

Original Research Article

A serological survey on cryptosporidiosis in Najran region, Southwestern Saudi Arabia with reference to some epidemiological features of the infection

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ABSTRACT

Background: *Cryptosporidium* infection remains a major public health issue, but its epidemiology in humans is still unclear, particularly in Southwestern Saudi Arabia. Hence, this study was designed to determine the infection status of *Cryptosporidium* and the reservoir hosts in southwestern Saudi Arabia community and to recognize the associated risk factors, with evaluating the diagnostic performance of the techniques used.

Methods: A total of 576 stool specimens and sera were screened for cryptosporidiosis using modified Ziehl neelsen technique and a newly developed enzyme-linked immunosorbent assay (ELISA) respectively, between September 2015 and December 2016.

Results: Of the 576 samples assayed, 9.5 and 20.8% were positive for *Cryptosporidium* oocyst and anti-*Cryptosporidium* antibody, respectively. The seropositivity was positively correlated with age, especially in the age group less than 6 years old. Likewise, the prevalence of *Cryptosporidium* infections in males was significantly higher than in female counterparts ($P < 0.05$). The current study revealed that the maximum number of cases, 22.4%, was observed during the months of January to April, indicating marked significant seasonal variation. Sensitivity and specificity of newly developed Elisa technique when compared to acid-fast were 100% and 88.24%, respectively.

Conclusions: This is the first study highlighting that *Cryptosporidium* infections are still an important public health problem in Southwestern Saudi Arabia. Therefore, health education would be the best way to prevent the infections and the serological assays proved to be useful mean to provide more accurate information regarding community levels of *Cryptosporidium* infection.

Keywords: *Cryptosporidium*, Epidemiology, Elisa test, Seroprevalence

INTRODUCTION

Cryptosporidium is a coccidian protozoan parasite that is widely spread in the environment and recognized as responsible for diarrheal disease in both, immunocompromised and immunocompetent individuals. This disease is self-limiting within a short-term period in immunocompetent hosts but can become severe in immunocompromised individuals, such as those with

acquired immunodeficiency syndrome (AIDS), cancer and congenital immunoglobulin deficiencies, or receiving immunosuppressive drugs, causing a chronic and debilitating disease.¹

Furthermore, many cases of non-bacterial, non-viral infectious human diarrhea are commonly attributed to *Cryptosporidium*.² This *Cryptosporidium*-associated diarrhea is not unique. Thus, diagnosis should be relied

on laboratory diagnostic tests. Diagnosis of cryptosporidiosis is frequently based on microscopic examination and morphological identification of parasite oocysts in the stool samples. However, microscopic examination of a single stool specimen has a low sensitivity (46%) due to intermittent shedding of the oocysts. Hence, at least three faecal samples have to be taken and examined over different days to achieve 85% accuracy in diagnosing positive cryptosporidium cases.³ The main drawback of this method is that it is tedious, time-consuming and depends on the skill of an experienced microscopist. Furthermore, low oocyst density severely lowers diagnostic sensitivity.⁴ Likewise, certain stains such as acid fast and auramine/rhodamine have facilitated oocyst detection but on the expense of time and costs.

In recent years, a number of immunodiagnostic tests, which incorporate antibodies against *Cryptosporidium* spp., such as enzyme-linked immunosorbent assay (ELISA) have been developed. Using these methods, it is possible to assess the immune response during the infection and realize epidemiological studies, using the frequency data of circulating antibodies in the population as an indicator of exposure to the parasite.⁵⁻⁷

In Saudi Arabia, diarrheal disease is an important cause of morbidity chiefly in children but the contribution made to it by coccidian parasites is largely unknown. Furthermore, few reports have studied the prevalence of *Cryptosporidium* infection in various populations.⁸⁻¹⁰ All the estimated prevalence rates were based on microscopic methods.

