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COMPARISON BETWEEN ELISA AND VARIOUS STAINS TECHNIQUES IN LABORATORY DIAGNOSIS OF CRYPTOSPORIDIOSIS

By

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Abstract

Cryptosporidium spp. is an important parasitic protozoa causing diarrhea which is a severe life-threatening diarrhea especially in immunocompromised hosts. We aimed to evaluate the usefulness of detection of *Cryptosporidium spp*. copro-antigen from fecal specimens by using enzyme linked immunosorbent assay (ELISA) test and comparing its sensitivity and specificity with some staining methods. The results revealed that Modified Acid-Fast stain is considered better than Giemsa in detecting *Cryptosporidium species* oocysts in faecal smears as their sensitivity were 67.5% and 53.75% respectively. On contrary, ELISA technique is considered the best method used for detection of cryptosporidial infection as its sensitivity is 90%.

Key words: Egypt, *Cryptosporidium parvum*, persistent diarrhea and ELISA technique

Introduction

Cryptosporidiosis is one of the infectious zoonotic protozoan diseases. Several species belonging to the genus *Cryptosporidium* are recorded in human and many vertebrates (Xiao *et al*, 2004). The first case of human cryptosporidiosis was reported in 1976 in a 3.5-year-old girl from rural Tennessee who developed self-limited gastroenteritis (Nime *et al,* 1976). Cryptosporidiosis is a worldwide infection with prevalence rates being higher in developing (5 to 10%) than in developed (1to 3%) countries (Current and Garcia, 1991). At the end of the twentieth century, *Cryptosporidium* species emerged as an important etiologic agent of diarrheal disease worldwide (Fayer *et al*, 2000).

Cryptosporidium species are protozoan parasites that cause infection and diarrheal illness in a wide range of mammalian species (Priest *et al*, 2006). The disease can affect both immunocompetent and immunocompromised individuals causing a wide spectrum of diseases ranging from asymptomatic carrier state to severe diarrhea. Infection with this parasite results in severe but self-limiting diarrhea in immunocompetent and often lethal diarrhea in the immunocompromised individuals (Chen *et al,* 2002).

Man and mammaks can be infected with *Cryptosporidium parvum.* Outbreaks have been described as a result of transmission in day care centers, swimming pools, public water supplies, and other water sources (Current and Garcia, 1991). Several methods for identification of cryptosporidial oocysts in fecal specimens include microscopy and ELISA, which detect cryptosporidial antigen in stool (Huang *et al*, 2004). Because of the difficult and time-consuming nature of conventional microscopic examination for the detection of *Cryptosporidium* oocysts, there is a need for a simple, rapid, and objective test for the copro-diagnosis of *Cryptosporidium* infection. This need is further underscored by a recent paper suggesting that all of the currently available microscopic methods have a low sensitivity for the detection of oocysts (Weber et al, 1991). The existence of a reliable and relatively inexpensive stool ELISA would obviate the current need for the intensive training and/or expensive equipment necessary to accurately diagnose *Cryptosporidium* infection.

The present study focused on detection of *C. parvum* oocysts in stool samples by the conventional microscopy (by wet mount

technique, by Acid-Fast and Giemsa stains) compared to the detection of cryptosporidial copro-antigen by ELISA technique.

Subjects, Material and Methods

A total of ninety patients suffered from persistent diarrhea (minimum of 3 loose stools per day with duration of more than 2 weeks) in and outpatient clinics of Minia University Hospitals, after oral acceptance to be enrolled in this study. This study was done from March 2012 to February 2013.

A clean, plastic container was given to each person. One stool sample per subject was collected. All information, including personal identification, the stage of disease, and clinical symptoms were recorded. Fecal specimens were transported as soon as possible to the Parasitology Department, to be examined by different techniques for detection of parasitic infections within 0.5 to 1 hr of collection. Samples were subjected to:-

Macroscopic examination identified color, odor, blood and mucus, round worms, pinworms, or tapeworm proglottids, as well as stool consistency.

