18S ribosomal RNA gene in stool samples. The use of a modified Ziehl-Neelsen stain enabled the discernment of the morphological characteristics of the oocysts, so assisting in the practice of micrometry. The oocysts have an almost elliptical morphology, characterized by a mean length of 6.9±0.72 µm and a width of 5.8 ± 0.87 µm, as determined by measurement. The parasite exhibited conspicuous pink to red structures with high density on a background of blue or purple, accompanied by a distinct halo around the oocyst..

Keywords: Cryptosporidium, Iraq, molecular.

The prevalence of Cryptosporidium infection in the population was determined using the modified Ziehl Neelsen technique, yielding a prevalence rate of 87%. The incidence of infection exhibited a slightly higher rate among males (88.1%) compared to females (84.4%), however this disparity did not reach statistical significance (P >0.05) between the two sexes. In relation to age, the demographic exhibiting the highest incidence of infection was those between the ages of 21 and 30. The statistical analysis indicated that there were significant variations seen across different age groups (P < 0.05). In regards to the attributes of fecal matter, it was revealed that the presence of formed stool specimens was associated with the highest infection incidence (89.2%), while the lowest rate (84.2%) was detected among liquid stool specimens. Nevertheless, the statistical analysis revealed that there was no statistically significant difference (P>0.05) seen between the two types of specimens. In relation to water sources, persons who drink unfiltered municipal tap water had a greater prevalence rate of water-related issues (94.2%). Conversely, those who use commercial bottled water or filtered municipal tap water revealed a comparatively lower incidence (62.4%). These findings indicate statistically significant differences (P< 0.05) between the groups. The incidence of infection was found to be greater among suburban inhabitants (87.4%) compared to urban residents (75.4%), and a statistically significant difference was seen between the two groups (P< 0.05). Individuals who had contact with animals had a higher infection rate (90.4%) compared to those who did not have any contact with animals (85.2%). This disparity between the two groups was found to be statistically significant (P< 0.05). Individuals with lower levels of education exhibited a much greater risk of infection (93.8%) compared to other demographic groups. This disparity in infection rates based on education level was found to be statistically significant (P<0.05).

Introduction:

The protozoan parasites of the genus *Cryptosporidium* can only survive inside of host cells. These parasites have what is known as a monoxenous life cycle, meaning that they only need one host to reproduce (1).

According to many studies (2,3,4), cryptosporidiosis is the most common cause of watery diarrhoea and the sixth most common food-borne parasite worldwide. This ubiquitous coccidian parasite reproduces by making oocysts with thick walls; these are ingested with tainted food or water and cause an infection in the host's digestive system (5,6).

About 120 different genotypes of *Cryptosporidium* have been found, resulting in a total of 44 recognised species (7). Multiple animal orders, including fish, reptiles, birds, mammals, as well as humans, have acquired infections from *Cryptosporidium* species (8,9).

The majority of human infections ascribed to this genus have been traced back to the species *C. hominis* and *C. parvum*, according to previous epidemiological investigations (10,11,12). These studies operate under the assumption that *C. hominis* is exclusively transmitted among humans, while *C. parvum* primarily resides in domestic livestock, particularly cattle. The transmission of *C. parvum* to humans occurs either through direct contact with infected cattle or indirectly through the consumption of contaminated water (13).

Recent epidemiological studies conducted in several African and Asian nations have provided evidence indicating that Cryptosporidium spp. is the second most prevalent parasite among children. This particular parasite has been shown to be associated with the development of severe diarrhoea and a significant burden of morbidity. (14,15,16). (17) found that Cryptosporidium infection rates are disproportionately high in low-income Asian nations, particularly among children and young adults. Rotavirus infection was found to be widespread across Iraq. The rate was found to be 66.95 percent among youngsters in Dohuk (18). In the region of Wasit, the prevalence among diarrheal patients was found to be 40.4% (19). In Erbil, the rate was recorded at 14.6% (20), while in Baghdad, it was 47.3% among children (21). Lastly, in Al-Najaf, the prevalence among diarrheal patients was reported to be 58% (22).

