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EVALUATION OF DIRECT FLUORESCENT ANTIBODY AND ENZYME LINKED IMMUNOSORBENT ASSAY VERSUS COPROMICROSCOPY IN DIAGNOSIS OF CRYPTOSPORIDIOSIS

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ABSTRACT

Background: *Cryptosporidium* oocysts detection methods include alternate bright-field stains and negative stains**.** These methods show high sensitivities but it may leave some oocysts unstained**.**

Objective: Evaluation of the direct fluorescence antibody (DFA) and coproantigens by ELISA versus modified Ziehl–Neelsen (MZN) stained smears in detection of *Cryptosporidium.*

Material and Methods: Eighty two immunocompromised patients having acute/ chronic diarrhea, were selected from the attendants of the pediatrics, oncology and nephrology clinics in Al-Azhar University Hospitals, during the period from August 2013 to May 2014. All cases were subjected to history taking and clinical examination**,** laboratory examination of their fecal smears by microscopic examination of MZN stained smears, detection of coproantigens by ELISA and DFA for diagnosis of *Cryptosporidium.*

Results: Fifteen (18.29%) of the individuals were positive for *Cryptosporidium* infection using modified Ziehl–Neelsen stain, and 17 (20.73%) were positive by direct fluorescent antibody, while ELISA detect crypto-coproantigen in 18 (21.95%). Statistically, there were highly significant relations between ELISA, DFA, and MZN. The sensitivity, specificity, and positive and negative predictive values of DFA test were 100, 97, 88.2 and 100 %, respectively, and for ELISA test were 100, 95.5, 83.3 and 100%, respectively, compared with MZN method as the gold standard test for detection of the *Cryptosporidium* .

Conclusion: Modified Ziehl-Neelsen staining remained the gold standard for the detection of *Cryptosporidium* spp., but it may leave some oocysts unstained. So, the immunofluorescence assays were the methods of choice for greatest sensitivity and specificity especially when oocyst numbers in stool specimens were low.

INTRODUCTION

Cryptosporidium infection is increasingly recognized as a major cause of diarrheal disease worldwide in all age groups. The range of people affected is broad including immuno-suppressed people and children, especially in developing countries. Symptoms of the disease are diverse, 90% of patients have diarrhea which is often associated with other gastrointestinal symptoms such as vomiting, nausea or abdominal pain **(Chalmers, 2010**).

The infective stage of *Cryptosporidium* (oocysts) is ubiquitous in the environment, being transmitted via the fecal-oral route either through the ingestion of contaminated water or food or direct contact with infected individuals or animals **(Karanis et al., 2007 and Smith et al., 2007).**

Diagnosis of cryptosporidiosis depends mainly on the acid-fast staining methods, with or without stool concentration which is the most frequently used in clinical laboratories. These methods include alternate bright-field stains, negative stains and fluorescent stains **(Garcia, 2001).** These methods show high sensitivities and may leave some oocysts unstained **(Zimmerman and Needham, 1995).** For greatest sensitivity and specificity, immunofluorescence assays are the methods of choice **(Garcia et al., 1992).** Although available antigen detection assays are superior to microscopic examination, these methods require multiple reagent additions, washing steps and incubations **(Chan et al., 2000).**

Light microscopy is recognized as the "gold standard" for definitive diagnosis of *Cryptosporidium* in a clinical setting, using various techniques for concentration of oocysts in fecal specimens. A number of staining have been developed, but many have problems of sensitivity and specificity often with variable results between laboratories **(Zajac et al., 2002).** Immunofluorescence methods have provided enhanced sensitivity and specificity over the conventional staining methods, especially when oocyst numbers in stool specimens were low. Prevalence studies should particularly gain benefit from immunofluorescence assays, since a symptomatically infected individuals may shed oocysts in small numbers **(Angus et al., 1981)**.