Microscopy: a- Direct wet smear Saline wet mount to detect worm eggs or larvae, protozoan trophozoites, and cysts, and the presence of RBCs and WBCs, b- Iodine wet mount (Garcia *et al*, 1983) for glycogen and nuclei of protozoan cysts and c- Two stains; Modified Acid Fast (Casemore et *al*, 1985), and Giemsa stains (Garcia *et al*, 1983)

For ELISA: Rida-screen® *Cryptosporidium* kits (Cat No: C1201), (Clini-Lab, Cairo. Egypt). *Cryptosporidium* test, specific antibodies was used in a sandwich-type. Extinction was measured at 450nm using a reference wavelength 600nm. Cut-off extinction for negative control+0.15, sample was positive if extended more than 10% above calculated cut-off, sample was considered equivocal and was repeated if extended within±10% of the cut-off, and samples with extinctions more than 1 % below the calculated cut-off was considered negative.

Statistical analysis: Package of SPSS version 16 for windows was used for data entry and analysis. Descriptive statistics were calculated. For qualitative data, χ^2-test was used, and z-test was used for proportions, a significant *P*-value if less than 0.05.

Results

The results are shown in tables (1 to 12).

Table 1: Parasites detected by saline and iodine examination:

Parasites	No.	Percentage
Cryptosporidium parvum	49	43.75%
Giardia lamblia	21	18.75%
Blastocystis hominis	10	8.9%
Entamoeba histolytica/dispar	12	10.7%
Entamoeba coli	10	8.9%
Iodamoeba butchilii	4	3.6%
Hymenolepis nana	3	2.7%
Ascaris lumbricoides	2	1.8%
Isospora belli	1	0.8%

Frequency distribution of qualitative data by number and%

Table 2: Protozoan parasites detected by Modified Acid-Fast stain.

Parasites	No.	Percentage
Cryptosporidium spp.	55	64%
Cyclosora cayetanensis	15	16.4%
Giardia lamblia	9	10.5%
Entamoeba histolytica	2	2.3%
Entamoeba coli	2	2.3%
Isospora belli	2	2.3%
Chilomastix	1	1.2%
Total	86	100%

Table 3: Protozoan parasites detected by Giemsa stain.

Parasites	No.	Percentage
Cryptosporidium parvum	44	78.6%
Blastocystis hominis	6	10.7%
Giardia lamblia	4	7.1%
Isospora belli	2	3.6%
Total	56	100%

Table 4: Distribution of single and mixed protozoan parasites detected by wet mount

Protozoan parasites	No.	Percentage
Cryptosporidium only	22	24.4%
Cryptosporidium with other parasites	27	30%
Parasites other than *Cryptosporidium*	16	17.8%
No parasites	25	27.8%
Total	90	100%

Table 5: Distribution of single and mixed protozoan parasites detected by modified acid-fast.

Protozoan parasites	No	Percentage
Cryptosporidium parvum only	35	38.9%
Cryptosporidium with other parasites	20	22.2%
Parasites other than *Cryptosporidium*	14	15.6%
No parasite	21	23.3%
Total	90	100.0%

Table 6: Distribution of single and mixed protozoan parasites detected by Giemsa stain.

Protozoan parasites	No.	Percentage
Cryptosporidium parvum only	38	42.2%
Cryptosporidium with other parasites	6	6.7%
Parasites other than *Cryptosporidium*	12	13.3%
No parasite	34	37.8%
Total	90	100.0%

Table 7: Relationship between age and *Cryptosporidium parvum* by acid fast stain

Age group	Positive *Cryptosporidium* infection		P value
	No.	Percentage	
Less than 1y to 15y	29	52.7%	0.7
More than 15y	26	47.3%	
Total	55	100%	

Z test of proportion to compare between two proportions

Table 8: Relation between age and *Cryptosporidium parvum* by Giemsa stain

Age group	Positive *Cryptosporidium parvum*		P value
	No.	Percentage	
Less than 1y to 15y	22	50%	0.7
More than 15y	22	50%	
Total	44	100%	

Table 9: Relation between sex and *Cryptosporidium parvum* by modified acid-fast.