However, to the best of our knowledge, no report is available about the prevalence of *Cryptosporidium* infection in Southwestern Saudi Arabia. Thus, the objectives of the present study are to determine the seroprevalence of cryptosporidiosis among participants in Southwestern Saudi Arabia and recognize the risk factors associated with *Cryptosporidium* infection within the population in order to understand the disease epidemiology, and consequently to convey a suitable integrated scheme for control. Additionally, the diagnostic performance of a newly developed enzyme-linked immunosorbent assay (ELISA) that detects *Cryptosporidium* antibodies in the serum was also estimated.

METHODS

The present research was carried out in the southwestern region of Saudi Arabia where almost 60% of the population lives in rural areas. The city of Najran; wherein the study was conducted, is located at latitude of 17° 30' 20" and longitude of 44° 11' 3" and at attitude of 1264 m above sea level. The climate for Najran is a hot desert climate type, typical of the Arabian Peninsula. The rainfall is very sporadic in occurrence and consists of light individual rainfalls.

Study population and sample collection

The present study was conducted over a period of one year from September 2015 to December 2016. Through this cross-sectional survey, a single blood sample (5ml of blood by venipuncture) was taken from each participant (n=576) under aseptic conditions with the approval of the relevant ethics committees. Furthermore, epidemiological information on the key demographics for each participant was collected via accompanying questionnaires. Attributes of humans (including marital status, education, occupation, history of diarrhea, site of residence), personal hygiene habits (including drinking unboiled water, having a house hold lavatory, washing hands before meals and washing before eating raw fruits and raw vegetables) were recorded using a standardized questionnaire that was completed by the senior investigator who asked all the patients for the information. For identification of risk factors, the age and gender of humans were also considered. Sera were separated after sedimentation of blood cells and were stored frozen at -20 °C and transported on dry ice to the Department of Applied Medical Sciences, Community College, Najran University, Najran, Saudi Arabia.

Serological methods

Enzyme-linked immunosorbent assay (ELISA)

The newly developed cryptosporidium (Cry) antibody, ELISA Kit (My-BioSource, Inc., San Diego, USA) is an enzyme immunoassay based on the detection of Anti-Cryptosporidium antibodies in the serum specimen. The test was carried out following the kit manufacturer's protocol at the laboratory of the Department of Applied Medical Sciences, Community College, Najran University, Najran, Saudi Arabia.

Experimental design

Comparison of conventional and immunological methods

The sensitivity and specificity of microscopic examination and the detection of Anti-Cryptosporidium antibodies in the serum samples from subjects were compared due to the absence of a standard method. For this purpose, fecal samples were also collected from the same inhabitants (n=576) whose blood was collected and residing in Najran region. Fecal smears were prepared from each sample by the formalin-ether concentration technique, stained by modified acid-fast procedure¹¹, and then examined for oocysts of *Cryptosporidium* species. Oocysts of 4-5 µm in diameter, red in color and containing sporozoites were considered positive for *Cryptosporidium*.

Data analysis

The significance of differences was analyzed using chi-square (χ^2) using the SPSS statistical package (version

20.0; IBM SPSS Institute, Inc., USA), and $p < 0.05$ was considered significant. Additionally, the degree of agreement between the results from microscopic stool examination (gold standard) and Elisa test was quantified using k statistics. Sensitivity (SE), specificity (SP), positive predictive values (PPV) and negative predictive values (NPV) of cryptosporidium (Cry) antibody test were calculated.¹²

RESULTS

In this study, a total of 576 participants provided both blood and stool specimens were included in the analysis. Of all 576 stool specimens screened by microscopy, *Cryptosporidium* oocysts were seen in 55 wet mount preparations stained with modified ZN method. The estimated prevalence rate was 9.5%.