This investigation was done for molecular detection of *Cryptosporidium spp.* From human in Iraq.

Materials and Methods:

Descriptive cross-sectional research was undertaken, whereby 300 faecal samples were collected from outpatients at a hospital over the period spanning from 2020 to the middle of 2022. Every new specimen was obtained in a sterile and clearly marked container with a screw-top lid. Subsequently, the samples were promptly frozen on a refrigerated pack that was appropriately labelled with the relevant patient information. Prior to sample collection, verbal agreement was obtained from each participant, as well as from the parents or guardians of babies and young children.

Demographic data, including age, gender, place of residence, level of education, as well as other relevant information, were obtained by the administration of a specifically constructed questionnaire for the purpose of this research.

Microscopic examination was conducted on stool specimens with the modified Ziehl Neelsen method (18).

Macroscopic Examination: The naked eye was used to assess the consistency, odour, as well as the presence of blood, mucus, and pus in each fresh sample.

Molecular detection:

DNA was extracted by using kit purchased from Genaid company, and method was done according to the manufacture instructions. Cryptosporidium spp. were detected in stool samples using a polymerase chain reaction analysis that focused on the 18S ribosomal RNA gene. The methodology used in this study was conducted in accordance with the approach outlined by (23). PCR run component: DNA 5µ, primer 1 µ of each, water 15 µ, master mix 5 µ. The thermocycler condition: this is done according to (23).

Statistical analysis was done by using SPSS version 23.

Results:

Oocyst morphological characteristics were more easily identified with the use of a modified Ziehl-Neelsen stain, which aided micrometry. The oocysts have a somewhat oval shape, with a measured length of 6.9±0.72 µm and a width of 5.8 ± 0.87 µm. The parasite seemed to have densely packed pink to red structures on a blue or purple background, and a halo could be seen out around the oocyst.

Table 1 summarises the population prevalence and linkage of Cryptosporidium oocysts and other risk variables. The study indicated that 87% of the population was infected with Cryptosporidium using a modified Ziehl Neelsen technique. "Although there was no statistically significant difference between the sexes in terms of infection frequency ($P > 0.05$)", men had a little higher infection rate (88.1%) than women (84.4%). Regarding age, the most significant infection rates were seen among those aged 21-30. The statistical analysis revealed significant differences across various age groups ($P < 0.05$). Regarding faecal characteristics, it was shown that formed stool specimens had the highest infection rate (89.2%), while liquid stool specimens had the lowest rate (84.2%). Nevertheless, no statistically significant difference ($P>0.05$) was found between the two types of specimens.

In relation to water sources, individuals who consume unfiltered municipal tap water exhibited a higher prevalence rate (94.2%), whereas those who consume commercial bottled water or filter municipal tap water demonstrated a lower rate (62.4%). These findings indicate statistically significant differences (P< 0.05) between the groups. The incidence of infection was found to be greater among suburban inhabitants (87.4%) compared to urban residents (75.4%), and a statistically significant difference was seen between the two groups (P< 0.05).

Individuals who had contact with animals had a higher infection rate (90.4%) compared to those who did not have any contact with animals (85.2%). This disparity between the two groups was found to be statistically significant (P< 0.05).

Individuals with lower levels of education exhibited a much greater risk of infection (93.8%) compared to other demographic groups. This disparity in infection rates based on education level was found to be "statistically significant (P<0.05)".

Table 1. The *Cryptosporidium spp.* Prevalence among the population in response to certain characteristics.