Sex	*C. parvum*	*C. parvum* & other parasites	Parasites with-out *C. parvum*	No parasites	Total	P value
Females	13 (37.1%)	11 (55%)	8(57.1%)	9 (42.9%)	41(45.6%)	0.4
Males	22 (62.9%)	9(45%)	6(42.9%)	12 (57.1%)	49(54.4%)	
Total	35 (100%)	20 (100%)	14(100%)	21(100%)	90(100%)	

Qui square test used to compare proportions

Table 10: Relationship between sex and *Cryptosporidium parvum* by Giemsa stain.

Sex	*C. parvum*	*C. parvum* & other parasites	Parasites without *C. parvum*	No parasites	Total	P value
Females	17(44.7%)	3 (50%)	7(58.3%)	14 (41.2%)	41(45.63%)	0.7
Males	21 (55.3%)	3 (50%)	5(41.7%)	20 (58.8%)	49 (54.4%)	
Total	38 (100%)	6 (100%)	12(100%)	34 (100%)	90(100%)	

Table 11: Detection of *C. parvum* copro-antigen by ELISA:

Cases	No	Percentage
Positive cases	80	88.9%
Negative cases	10	11.1%
Total	90	100%

Frequency distribution of qualitative data by number and%

Table 12: Comparison between Modified Acid-Fast and ELISA

Acid fast	ELISA		Total	P value
	Positive	Negative		
Positive	54 (67.5%)	1 (10%)	55 (61.1%)	0.0007
Negative	26 (32.5%)	9 (90%)	35 (38.9%)	
Total	80 (100%)	10 (100%)	90 (100%)	

Qui square test used to compare proportions, * significant test

Table 13: Comparison between Giemsa and ELISA

Geimsa	ELISA		Total	P value
	Positive	Negative		
Positive	43 (53.8%)	1 (10%)	44 (48.9%)	0.01
Negative	37 (46.3%)	9 (90%)	46 (51.1%)	
Total	80 (100%)	10 (100%)	90 (100%)	

Table 14: Sensitivity and specificity for different stains and ELISA

Sensitivity of ELISA	90%
Specificity of ELISA	67.5%
Positive predictive value of ELISA	98.18%

Acid fast stain	Value	95% confidence interval
Sensitivity	67.5%	0.561- 0.776
Specificity	90%	0.555- 0.997
Predictive value positive	98.18%	0.903- 0.999
Predictive value negative	25.71%	0.125- 0.432
Geimsa stain	Value	95% confidence interval
Sensitivity	53.75%	0.422- 0.649
Specificity	90%	0.555- 0.997
Predictive value positive	97.73%	0.879- 0.999
Predictive value negative	19.57%	0.093- 0.339

Sensitivity = true positive/ true positive+ false negative, Specificity = true negative/ true negative + false positive

Discussion

The present study confirmed that ELISA is superior to the light microscopic examination for the detection of *C. parvum* in stool samples. The sensitivity, specificity, and positive predictive value of the ELISA (in relation to the microscopic examination) were 90, 67.5% and 98.18% respectively. In the present study *C. parvum* was the most protozoan parasite detected in the stool samples examined by both techniques. By using the wet mount technique, *C. parvum* can be detected in 49 stool samples (54.4%) out of 90 stool samples. While, this number of positive samples for *C. parvum* increased to 55 samples (61%) out of 90 stool samples by using the Modified Acid-Fast stain. One the other hand, the number of *C. parvum* de-

creased to 44 (48.8%) out of 90 stool samples by using Giemsa stain.

These results indicated that Modified Acid-Fast stain is the most effective stain in the detection of *C. parvum* oocysts in fecal samples. These results agreed with those of Kamal *et al*. (2008) who stated that Modified Acid-Fast stain was more effective in the detection of *C. parvum* oocysts. Also, the use of ELISA technique results in increased the number of *C. parvum* positive in 80 (88.9%) out of 90 samples.

