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COMPARISON BETWEEN AURAMINE PHENOL STAIN, MODEFIED ZIEHIL-NEELSEN AND ELISA FOR DETECTION OF INTESTINAL *COCCIDIA*

By

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Abstract

Coccidia had been implicated as the most important opportunistic parasites in patients with acquired immune deficiency syndrome. It is transmitted via the feco-oral route. The study aimed to compare the efficacy of microscopic tests with ELISA assay for detection of oocysts in feces. This study assisted the presence of *Coccidia* oocyst in fecal samples of 300 patients attending Zagazig University Hospital by using modified Ziehl-Neelsen stain (MZN) ,Auramine phenol stain (AP) and Enzyme-linked immunosorbent assay. *Cryptosporidium* infection rate was the highest (20.7%) among other Coccidia. Mixed infection of *Cryptosporidium* and *Cyclospora* either alone or with *Cytoisospora* and that of *Cryptosporidium* and *Cyclospora* with concomitant *Microsporidia* were recorded (9.7%, 1.7% and 5% respectively) by AP and (17.7, 6.7, 1 and 3.3 % respectively) by MZN. The specificity of modified Ziehl-Neelsen was 100% for both, while the sensitivity was 77.5%, while they were 100% and 100% respectively for AP, and 100% and 91.9% for ELISA. Conclusion, this study shows that Auramine phenol is a simple fluorescent staining, promising technique in diagnosis of intestinal *Coccidia* as it has high sensitivity and specificity and it is less time-consuming method.

Keywords: Patients, Stool staining techniques, *Apicomplexa, Cryptosporidium,*

Introduction

Coccidia are well known protozoans belonging to *phylum Apicomplexa* which are pathogenic mainly for animals (Norman *et al,* 2006). They include Cryptosporidia, *Isospora, Cyclospora, Sarcocystis and Microsporidia.* They are considered obligate intracellular spore-forming parasites of intestinal epithelium (Lawrence *et al,* 2005).The infective stage of these parasites are oocysts or spores that can be transmitted to man through consumption of contaminated food and contact with infected animals or man (Inabo *et al,* 2012). Laboratory diagnosis of intestinal parasitic infections depends mainly on microscopic examination of stool samples for identification of trophozoites or cysts (Moges *et al,* 2010). Despite this technique has some limitation as it is time consuming, some oocysts not stained and need experienced individuals, it is considered the corner stone in the diagnosis of intestinal *protozoa* (McHardy *et al,* 2014). Auramine phenol stain (AP) stains the cyst wall of coccidian, where stained *Cryptosporidium* oocysts appeared as fluorescent round bodies (Casemore *et al,* 1985).

Antigen detection using the immunoassays is used in diagnosis of *Coccidia* because it is thought to be highly sensitive than other staining methods and give best results in cases with few oocysts (Fletcher *et al,* 2012). Immunofluorescence assays (IFA) are more expensive and can be affected by the stool consistency (Johnston *et al,* 2003). The fecal Enzyme linked Immunosorbent assay (ELISA) proved to be easy, quick and convenient technique widely application in clinical settings (Marques *et al,* 2005). There is an increasing demand for low complexity, highly sensitive and low cost methods to replace microscopy based approaches for diagnosis of protozoa (McHardy *et al,* 2014).

This study aimed to evaluate Auramine phenol stain (AP), and Enzyme Linked Immunosorbent Assay technique (ELISA) for the diagnosing human intestinal coccidia and to compare these techniques with the other methods**.**

Patients and Methods

The current study was carried out on 300 patients aged from 0 months up to 50 years, of 62 male and 54 female. The stool samples were collected from patients complaining of GIT troubles with or without diarrhea admitted to different inpatient departments (Oncology, Pediatric, Tropical diseases, Internal Medicine and Surgery) and Outpatient Clinics of Zagazig University Hospital from March 2015 to September 2015.

All subjects were submitted to the following:

I. History taking: includes personal data (name, age, sex, residence and occupation), sanitary habits (washing hands before eating and after getting out the bath), source of food and water, history of contact with animals. Complaint: (long standing diarrhea, abdominal pain or abdominal distension) also presence of systemic troubles as liver disease, nephritis, leukemia and cancers. History of present illness: onset of diarrhea, duration, number of motions per day, similar attacks, history of receiving immunosuppressive therapy as corticosteroid, chemotherapy and/or radiotherapy. The questionnaire used was quoted from Mor *et al.* (2010). Patients taking anti-parasitic drugs were excluded.

II. Stool analysis: Samples collected were assessed in the laboratory of medical Parasitology department, Faculty of Medicine, Zagazig University.

Ethical considerations: The study had been approved by the Ethical Committee of Faculty of Medicine, Zagazig University. Aim and procedures of study were demonstrated to all patients and a written consent was taken from all of them. Parents provided informed consent on behalf of all child participants.