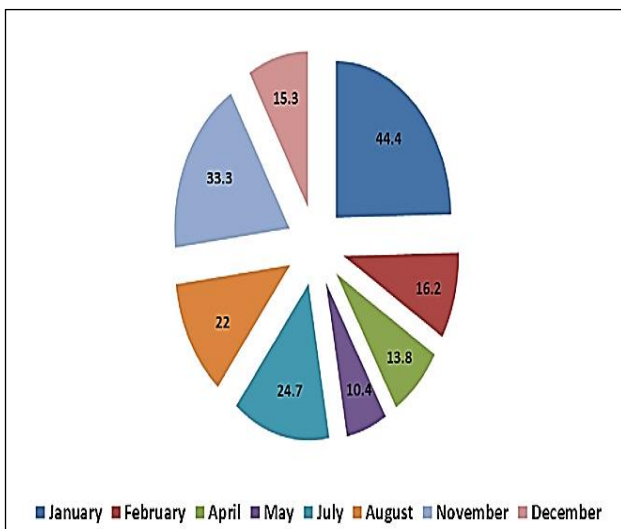


Figure 1: Monthly dynamics of Cryptosporidium seropositivity among subjects.

Furthermore, the present investigation revealed that Anti-Cryptosporidium antibodies were detected in the sera of 120 out of 576 participants corresponding to an overall Seroprevalence of 20.8%. There was a significant

difference between positive and negative results when comparing microscopy and Elisa results with chi square test ($p < 0.0001$). Likewise, the strength of agreement between the 2 methods was considered to be moderate. Therefore, not only did the Elisa test using antibody give 87.5 % specificity but it also exhibited 100 % sensitivity for identifying infected individuals (Table 3).

Considering the results with respect to age of subjects, the distribution of positive serum samples among different age groups for anti-Cryptosporidium antibody showed that participants in the age group less than 6 years had the highest percentage (30.9 %) of positive results followed by the age group of 20-30 years (23.4 %), while the age group of over 40 years showed the lowest percentage (12.7 %).

The correlation between age groups and percent of positivity is illustrated in Table 1, and this marked difference was found to be statistically significant ($\chi^2 = 13.59, p = 0.008$).

Similarly, the frequency of infection was higher among the male participants (75/288) 26.04 % compared to the female counterpart (45/288) ($\chi^2 = 9.47, p = 0.002$; Table 2).

With regards to education level, the current study showed that the prevalence of cryptosporidiosis was higher among participants of university educated subjects (26.1%) compared to the other education levels. However, the difference was not statistically significant ($\chi^2 = 5.89, p = 0.052$).

Similarly, there were no significant differences in epidemiologic parameters between Cryptosporidium-infected cases with history of diarrhea ($\chi^2 = 1.59, p = 0.206$, Table 2).

In the Saudi Arabia, as the result of monthly observations in Najran area, the Cryptosporidium seropositive rate was lower during summer (11.8%) than in the winter (36.8%), spring (24%) and autumn seasons (15.7%). This marked difference was found to be statistically significant ($\chi^2 = 28.0788, p < 0.0001$) (Figure1).

Table 1: Frequency of anti-cryptosporidium antibody among different age groups of subjects.

Age range (years)	Number examined	Positive	%	Negative	%	OR (95% confidence)	95% ci	P-value
<6years	110	34 ^a	30.9	76	69.1	3.0677	1.5366 - 6.1243	0.008
7-19 years	90	20 ^b	22.2	70	77.8	1.9592	0.9261 to 4.1445	
20-30 years	124	29 ^c	23.4	95	76.6	2.0932	1.0415 - 4.2072	
31-40 years	142	23 ^d	16.2	119	83.8	1.3253	0.6472 - 2.7140	
>40 years*	110	14 ^e	12.7	96	87.3	-	-	

a, b, c, d, e value with different superscript in the same column differ at $p < 0.05$, *Reference category, OR- odds ratio, CI- confidence interval.

Table 2: Univariable and multivariable analyses for risk factors associated with Cryptosporidium infection.