The present study showed that Modified Acid-Fast stain diagnosed 55 cases (61.1%) and Giemsa stain can diagnose only 44 cases (48.9%) with P= 0.1. This indicated that both stains detected *C. parvum* oocysts. Although the number of positive cases detected by Modified Acid-Fast stain was more than that detected by Giemsa stain, but without significant (P=0.1). The present results agreed with Garcia *et al.* (1093) who reported that both Giemsa and modified Acid-Fast stains were the most effective methods for *C. parvum* identification in examined stool samples. Kamal *et al*. (2008) reported that Modified Acid-Fast stain is more effective in the detection of *Cryptosporidium* spp. On the other hand, the results obtained by Fagbenro-Beyioku *et al*. (2006) were partially compatible with the present results as Modified Acid-Fast stain was more effective in diagnosing of *C. parvum* oocysts (35.7%) than Giemsa stain (3%). They concluded that wet mount technique was ineffective for diagnosis of cryptosporidiosis.

In the present study, *C. parvum* oocysts were detected either alone in 35 cases (38.9%) or mixed with other parasites in 20 cases (22.2%) by using Modified Acid-Fast stain, while Giemsa stain detected *Cryptosporidium* alone in 38 cases (42.2%) or mixed with other parasites in 6 cases (6.7%). This agreed with the detection of mixed infections of 21.7% (5/23) in positive patients (Abd El Kader *et al*, 2012). No doubt, cryptosporidiosis is a common pathogen present in cases with mixed infections. This agreed with Philips *et al*. (1992) in London, but in Jakarta and Ethiopia mixed infection occurred in 7% and 2.6% respectively (Kurniawan *et al*, 2009; Wegayehu *et al*, 2011).

The high percentage of mixed parasites detected by modified acid-fast stain (22.2%) (*C. parvum* with other parasites) may be explained by the fact that an established parasite paves the way for flourishing of other parasites (MacKenzie *et al*, 1994).

In this study showed no age predilection for cryptosporidial infection as 52.7% of cases were below 15 years and 47.3% were above 15 years old. The present results come in agree with the results revealed by a study done in western Venezuela which reported that there is no age predilection for cryptosporidiosis i (Chaci´n-Bonilla and Sa´nchezcha´vez, 2000). In the United States from 2006 to 2008 the majority of *Cryptosporidium* were from children aged 1-9 years and adults aged 25-39 years (Yoder *et al,* 2010).

In Iraq higher infection appeared in age below one year (Rahi *et al*, 2013). Another study on acute diarrheic patients in North-Eastern India reported that the most commonly infected age group was 16-45 years. and no significant difference in the occurrence of cryptosporidiosis was observed in children under the age of 5 years (Nath *et al*, 1999). In Kano state, Nigeria highest prevalence rate of *C. parvum* was in patients aged 46 to 55 years old (Kumurya and Gwarzo, 2013). In Plateau State, North-Central Nigeria cryptosporidiosis was the highest 11.0% (33/300) in ages 0-10 years and lowest 1.7% (5/300) in ages; 31- 40 years (Pam *et al*, 2013).

In the present study, no significant difference was recorded between males and females as 63.3% of the cryptosporidiosis cases were males while 58.5% were females. This might be due to environmental conditions as over crowdedness, water sources, improper sewage disposal and resistance of the oocyst to the routinely used disinfectant watersheds (Philip *et al,* 2008). These results are in agreement with another study done in