The big advantage of microscopy is that it is not specific and, therefore, other parasite can be detected which may be important in determining the cause of non specific symptoms such as diarrhea. It should be remembered that *Cryptosporidium* can be found in stool in the absence of clinical signs **(Kaushik et al., 2008).**

Sometimes, the standard diagnostic laboratory procedures may not be sufficient to confirm infection, or specimen collection may not be practical. In these circumstances, alternative methods may be helpful including antigen, antibody and nucleic acid detection **(Garcia, 2001).**

As a result, immunoassays for the detection of *Cryptosporidium* stool antigens have replaced microscopy as the routine diagnostic procedure of choice in many hospitals and public health laboratories **(Garcia et al., 1997).** The most widely used antigen detection immunoassays for *Cryptosporidium* are the direct fluorescent-antibody (DFA) tests which detect intact organisms (**Garcia et al., 1992**), and enzyme immunoassays which detect soluble stool antigens (**Garcia and Shimizu**, **2000**).

Much attention should be focused on the specific pathogens as causes of chronic or intermittent diarrhea in immunocompromized patients, since its correct treatment could improve the patient general well being **(Alemu et al., 2011).**

The aim of this work was to evaluate the direct fluorescent antibody and coproantigen (ELISA) versus modified Ziehl–Neelsen staining method in detection of *cryptosporidium*.

SUBJECTS AND METHODS

Eighty two immunocompromised patients (40 males and 42 females), ranging in age from 6 months to 60 years and having acute/chronic diarrhea, were selected from the attendants of the pediatrics, oncology and nephrology clinics in Al-Azhar University Hospitals, during the period from August 2013 to May 2014. All subjects had to fulfill one of the following criteria: Children with protein energy malnutrition, diabetes of more than one year, corticosteroids therapy for more than one year, malignancy or end stage renal failure. Informed consent was obtained from all patients or their parents when patients were under 18 years old.

All individuals were subjected to :

1. **History taking and clinical examination:** Name, sex, age, occu-pation, address, traveling, duration of symptoms, frequency of symptoms, complaint taking for presence of gastro - intestinal symptoms (nausea - vomiting - dyspepsia - constipation - diarrhea - dysentery - abdominal distention or enlargement) and gastro-intestinal signs as abdominal tenderness, hepatomegaly, ascites or signs of dehydration.

2. Laboratory examination:

Stool samples examination: All stool samples collected were examined microscopically by direct smear (with and without iodine staining) and by formol-ether concentration for the presence of Cryptosporidium oocysts and for detection of other parasites. For detection of Cryptosporidium oocysts, samples were examined by direct smearing and concentrating with formol-ether technique, Microscopic examination of modified Ziehl- Neelsen stained smears, RIDA screen Cryptosporidium coproantigene (ELISA) and direct immunoflurescence antibody (DFA).

Coproantigene (ELISA) (R-Biopharm AG, Darmstadt,Germany): Diagnosis of *cryptosporidium* was performed in all stool samples using the method described by the manufacturer**.**

Direct immunofluorescent antibody (DFA) (Sterling et al., 1986): In direct immunofluorescence, a FITC-labelled MAb reactive with genus-specific surface-exposed epitopes on *Cryptosporidium* oocysts binds to oocysts present in the sample.

Statistical analysis: Data were collected, revised, coded and entered to the Statistical Package for the Social Science (IBM SPSS) version 20. Qualitative data were presented as number and percentages, while quantitative data were presented as mean, standard deviations and ranges. The comparison between two groups with qualitative data were done using ***Chi-square test; Fisher exact test*** was used instead of Chi-square test when the expected count in any cell was found less than 5. Receiver operating characteristic curve (ROC) was used to assess the sensitivity, specificity, positive prediction value (PPV), negative prediction value (NPV), false positive rate (FPR), and false negative rate (FNR) of DFA and ELISA using MZN as the gold standard test.