Variables	Cryptosporidiosis		P- value
	No. examined	Infected, n (%)	
Gender			
Male	288	75 (26.04)	0.002
Female	288	45 (15.6)	
Marital status			
Not married	192	32 (16.7)	0.017
Married	192	35 (18.2)	
Widowed	192	53 (27.6)	
History of diarrhea			
Yes	380	85 (22.4)	0.206
No	196	35 (17.9)	
Site of residence			
Aluraysah	144	44 (30.6)	0.004
Al Khaledia	144	20 (13.9)	
Alfyselia	144	30 (20.9)	
El-ballad	144	26 (18.1)	
Educational levels			
Primary	320	66 (20.6)	0.052
Secondary	103	14 (13.6)	
University	153	40 (26.1)	
Occupation			
Student	306	53 (17.3)	0.0270
Farmer	270	67 (24.8)	
Drinking unboiled water			
Yes	390	82 (21.01)	0.869
No	186	38 (20.43)	
Washing fruits and raw vegetables before eating them			
Often or always	283	45 (15.9)	0.004
Never or occasionally	293	75 (25.6)	
Having a household lavatory			
Yes	337	48 (14.2)	< 0.00001
No	239	72 (30.1)	
Washing hands before meals			
Often or always	362	70 (19.3)	0.250
Never or occasionally	214	50 (23.4)	

Table 3: Diagnostic performance of microscopy (modified ZN) and enzyme-linked immunosorbent assay (ELISA) of participants (n=576) with antibodies against cryptosporidiosis.

Variables	Microscopy			Sensitivity (95% CI)	Specificity (95% CI)	K value	PPV	NPV	SE of kappa
	+VE	-VE	Total stool samples tested						
ELISA IgG Serological test: (Positive)	55	65	120	100% (93.51% - 100%)	87.52% (84.38% - 90.24%)	0.573	45.83%	100%	0.045
(-VE)	0	456	456						
Total stool samples tested	55	521	576						

sensitivity= true positives detected/ (true positives+false negatives); specificity= true negatives detected/ (true negatives+ false positives), SE- Standard error.

Risk factors of being anti-Cryptosporidium antibodies seropositive

The results of the univariable and multivariable analysis for risk factors associated with Cryptosporidium infection

are summarized in Table 2. The significant risk factors for acquiring Cryptosporidium infection were site of residence, keeping livestock, owning a household lavatory, washing fruits and raw vegetables and occupation. No significant associations were observed

between *Cryptosporidium* infections and any hygienic habits including drinking unboiled water, washing hands before meals.

Diagnostic performance of the used methods

Taking the modified ZN-stained microscopy test results as the gold standard, the newly developed Elisa test showed SE, SP, PPV, and NPV of 100%, 87.52%, 45.83%, and 100%, respectively (Table 3).

DISCUSSION

Most reports concerning the epidemiology of cryptosporidiosis have been concerned with outbreaks of disease, particularly those caused by drinking from piped water supplies.¹³ However, recognized outbreaks represent only a small proportion of cases of disease, and the majority of cases reported are sporadic, in that they are not linked to other known cases.

Through this study, 9.5% of the diagnosed participants were positive for *Cryptosporidium* infection by using stool analysis. A large variation in the prevalence of *Cryptosporidium* infection was noticed by comparing our result to those already reported in many regions of Saudi Arabia. Indeed, in Saudi Arabia, previous stool surveys have indicated that approximately 1-11.5% of the various studied groups were infected with *Cryptosporidium* infection.¹⁴⁻¹⁶ This large discrepancy in the prevalence of *Cryptosporidium* could most likely be explained by the group of examined patient.

Nevertheless, by comparing the overall prevalence of *Cryptosporidium* infection found in the current study to the previous studies examining the stool of participants, the present finding is nearly consistent with other studies conducted in Riyadh region (8%), the capital of Saudi Arabia indicating that *Cryptosporidium* infection is an important public health problem in Saudi Arabia since the last century.¹⁷ On the contrary, the present figures were higher than previous investigation carried out in Dammam, Al Khobar, Jeddah, and Makah.⁸⁻¹⁰ Furthermore, worldwide high prevalence rates were reported from developing countries such as southern India where the overall period prevalence of intestinal parasites was 97.4%.¹⁸ Another study in Sierra Leone showed a prevalence rate of 73.5%.¹⁹ The higher rates in these communities may be attributed to improper hygiene and agricultural backgrounds.

No data was available regarding the Seroprevalence of cryptosporidiosis in Saudi Arabia. Therefore, the present investigation was the first to consider them.