Samples: Fresh stool samples were collected during the spring and summer months from all patients. A rectal tube was used for taking stool sample from infants below 2 years.. Each stool sample was divided into two parts: one for Ag-capture ELISA which kept at -20˚C and the other for staining with (MZN) and Auramine phenol stains which fixed in 10% formalin.

Stool examination: A-Macroscopic examination: For determination of consistency, color, odor and presence of blood or mucus. B-Microscopic examination: For detection of parasitic infection by direct wet mount smear and iodine stained smear. Fixed smear were prepared for MZN and AP staining (Jafari *et al*, 2015). C- Detection of *Cryptosporidium* antigen with ELISA. This was performed using Ag-capture ELISA kit (RIDASCREEN® *Cryptosporidium* test, R-Biopharma, Germany). The stool samples were processed according to manufacturer's recommendations.

Evaluation: Cut-off value = extinction for the negative control +0.15 Samples are reported as a positive case if their extinction is higher than 10% above the calculated cut-off value. Samples were equivocal and repeated when the extinction was in the range of 10% above to 10% below the cut-off value. Samples were negative if their extinction is more than 10% below the calculated cut-off value.

Statistical methods: SPSS version 17 was used for statistical analysis. Numerical data were expressed as mean and SD or median range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test used for detection of the relation between qualitative variables, $P<0.05$ considered as Significance

Results

In the present study, AP was the most effective in detection of human intestinal *Coccidia* followed by ELISA (37%& 34% respectively)**.** *Cryptosporidium* infection rate was the highest (20.7%) among other *Coccidia.* Mixed infection of *Cryptosporidium* and *Cyclospora* either alone or with *Cytoisospora* and that of *Cryptosporidium* and *Cyclo spora* with the concomitant *Microsporidia* were recorded (9.7%, 1.7% & 5% respectively) by AP and (17.7%, 6.7% & 3.3% respectively) by MZN. ELISA was significant than MZN in *Cryptosporidium* diagnosis.

Auramine-Phenol staining was significant in *Cryptosporidium* diagnosis as compared with ELISA and in diagnosis of intestinal coccidia as compared with MZN.

For sensitivity, truly positive cases were positive by more than two methods. Also, 92 cases were truly negative by all tests. The AF was 100% sensitive with 91.28% specificity, 100 negative predictive values and 92.5 positive predictive values, while ELISA was 100% sensitive with 95.2% specificity, 100% negative predictive value and 91.07 positive predictive values. Details were represented in tables (1-6) and figures (1-6).

Table 1: MZN, AP and ELISA in detection of intestinal Coccidia.

Technique	Result	300	
		No	%
MZN	*+ve*	86	28.7
	-ve	214	71.3
AP:	*+ve*	111	37
	-ve	189	63
ELISA(*Crypto)*	*+ve*	102	34
	-ve	198	66

Table 2: Coccidial infection% among groups according to and

Type of *Coccidia*	Auramine phenol stain (AF)		Modified Zeil Neelsen (MZN)	
	No.	%	No.	%
Crypto only	62	20.7	53	17.7
Crypto + Cyclo	29	9.7	20	6.7
Crypto + Cyclo + Cytoiso	5	1.7	3	1
Crypto + Cyclo + Micro	15	5	10	3.3

Table 3: Sensitivity and specificity of ELISA for detection of *Cryptosporidium* in relation to AP stain.

ELISA	AP +ve	AP –ve	Total	Kappa	P
+ve	102	0	102	0.94	<0.001**
-ve	9	189	198		
Total	111	189	300		
Validity	Sensitivity: 91.9 %, Specificity: 100 %, PPV: 100 % , NPV: 95.5 %				
Accuracy	97 %				

P <0.001**: highly significant difference.

Table 4*:* Sensitivity and specificity of MZN for detection of *Coccidia* in relation to AP stain.

MZN	AP +ve	AP –ve	Total	Kappa	P
+ve	86	0	111	0.31	<0.001**
-ve	25	189	214		
Total	111	189	300		
Validity	Sensitivity: 77.5 % ,Specificity: 100 %, PPV: 100 %, NPV: 88.3 %				
Accuracy	91.7 %				

P <0.001**: highly significant difference.