Sensitivity of the test =

$$\frac{\text{Number of true positives}}{\text{No. of true positives + No. of false negatives}} \times 100$$

Specificity of the test =

$$\frac{\text{Number of true negatives}}{\text{No.of true negatives} + \text{No. of false positives}} \times 100$$

PPV of the test =

$$\frac{\text{Number of true positives}}{\text{No.of true positives} + \text{No. of false positives}} \times 100$$

NPV of the test =

$$\frac{\text{Number of true negative}}{\text{No.of true negatives} + \text{No. of false negatives}} \times 100$$

FPR of the test =

$$\frac{\text{Number of false positives}}{\text{No.of false positives} + \text{No. of true negatives}}$$

FNR of the test =

$$\frac{\text{Number of false negatives}}{\text{No.of true positives} + \text{No. of false negatives}}$$

The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant when P > 0.05. Kappa statistic was applied to investigate the consistency of results between DFA, ELISA test kit, and microscopic examination of MZN stain method. The strength of agreement was categorized based on the kappa values as follows: poorly correlated (<0), slightly correlated (0-0.20), fairly correlated (0.21-0.40), moderately correlated (0.41-0.60), substantially correlated (0.61-0.80), and perfectly correlated (0.81-1.0).

RESULTS

This study showed that 15 (18.29%) of the cases were positive for *Cryptosporidium* infection using modified Ziehl-Neelsen stain and 17 (20.73%) were positive by direct fluorescent antibody while ELISA detect crypto-coproantigen in 18 (21.95%). There was a statistically significant relation between both ELISA, DFA, and MZN as the gold standard test **(P** < 0.001) **(Table 1).**

The sensitivity, specificity, and positive and negative predictive values of DFA test were 100, 97, 88.2 and 100 %, respectively, and for ELISA test were 100, 95.5, 83.3 and 100%, respectively, compared with MZN method as the gold standard test for detection of the *Cryptosporidium* . The strength of agreement between DFA test and MZN method for detection of *Cryptosporidium* was categorized as perfect correlation with a kappa value of 0.92. Also, the strength of agreement between ELISA test and MZN method for detection of *Cryptosporidium* was categorized as perfect correlation with a kappa value of 0.88 **(Table 2)**.

There was no statistically significant difference between the three tests regarding sex **(Table 3)**.

Prevelance of cryptosporiodiosis was significantly higher among age group up to 5 years using MZN stain ($\chi 2$ = 9.034, p= 0.028), DFA ($\chi 2$ = 10.381, p= 0.015) and by coproantigen detection ($\chi 2$ = 16.113, p= 0.001) **(Table 4)**.

Cryptosporidium was present alone in 60% of positive cases, with one parasite in 20%, with two parasites in 13.33% and with more than two parasites in 6.67% **(Table 5)**.

One asymptomatic case occured (11.11%), while symptomatic cases were 8 cases (88.88%), and distributed as having abdominal pain in 7 cases (77.77%), jaundice in 2 cases (22.22%), abdominal distension in 5 cases (55.55%) and with diarrhea in 6 cases (66.66%) (**Table 6).**

Signs of symptomatic cases distributed as having tender abdomen in 7 cases (87.5%), hepatomegaly in 5 cases (62.5%), dehydration in 6 cases (75%) and with ascites in 3 cases (37.5%) **(Table 7)**.

Table (1): Diagnosis of *Cryptosporidium* cases by Modified Ziehl–Neelsen stain (MZN), Direct fluorescent antibody (DFA), Coproantigen (ELISA)..

Other tests \ MZN		Positive MZN (no. = 15)		Negative MZN (no. = 67)		Chi-square test	
		No.	%	No.	%	X^2	P-value
ELISA	Positive	15	100.0%	3	4.5%	65.274	< 0.001
	Negative	0	0.0%	64	95.5%		
DFA	Positive	15	100.0%	2	3.0%	70.193	< 0.001
	Negative	0	0.0%	65	97.0%		

Table (2): The test performance of DFA and coproantigen (ELISA) test kit for detection of *Cryptosporidium* cases in comparison with (MZN) method.

Parameters \ Tests	DFA	ELISA
Sensitivity	100%	100%
Specificity	97.0%	95.5%
PPV	88.2%	83.3%
NPV	100%	100%
FPR	3.0%	4.5%
FNR	0%	0%
Agreement between tests kappa	0.92	0.88

Table (3): Sex distribution among *Cryptosporidium* cases.