Cairo, Egypt which reported that 55.6% of infected cases of cryptosporidiosis cases were males and 44.4% were females (El-Helaly *et al,* 2012). Also, the results agreed with Abd El Kader *et al*. (2012) in Cairo, Egypt. The results are also more or less similar to a study in Iraq which showed that the prevalence of *Cryptosporidium* infection was 33.74% in the males and 33.93% in females (Rahi *et al*, 2013). A study of cryptospordiosis in Sulaimani pediatric teaching hospital recorded that there was no significant difference between males and females (Ali and Ali, 2013). Gatei *et al*. (2006) found that infection rates did not vary with gender distribution. Another recent study done in Nigeria reported that no significant difference was present between males and females (Pam *et al,* 2013). Mumtaz *et al.* (2010) observed high prevalence rate of cryptosporidiosis in males. Moreover, the study carried in Iran found that there was a statistically significant relationship between *Cryptosporidium* and sexes. They recorded that boys are more susceptible to cryptosporidiosis than girls (Khalili and Mardani, 2009). The present results revealed that ELISA technique increased the detected number of *C. parvum* to 80 (88.9%) out of 90 stool samples when compared to positive number detected by Modified Acid-Fast stain (55 or 61.1%) or by Giemsa stain (44 or 48.9%) with statistical significant (p= less than 0.05). The positive predictive value of Acid-Fast stain was 98.18% while that of Giemsa stain and ELISA were 97.73% & 98.18% respectively. The present results also revealed that modified acid-fast stain proved to be good negative test but not good positive one, but better than Geimsa in detecting the parasite with sensitivity of 67.5%. Geimsa stain proved to be good negative test but not good positive one with sensitivity of 53.75%. ELISA proved to good positive and good negative test with a sensitivity of 90%. These results agreed with those of Abdel-Messih *et al*. (2005) among children and those of Nautiyal *et al,* (2013) among adults.

The copro-antigen detected 97 (4.9%) of 2000 Turkish children by ELISA, and cryptosporidial oocysts were in 39 children (1.95%) by light microscopy (Yilmaz *et al*, 2008). In Brazil a study compared Modified Acid-Fast stain with ELISA reported that the sensitivity of ELISA was 100%, with a specificity of 96%; positive and negative predictive values were 89% and 100%, respectively (Marques *et al*, 2005). In India the sensitivity and specificity for ELISA for detection of cryptosporidial copro-antigin in stool samples were 90.9% and 98.7% respectively (Sarkar *et al*, 2013). In Iraq a study showed 100% positive by microscopic examination and 72.5% by ELISA (Rahi *et al*, 2013). Although ELISA test is expensive and has the possibility of false positive results (Vohra *et al*, 2012), it is simple, rapid, reliable, and specific test and may be useful for large-scale epidemiological studies of cryptosporidiosis, and also confirmed (Jayalakshmi *et al*, 2008).

In Egypt, cryptosporidiosis is one of the serious zoonotic parasites (Helmy *et al*, 2014). The first *C. parvum* case was 18 month old child (Azab *et al,* 1985), in outpatient clinic of Abu El Riche Children's Hospital with acute diarrhea. Since then, so many Egyptian authors too much to mention described zoonotic human cryptosporidiosis. Youssef *et al.* (2008) reviewed more than 61 Egyptian publications on cryptosporidiosis and stated that *C. parvum* diarrhea was but one of the many causes of diarrhea among Egyptians, but efforts to control this disease should also serve well to mitigate a number of infectious causes of diarrhea especially among children. Massoud *et al*. (2008) stated that zoonotic cryptosporidiosis is one of the public health and veterinary importance protozoa not only in Egypt, but also worldwide at least in the developing countries. They treated patients in Mansoura University Hospitals by a combination of Mirazid® and Paromomycin®. In Ismailia Governorate, Shoukry *et al*. (2009) reported infection rate of 88.2% among cryptosporidiosis children

compared to those 11.8% that not in contact with animals. Water samples examined showed 0.0% in bottled water up to 9.33% in water tank. *C. parvum* in farm animals was 20.9% in sheep, 22.5% in buffaloes, 23.7% in cows and 25.9% in goats.

Conclusion

Cryptosporidiosis causes diarrheal disease with 10 *Cryptosporidium* species infecting nearly all mammals worldwide. *C. parvum*, the most important species, was an endemic zoonotic coccidian highly prevalent in many of the developing countries, and responsible for up to 20% of the childhood diarrhea. The common mode of transmission included waterborne, animal contact, food-borne, or man to man contact with oocysts. ELISA is simple, rapid, reliable, sensitive and specific for routine diagnosis and may be useful for large-scale epidemiological studies of Cryptosporidiosis. In the present study, the sensitivity, specificity, and positive predictive value of the ELISA in relation to microscopy were 90, 67.5%., and 98.18% respectively.

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