Variable		No. of infected individuals	Percentage
Gender	Male (210)	185	88.1
	Female (90)	76	84.4
Age	1-10 years (73)	59	80.8
	11-15 years (61)	52	85.2
	16-20 years (96)	87	90.6
	Above 21 years (70)	64	91.4
Stool consistency	Liquid (114)	96	84.2
	Formed (186)	166	89.2
Drinking water source	Tap water (207)	195	94.2
	Bottled water (93)	58	62.4
Residency	Urban (118)	89	75.4
	Suburban (182)	159	87.4
Presence of contact with animals	Yes (104)	94	90.4
	No (196)	167	85.2
Education levels	No education (65)	61	93.8
	Primary (97)	85	87.6
	Secondary (86)	73	84.9
	University (52)	43	81.1

Through the use of molecular techniques, the findings revealed that the overall infection rate of Cryptosporidiosis amounted to 276 cases, representing a prevalence of 92%, it is higher than those of traditional method which represent 87% (Table 2, Fig. 1).

Table 2. Molecular method VS traditional method

Method	Rate of infection	percentages
Traditional method	261	87
Molecular method	276	92

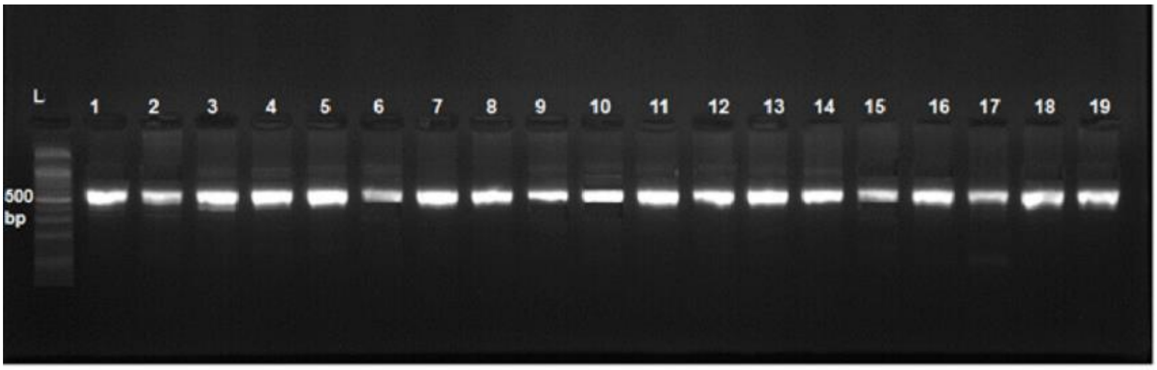

Figure 1. The provided picture depicts the examination of the PCR product of the 18S rRNA gene in *Cryptosporidium* spp. obtained from human faecal samples, using agarose gel electrophoresis. Positive *Cryptosporidium* spp. specimens were detected in Lane 1-10, as shown by the presence of a PCR product measuring 540 base pairs (bp) on the M: marker (1500-100bp).

Discussions:

Cryptosporidium species are distributed globally, with a particular prevalence in underdeveloped and emerging nations (24). The fecal-oral route is the most common means of transmission, and it often involves eating and drinking infected items. This may happen if someone comes into touch with animal excrement, either directly or by cross-contamination. *Cryptosporidium* is often spread when hosts ingest infected oocysts (24,25).

This study is the first to use a modified Ziehl Neelsen stain method to look for *Cryptosporidium* oocysts. Cryptosporidiosis was initially reported in Iraq by (26) and was found in 1996. Research on the prevalence of Cryptosporidiosis in urban and rural areas has since been conducted in a number of different locations around the country (27,28). Consistent with earlier studies conducted in other locations of Iraq, the latest study found a high infection incidence of 87%. Research conducted in the Mid-Euphrates Region, for instance, reported an infection rate of 92.2% (29), which is somewhat higher than the percentage shown here. Infection rates have been shown to vary throughout Iraq, according on studies conducted in different areas. For instance, in Al-Najaf City, (30) discovered a much reduced infection incidence of 58%. In Baghdad Province, (31) discovered a rate of 47.33 percent, whereas in Basra Province, (32) found a rate of 23.8 percent. The rate in Kirkuk City was estimated to be 22.68% by (33). Possible causes for the observed differences in infection rates include regional and cultural differences, differences in sample sizes, the fact that most studies have focused on infants and young children, and the use of different diagnostic techniques.