Sex \ Methods	MZN	DFA	ELISA	X^2	P-value
Male (40)	7 (17.5%)	6 (15%)	8 (20%)	0.492	0.781
Female (42)	8 (19.05%)	11 (26.19%)	10 (23.81%)		
Total (82)	15(18.29%)	17 (20.73%)	18 (21.95%)		

Table (4): Age distribution among the *Cryptosporidium* cases:

Methods / Age	N/Z	DFA	Coproantigen
Up to 5 (15)	4 (26.67%)	4 (26.67%)	5 (33.33%)
> 5- 20 (16)	4 (25%)	4 (25%)	5 (31.25%)
>20-40(25)	3 (12%)	3 (12%)	3 (12%)
> 40 (26)	4 (15.38%)	6 (23.08%)	5 (19.23%)
Total(82)	15 (18.29%)	17 (20.73%)	18 (21.95%)

Table (5): Co-existing of *Cryptosporidium* with other parasites.

	No. of patients	% (n=15)	Other parasite detected
Cryptospori-dium alone	9	60%	No one
With one parasite	3	20%	- with *E. histolytica* (1) - with *G. lamblia* (1) - with *Isospora* (1)
With two parasite	2	13.33%	- with *G. lamblia* + *B. hominis* (1) -with *Strongyloides spp. + E. histolytic (1)*
With more than two parasite.	1	6.67%	- with *H. nana* + *G. lamblia* + *B. hominis* (1)

Table (6): Clinical presentation among cryptosporidiosis patient (single infection).

Clinical presentation	Numbers	(%) n=9
Asymptomatic	1	11.11%
Symptomatic	8	88.88%
Abdominal pain	7	77.77%
Jaundice	2	22.22%
Abdominal distension	5	55.55%
Diarrhea	6	66.66%

Table (7): Signs of cases of cryptosporidiosis.

Signs of cases	Numbers	(%) n= 8
Tender abdomen	7	87.5%
Hepatomegaly	5	62.5%
Dehydration	6	75%
Ascites	3	37.5%

DISCUSSION

In the present study, by using MZN stain method, DFA assay and ELISA technique, a total of 15 (18.29%), 17 (20.73%) and 18 (21.95%) positive samples were detected respectively. DFA assay and ELISA technique showed statistically highly significant relation with MZN method as a gold standard test. These results agreed with that reported by **Parghi et al. (2014)** who found that 17.7% were positive for *Cryptosporidium* by stool ELISA, and **Abdel Messeh et al. (2005)** who detect *Cryptosporidium* infection in 17% of diarrheic children. Similar to the present study, there was a study by **Aghamolaie et al. (2014)** who showed statistically highly significant relations between the results of MZN method and DFA assay and detected *Cryptosporidium* infection in 1.2 % and 1.1% respectively. But the results was different in a study by **Yilmaz et al.(2008)** who recorded that only 1.95% of 2000 children were positive on microscopy of acid fast stained smears and 4.9% were positive by ELISA. Moreover, **EL-Shazly et al. (2002**) diagnosed *C. parvum* in stool samples by MZN stain as 5.3% and ELISA as 8.3% . On other hand, the current results were lower than that reported by **AL-Shamiri et al. (2010)** in Yemen who recorded that 34.7% were positive by microscopy and 26.1% were positive by ELISA. In general, surveys indicated prevalence rates of *Cryptosporidium* spp. infection ranged from less than 1% to more than 30% worldwide. Most of these variations may be attributed to geographic differences, demographic, temporal, and methodological factors **(Casemore et al., 1985).**

According to the results in this study, the sensitivity, specificity, and positive and negative predictive values of DFA assay compared with the microscopic method for detection of the *Cryptosporidium* spp. were 100% , 97%, 88%, and 100%, respectively, and for ELISA were 100%, 95.5%, 83.3%, and 100 %, respectively. There were other studies more or less similar to these results. A study by **Parghi et al.(2014)** reported that sensitivity and specificity of stool ELISA for detection of *Cryptosporidium* compared with modified AF staining was 100% and 92.7%, respectively. Other study from southern India by **Jayalakshmi et al. (2008)** had reported sensitivity and specificity of ELISA to be 90.9% and 98.7%, respectively. Also, there was a study by **Ungar (1990)** who reported that the sensitivity of the ELISA was 82.3%, and specificity was 96.7%. He stated that the ELISA may have an advantage over microscopy especially in large scale epidemiological studies, as all microscopic diagnosis rely on direct visualization and morphologic recognition of small-sized oocysts which may be scant in number, intermittently shed, or inconsistently stained. However, the value of microscopy in detecting other parasites in immunocompromised patients cannot be overlooked. Regarding DFA assay, the study by **Aghamolaie et al. (2014)** detected that the sensitivity, specificity, positive and negative predictive values for DFA assay were 87.5, 100, 100, and 96 %, respectively. The sensitivity and specificity of DFA test have been reported to be 96 to 100% and 99.8 to 100%, respectively for *Cryptosporidium* **(Garcia et al., 1992, Kehl et al., 1995,**