Based on worldwide revisions, a number of studies have investigated on serum antibody responses to *Cryptosporidium* spp. in individuals residing in both developed and developing countries.^{20,21} Similarly, antibodies against *C. parvum* had previously been

identified in 20% and 16% of study populations in Peru and Venezuela, respectively²², and in 17-70% of the US population.^{20,21} In southern Europe, 62-83% of studied populations were detected to have the IgG antibody to *Cryptosporidium* antigens.²³

The present figures also revealed a significant positive correlation between the prevalence of infection among different age groups with peak values among children under 6 years age group. The high seropositivity of *Cryptosporidium* sp. in the young population (6-19 years) is similar to previous reports^{24,25}, but much lower compared with levels reaching 70% in rural China and 90% in Brazil.²⁶ The explanation for the higher seropositivity in younger subjects is not clear, but this may be influenced by physiological, immunological, and behavioural characteristics. However, all antibody responses decreased in those older than 40 years, which may have been due to weakened immunity of these subjects.

The gender has not been considered as an actual risk for *Cryptosporidium* infection in many previous reports^{10, 27}, although elsewhere, higher infection in females than in males has been reported.^{28,29} Interestingly, in this study, *Cryptosporidium* infection was significantly higher in males than females. This could be explained by the fact that males, especially in this community, have more freedom than females to go outdoors and practice activities such as dealing with farm animals, drinking unprotected underground well-water, and swimming in public pools.

For instance, only 12.3% of the participants would boil water before drinking it and nearly half of them had no access to a household lavatory. Not using sanitation facilities and water treatment and poor hygiene habits of some of the participants might increase the general environmental contamination and thereby increases the risk of *Cryptosporidium* infection for all other persons living in the same setting.³⁰ Additionally, the current data had established that the prevalence of *Cryptosporidium* in the subjects with history of diarrhea was higher than the other free participants. Similarly, previous work had demonstrated that the prevalence of *Cryptosporidium* in the young children with prolonged diarrhea was significantly higher than the other control and acute diarrheal groups.³¹ Therefore, *Cryptosporidium* should always be included in the differential diagnosis.

Considering the seasonal and monthly dynamics, several factors could account for seasonal variations in the occurrence of cryptosporidiosis, including: factors affecting the number of oocysts present in the environment, such as rainfall or agricultural practices; factors affecting oocyst survival, such as humidity or temperature; and factors promoting exposure to oocysts, such as contact with animals or attendance at child care centers. However, in most studies, the highest numbers of cases have been detected during the rainy season.^{32,33}

Because of its location in a desert geographic region; the climate of Najran is characterized by long, dry, hot summers (30-40 °C) and short, warm winters. There is a light individual rainfall in Najran as such. During the months January to April, the weather is pleasant; many Saudi people spend time in the desert living in tents, and use drinking water stored in overhead water tanks. Similarly, our results were in line with a former study in Kuwait which found the highest prevalence was observed during winter season (from January to April).³⁴

The sensitivity and specificity of newly developed Elisa test in comparison with direct wet mount microscopy (modified ZN) was found to be 100% and 87.5% respectively. This is comparable to another study which showed sensitivity and specificity of Elisa coproantigen test in comparison to microscopy as 98.8% and 100% respectively.³⁵ In another study sensitivity and specificity of Elisa test was found to be 76.4% and 100% respectively.³⁶ This reduced sensitivity could be due to less number of samples included in their study, as the sensitivity of Elisa test has been found to improve with increase in the number of specimens.³⁷

CONCLUSION

The current seropositivity rate of *Cryptosporidium* in this study is quite high (20.8%). Furthermore, *Cryptosporidium* spp. infection occurred most commonly in young people especially those less than six years of age. Likewise, the newly developed Elisa technique was found to be simple to perform and has been demonstrated to have a higher sensitivity than traditional staining procedures. Subsequently, this disease remains underdiagnosed in current routine laboratory procedures. It is recommended that assessments for *Cryptosporidium* be done as part of a general diarrhea screen during standard serological tests in diagnostic laboratories. This is particularly important since there is now an effective drug, nitazoxanide, available for therapy. Based on our findings, future studies of cryptosporidiosis should aim to further study genotypic differences.

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