According to the data shown here, males have a slightly higher infection rate than females do, however the difference is not statistically significant ($P>0.05$). This confirms the results of studies in other parts of Iraq, such as Erbil and Basra cities and the Karbala province (34,35). Infection rates in these investigations ranged from 20.95% to 23.85%, 24.2% to 23.5%, and 27.41% to 23.68%. Males tend to be more outgoing and gregarious than females, which may help explain why men and women have different infection rates. It has also been hypothesised that girls' immune systems are superior than men', a difference that may be attributable to microRNAs found on the X chromosome (36). On the other hand, women tend to be more conscientious about personal cleanliness.

Researchers report that in “Al-Najaf City and Thi-Qar Province”, males outnumbered females 55.2% to 44.8% and 58% to 42.2%, respectively (37,38). (39) Research in Yemen also found a gender gap in the prevalence rates among youngsters. The research indicated that incidence was higher among boys than girls; 30.7% of male children were impacted vs 20% of female infants. The potential cause of this phenomenon might be the frequent interaction between young boys and the polluted outdoor play areas, where they engage in various outdoor activities and touch animals and dirt. Such interactions may increase the likelihood of parasite transmission.

Current research established an age-related correlation between cryptosporidiosis. There was a higher rate of infection in age groups older than 21. The findings presented here are consistent with the results obtained from similar investigations conducted in southwest Uganda, China, and Egypt (40). The studies as mentioned above also noted a greater occurrence of cryptosporidiosis in adults between the ages of 41 and 50, as well as those aged 31 to 40, with prevalence rates of 17.3%, 25%, and 37.7% respectively, in comparison to other age cohorts. The precise etiology of this observed rise remains uncertain; nonetheless, it is plausible that variations in immuno-physiological and ethological factors, namely a diminished immunological response beyond the age of 40, may contribute to this phenomenon.

The infection rate among children was lower than that among adults, but it was still rather high. This might be because kids spend more time in water during the summer, engaging in risky behaviours like swimming in rivers and drinking water, which exposes them to the parasite's infective stage. The greater infection incidence may also be attributable to children's general lack of understanding. In addition, a large percentage of those who caught the virus were adults over the age of 21. Concerns about the transmission of illness were heightened since persons of this age group had more frequent contact with domestic animals. Transmission from animals to humans occurs when infected animals release *Cryptosporidium* oocytes (28). Other studies have shown higher infection rates among children under the age of 6 in Iraq and Pakistan than what was seen in this one, at 25.45 and 41 percent, respectively (41,42).

We found no indication in this study that stool consistency was associated with the prevalence of *Cryptosporidium spp*. Formed stool samples, however, showed a somewhat greater incidence. Parasite invasion of the jejunum and ileum, which results in the presentation of liquid diarrhoea in immunocompromised persons, may explain this phenomena. It's also important to note that the parasite may infect healthy people with no outward manifestations of illness. Alternatively, the results may have been influenced by the fact that formed stool specimens were the norm in the samples studied for this investigation. The findings presented in this study are inconsistent with the results given by (43) in their research done in Erbil city. (43) found that the prevalence of infection was greater among those with diarrheal stool compared to those with formed stool, with rates of 36.36% and 6.66% respectively. Higher infection rates in liquid stools compared to formed stools have been observed in many research carried out in Asia, particularly in Pakistan and Cameron. Infection rates were lower in the former group (35%) than in the latter (15.6%), but higher in the latter (41%) and in the former group (13.40%) than in the latter (2.2%) (44).