Zimmerman et al., 1995 and Garcia et al., 1997).This test had a sensitivity equal to or greater than that of traditional examination of perma-nent smears prepared from concentrated stool specimens for *Cryptosporidium* **(Kehl et al., 1995)**.

Regarding gender variation in the present study, cryptosporidiosis was found to be relatively higher in females than males, but the difference was statistically insignificant with all used tests. This result varied with **Park et al. (2006)** who recorded 1.9% in males and 1.2% in females but also with statistically insignificant difference. Also, **Al-Shamiri et al. (2010)** recorded that cryptospori-diosis was 36.2% in males and 32.7% in females. Higher prevalence in males could be attributed to higher sample size of males in the study, or due to the presence of males in outdoor areas as farms and contact with animals more than females which increase the risk of parasite transmission. However, other studies suggested that distribution of cryptospori-diosis cases by sex indicates that males and females appear to be equally susceptible to infection **(Fayer and Ungar, 1986).**

As regard the age of studied groups, cryptosporidiosis was recorded in the present study to be relatively higher in the age group up to 5 years old by MZN, DFA and coproantigen detection. These results agreed with a study by **Abou El-Magd** and **Abou-Shady (1986),** who stated that cryptosporidiosis was more common in the age of 2-12 years old. Also, **Al-Shamiri et al. (2010)** detected the highest rate of infection 40.3% , in preschool age group between 2-6 years. In Korea also, the peak of infection was in children aged 1-5 years **(Casemore, 1990).** Thus, at age of 2 to 6 years, children may be more exposed to the infection by *Cryptosporidium* spp. because they have lack of the knowledge about the good food and water. They eat without washing their hands, play in soil and sewage water, exposed to more fecal/oral contact or through contaminated food or water, or may be attributed to their weak immune responses **(Mirzaei, 2007).** A small secondary peak in laboratory-confirmed incidence has also been described in young adults aged 20-40 years which has been commonly attributed to familial contact with children or occupational exposure **(Casemore, 1988).** Clinical infection is less common after the age of 40 years, and there is apparently no evidence of elevated incidence rates in the elderly **(Casemore, 1988 and 1990).** However, incidence in adults may increase dramatically during waterborne outbreaks of cryptosporidiosis, and, therefore, may provide an early indication of the likely route of transmission of *Cryptosporidium* to the community **(Casemore, 1995).**

Certad et al. (2005) reported that 34% of *Cryptosporidium* infected patients had mixed infections with other parasites, mostly with *B. hominis* in 19% and *S. stercoralis* in 7%. Concurrent infection with *Cryptosporidium* spp. and a variety of other enteric microorganism, including *Giardia* and *Campylobacter* has also been reported **(Casemore, 1987)**. Descriptions of mixed enteric infections may reflect overlapping sequential infections with other enterpathogens or common sources of infection and mode of transmission with *Cryptosporidium* spp. co-infection with *Giardia* has been noted. These suggest the possibility of contaminated water or food as a common source of exposure, as well as person-to-person transmission **(Isaac-renton et al., 1987)**.

In the present study, diarrhea were recorded in 66.66 % of the cases. This result was lower than that reported by **Hassan et al. (1995)** who recorded that 91.7% of children suffering from diarrhea were positive for *Cryptosporidium* coproantigen by ELISA. On the other side, it was higher than that reported by **Al-Shamiri et al. (2010)** who recorded that only 38.45% of children infected by *Cryptosporidium* spp. had diarrhea. Also, **Mirzaei (2007)** recorded 25.6% of cryptosporidiosis cases had diarrhea. Moreover, Egyptian study revealed that only 13.9% of children were with diarrhea **(Rizk and Soleiman, 2001).** According to **Abdel Messeh et al. (2005),** vomiting and persistent diarrhea are important clinical findings associated with *Cryptosporidium* spp. and the need for hospitalization.