Table 5: Effectiveness of different techniques in diagnosis of *Cryptosporidium*

Technique	Positive No.	Positive %	Sensitivity	Specificity	NPV	PPV
MZN	86	28.7	77.5%	100 %	97.1%	100 %
AP	111	37	100 %	100 %	100 %	100 %
ELISA	102	34	91.9 %	100 %	100 %	91.07 %

Discussion

In this study, intestinal *Coccidia* were detected with predominance of *Cryptosporidium.* This agreed with Sadraei *et al.* (2005), Tuli *et al*. (2008), Basak *et al.* (2010) and Das *et al.* (2013). *Cryptosporidium* infection alone was detected in 20.7% of the samples, whereas combined infection with *Cyclospora* oocysts along with *Cryptosporidium* were detected in (9.7%). *Cytoisospora belli* mixed with both *Cryptosporidium* and *Cyclospora* were detected in (1.7%), which were more or less similar results of Gupta (2013) who examined 310 stool samples and detected *Cryptosporidium* in (66%) and *Cyclospora* with Cryptosporidium in (31%). *Microsporidia* was concomitantly found with *Cryptosporidium* and *Cyclospora* (5%). Lee *et al*. (2007) explained this association by the possibility of the presence of a common source of infection. Mixed infection of intestinal coccidia with other pathogenic parasites was

prominent in this study. Wongstitwilairoong *et al*. (2007) stated that coccidian infections might increase susceptibility to parasitosis.

In the present study, by using MZN stain intestinal *Coccidia* was 28.7% patients with 77.5% sensitivity, 100% specificity, 100% PPV & 88.3% NPV. Khurana *et al.* (2012) reported that MZN gave 100% specificity & 79.06% sensitivity. Tuli *et al.* (2010) reported that MZN staining method gave 98.9-100% specificity with 37-90% sensitivity. Uppal *et al.* (2014) reported that MZN staining proved effective diagnosis for *Cryptospridium* oocysts in fecal samples, but sensitivity & specificity were not 100%. Omoruyi *et al*. (2014) reported that MZN staining technique was less sensitive for *Cryptosporidium* diagnosis.

In the present study, detection of *Cryptosporidium* copro-antigens in fecal samples by ELISA was done. The infection was detected in 34 % of the examined samples, and gave sensitivity 91.9%, specificity100 %, positive predictive value 100% and negative predictive value 95.5 % in comparison with AP as the gold test. There was high significant difference between the two tests. Similar results were obtained by Khurana *et al.* (2012) who reported 95.35% sensitivity and 100% specificity of *Cryptosporidiu*m antigen detection using ELISA. However, Uppal et al. (2014) found that *C. parvum* antigen detection by using ELISA had a sensitivity of 86.6% using different kits for antigen detection ELISA. In contrary to the present results, Abd El Kader *et al.* (2012) indicated that the immunodiagnostic methods such as ELISA are more sensitive than microscopic methods. While, Ali and Ali (2013) reported that after examination of 250 stool samples the higher rate of infection was 15.2% using MZN stain, while by using ELISA test it was 6.8%.

As regard AP stain in detection of intestinal Coccidia, the present study revealed infection in (37%) of the stool samples. This is in accordance with Abou El-Naga and Gaafar (2014) who found that the detection and the identification of Coccidia with the AP stain was easy, rapid, and required less interpretive time.

In the present study, AP stain detected 111 positive samples of 300, but ELISA detected 102 positive samples. This agreed with Khurana *et al.* (2012) who found that AP staining gave higher positivity than ELISA. But, Jafari *et al.* (2015) showed that AP detected three positive samples compared to ELISA that detected eight positive *Cryptosporidium* spp. oocysts. Brook *et al.* (2008) had evaluated MZN, AP and ELISA techniques in diagnosis of *Cryptosporidium* found that they were all effective in diagnosing infection in frozen and fresh fecal specimens of cattle.

Generally speaking, many apicomplexan parasites are zoonotic pathogens. This makes therapeutic target development very difficult, a drug that harms an apicomplexan parasite can also likely to harm its human host.

Conclusion

The outcome results showed that AP is a simple fluorescent staining, highly sensitive and specific. Consequently, the AP staining technique could be promising in diagnosis of intestinal Coccidia.

Recommendations

Physicians can rely on Auramine phenol stain in diagnosing intestinal Coccidia. Fluorescent microscopy should be available in the central labs, as a good, dependable and rapid diagnostic tool for intestinal Coccidia.

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Explanation of figures

Fig. 1: Stool smear stained with MZN, showing mixed infection with *Cryptosporidium* (1 arrow) and *Cyclospora* (2 arrows, ×1000).
Fig. 2: Stool smear stained with Auramine phenol, showing *Cryptosporidium* (1 arrow) and *Cyclospora* (2 arrows, ×1000).
Fig. 3: Stool smear stained with Auramine phenol stain, showing *Cryptosporidium* (1arrow) and *Cytoisospora* (2 arrows, ×1000).
Fig. 4: Stool smear stained with MZN, showing *Cryptosporidium* (1arrow) and *Cytoisospora* (2 arrows, ×1000)
Fig. 5: Stool smear stained with MZN showing *Cryptosporidium* oocysts (1 arrow) and *Microsporidia* (2 arrows, ×1000).
Fig. 6: Stool smear stained with Auramine phenol stain, showing Cryptosporidium oocysts (1 arrow) and Microsporidia (2 arrows, ×1000).