This study's results show that the prevalence of *Cryptosporidium* infection is correlated with where people get their drinking water. People who drink unfiltered tap water from their local water supply are more likely to become sick than those who use commercially bottled water or home water filters. These results are consistent with those found in studies done in Beni-suef, Egypt (45), Sao Paulo City, Brazil (46), and the southern part of Cameroon (47).

According to this research, drinking tap water is linked to a greater infection rate than drinking bottled mineral water of the same volume. Specifically, the rates of infection were found to be 55% versus 12% in Sao Paulo City, 22.5% versus 18.8% in Beni-suef, and 7.14% versus 0.89% in the Southern region of Cameroon, respectively. This phenomenon may occur due to the ability of *Cryptosporidium* oocysts to endure chlorine treatment in water for extended periods, lasting many months. Additionally, these oocysts have been shown to exhibit resilience for up to 180 days in water and up to one year when stored at a temperature of 4 °C (48). I have a different perspective on the research done in Wasit

Province, Iraq by (34), whereby they observed a greater prevalence of water consumption-related issues among those who used bottled water compared to those who consumed tap water (54% against 52%).

In terms of residence, there was a notable correlation between suburban inhabitants and a greater incidence of cryptosporidiosis compared to their urban counterparts. The aforementioned observation aligns with the research conducted in Egypt, as well as 2 studies in Iraq (37,41). These studies reported a higher prevalence of infection among individuals residing in rural areas compared to their urban counterparts, with infection rates of 59.5% versus 40.5%, 63.3% versus 36.6%, and 64.7% versus 40%, respectively.

The potential cause of this phenomenon may be attributed to the perception that rural settings are conducive to the transmission of intestinal parasites. This is primarily due to the absence of adequate sanitation facilities, restricted availability of potable water, and frequent contact with animals (49).

Nevertheless, metropolitan areas are susceptible to the potential pollution of their water supply systems. Contrary to the findings of (50) in their research done in Erbil City, Iraq, which did not see a significant correlation between cryptosporidiosis and place of residence (rural 21.1% vs. urban 19.7%).

The findings of the present research revealed a statistically significant association between the incidence of *Cryptosporidium* infection and educational attainment. Specifically, the study noticed a higher prevalence of infection among those with lower levels of education, particularly those who were illiterate, compared to those who were literate. The findings of this study are consistent with the research done by (51) in southern Egypt. Their research showed that the prevalence of infection was much higher among people with lower levels of education, in contrast to those with higher levels of education, such as a college degree. The occurrence may be explained by differences in hygienic practises between educated and illiterate people, which contribute to the prevention and decrease of sickness transmission among the former group. While (52) found one set of outcomes in their research on Wasit City, Iraq, this study found the opposite. According to the findings (52), the prevalence of infection is higher among individuals with higher levels of education than among those with lower levels of education (80% vs. 50.8%).

According to the results, the microscopic test is not very sensitive. Expertise is required for the detection of oocysts in faeces samples. The diverse kinds of parasites cannot be distinguished by a simple microscopic inspection. Due to its great sensitivity, specificity, and speed in differentiating between species and genotypes in various samples, molecular tests like PCR have been widely used despite their high cost. Previous research has shown that the 18S rRNA gene may be successfully targeted by polymerase chain reaction (PCR) for efficient genotyping of *Cryptosporidium spp*. (53).

Conclusion:

Using a modified Ziehl Neelsen stain method, this study showed how useful it is for detecting and diagnosing cryptosporidiosis at the microscopic level.

The study also revealed a high incidence of cryptosporidiosis among the population, with an observed rate of 87%. Factors like as age, water supply, contact with animals, place of residence, and level of education all have a role in determining the prevalence of this ailment. Hence, the implementation of measures to enhance the quality of family living situations, promote environmental cleanliness, provide access to safe water supply for the people, prevent animal intrusion into human settings, and provide comprehensive health education would significantly contribute to the reduction of infection rates.

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