CONCLUSION

Modified Ziehl–Neelsen staining remained the gold standard for the detection of *Cryptosporidium spp.*, but it may leave some oocysts unstained. So, the immunofluorescence assays were the methods of choice for greatest sensitivity and specificity especially when oocyst numbers in stool specimens were low.

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أحمد عبد العزيز عز الدين البحيرى

قسم الطفيليات – كلية طب الأزهر

خلفية البحث : تشتمل طرق إكتشاف طفيل الكريبتوسبوريديوم على الصبغات المستضيئة والسلبية . ولقد أوضحت هذه الطرق حساسيات عالية ولكنها قد تترك بعض البويضات غير مصبوغة .

هدف الدراسة : تقييم طريقتى إكتشاف طفيل الكريبتوسبوريديوم بإستخدام الأجسام المضادة الفلوريسينية وطريقة تحديد الأنتيجينات بإستخدام إختبار الإليزا مقارنةً بالفحص الميكروسكوبى لعينات البراز المصبوغة بصبغة زيل نيلسن المعدّلة.

الأشخاص ومنهجية البحث : أُجريت هذه الدراسة على إثنين وثمانين مريضاً مصابون بنقص المناعة و يعانون من وجود الإسهال الحاد أو المزمن . تم إختيار الحالات من عيادات طب الأطفال والأورام وأمراض الكلى في مستشفيات جامعة الأزهر في الفترة من أغسطس 2013 إلى مايو 2014 . تم أخذ التاريخ المرضى والفحص السريرى لجميع الحالات كما تم فحص مختبرى لعينات براز منهم عن طريق الفحص الميكروسكوبى للعينات المصبوغة بصبغة زيل نلسن المعدّلة وتحديد الأنتيجينات فى البراز بإستخدام إختبار الإليزا و طريقة الأجسام المضادة الفلوريسينية المباشرة لتشخيص طفيل الكريبتوسبوريديوم .

النتائج : بينت النتائج أن 15 (18.29%) حالة كانت إيجابية لطفيل الكريبتوسبوريديم بإستخدام الفحص الميكروسكوبى بطريقة صبغة زيل نلسن المعدّلة و 17 (20.73%) حالة إيجابية بإستخدام طريقة الأجسام المضادة الفلوريسينية المباشرة بينما إختبار الإليزا بيَّن أن(21.95%) 18 حالة مصابون بطفيل الكريبتوسبوريديوم . وهناك علاقة ذات دلالة إحصائية عالية بين كلاً من طريقة الأجسام المضادة الفلوريسينية المباشرة وطريقة إختبار الإليزا مقارنةً بالفحص الميكروسكوبى للعينات المصبوغة بصبغة زيل نلسن المعدّلة. كما بيّنت النتائج أن الحساسية، والتخصصية، والقيم الإيجابية والسلبية التنبؤية لطريقة الأجسام المضادة الفلوريسينية كانت 100 ، 97 ، 88.2 و 100٪ على التوالي، وكانت لإختبارالإليزا 100، 95.5، 83.3 و 100٪ على التوالي، مقارنة مع طريقة الفحص الميكروسكوبى للعينات المصبوغة بصبغة زيل نلسن المعدلة كإختبار أمثل للكشف عن الكريبتوسبوريديوم.

الإستنتاج : أوضحت الدراسة أن طريقة الفحص الميكروسكوبى للعينات المصبوغة بصبغة زيل نلسن المعدّلة تبقى الأمثل لتشخيص طفيل الكريبتوسبوريديوم ولكن لأنها ربما تترك بويضات الطفيل غير مصبوغة فستكون طريقة الأجسام المضادة الفلوريسينية المباشرة هى الأعلى حساسية و تخصصيه خصوصاً عندما يكون عدد البويضات في عينات البراز قليل .