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Recognition of *Cryptosporidium oocysts* in fresh and old stool samples: comparison of four techniques

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ABSTRACT

Objective: To perform a comparative diagnosis of *Cryptosporidium* infection in fresh and old stool specimens.

Methods: A total of 518 stool samples including 464 diarrheic human samples, 53 samples from calves with or without diarrhea and one sample from a sheep were screened. Initially, the auramine phenol, modified Ziehl-Neelsen and ELISA techniques, were preformed. Subsequently, PCR of the 18S rRNA gene of *Cryptosporidium* was carried out on all positive samples obtained by microscopy and ELISA.

Results: Auramine phenol and Ziehl-Neelsen identified *Cryptosporidium oocysts* in 49 (9.5%) and 56 (10.8%) out of 518 samples, respectively. *Cryptosporidium* specific antigen was found in 97/518 (18.7%) of the samples by the ELISA test. The 18S rRNA gene of the parasite was amplified in 79 out of 119 (66.4%) samples by PCR. Comparison of the detection rates between the diagnostic methods, *i.e.* PCR and ELISA, showed that there was a moderate level of agreement between these tests (Kappa=0.55).

Conclusions: The current study suggested that PCR or ELISA methods with one of microscopy technique should be used for screening of *Cryptosporidium* infection in a stool sample collection.

1. Introduction

Cryptosporidiosis is a diarrheal disease caused by *Cryptosporidium* species in animals and humans[1]. In human, several groups of humans are susceptible to *Cryptosporidium* infection and it has high endemicity in immunocompromised patients particularly those with human immunodeficiency virus (HIV)[2]. The parasite is shedding millions of oocysts in the stool and hence, the diagnosis of cryptosporidiosis is generally made by assessment of stool samples. Mainly, stool specimens are examined microscopically by different staining methods such as auramine phenol (AP) and acid-fast Ziehl-Neelsen (ZN)[3]. In addition, several immunological techniques such as enzyme immunoassays have been developed for detection of cryptosporidiosis[4]. These methods are useful for screening large numbers of samples for the presence of *Cryptosporidium* copro-antigens[5]. Moreover, molecular methods, including the polymerase chain reaction (PCR) such as 18S rRNA gene

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used in most research and reference diagnostic centers, since they can help to identify *Cryptosporidium* parasites at the species level and the possible source of infection[6]. On the other hand, the specificity and sensitivity of above procedures show a discrepancy on the base of the protocols utilization. The available studies have produced incompatible outcome and it is reasonable to conclude that there is no available gold standard diagnostic technique. In fact, it is complicated to evaluate the results because of the difference in the methods and the applied reference standards[3,7]. Even though, extensive epidemiological studies have been carried out in many countries; surprisingly, there has been little research on the comparative laboratory and molecular study of cryptosporidiosis in humans and cattle[3,7-10]. The current study conducted to evaluate results obtained by staining methods, enzyme linked immunosorbent assay (ELISA) and molecular technique in diagnosis of *Cryptosporidium* infection in fresh and old stool samples.

2. Materials and methods

2.1. Sample collection

A total of 518 stool samples were obtained from Kenya, Malawi, Nigeria, Vietnam, Iran and UK. The samples were collected in tubes containing 75% ethanol, or 2.5% potassium dichromate or 10% formalin and some of them were collected in tube without preservative. All samples were transferred through cool chain and stored at 4 °C. This collection includes 464 diarrheic human samples and 52 samples from calves with or without diarrhea from Liverpool, Northwest England and one sample from a sheep and one from a calf both from Iran.

With the exception of 61 samples from children from Alder Hey Hospital of Liverpool which were collected during 1983 till 1990, the rest of the samples were collected between 1999 and 2004. These samples were stored at 4 °C with or without preservative (75% ethanol, 2.5% potassium dichromate or 10% formalin).

2.2. Cryptosporidium identification methods

2.2.1. AP staining method

Stool specimens were examined by AP staining as described previously with minor modifications[7]. Briefly, thin smears were set from the stool and allowed to air dry for at least 1 h. The old and dried specimens were processed by adding about 0.5 mL of normal saline overnight before testing. The slides were fixed in absolute methanol for 3 min on a staining rack followed by staining with auramine phenol for 10 min. The slides were washed with tap water and differentiated with 1% acid alcohol (1% HCl in 95% ethyl alcohol v/v) for 5 min until decolorized and then rinsed with tap water. The slides were counterstained with 1% w/v potassium permanganate (diluted with distilled water in the ratio of 1:10 before use) for 30 seconds, rinsed with tap water and followed by air drying. The slides were examined under the fluorescent microscope with ×40 objective and filtered at 490 nm.

2.2.2. ZN staining method

This method was carried out according to standard techniques as previously described[7]. Slides were then mounted with a cover slip using immersion oil and scanned at ×10 and ×40 and confirmed at ×100 magnification.

2.2.3. Antigen-EIA/ELISA technique

The ProSpecT® *Cryptosporidium* microplate assay (Alexon-Trend, now Remel, USA) for the detection of *Cryptosporidium* specific antigen (CSA) was used according to the manufacturer's instructions.

2.3. Molecular study

PCR analysis of 18S rRNA gene was performed for 119 positive samples identified by either microscopy or ELISA. DNA was extracted from whole feces using primers and PCR conditions based on a method which is previously described[11].

2.4. Statistical analysis and ethical considerations

The data in this study were analyzed using the StatsDirect and Minitab programs version 14.13. Full ethical agreement was obtained from the relevant National Research Ethical Committees.

3. Results

3.1. Microscopy

The number and percentage of *Cryptosporidium* infection based on their origin are shown in Table 1.

Table 1

The number and percentage of *Cryptosporidium* infection in fecal samples based on their origin using microscopy and ELISA techniques.

Locality	No. of tested samples	Positive samples by microscopy [*n* (%)]	Positive samples by ELISA [*n* (%)]
Iran	214	5 (2.34)	6 (2.5)
Iran (Calves)	1	1 (100.00)	1 (100.00)
Iran (Sheep)	1	0 (0.00)	1 (100.00)
Kenya	40	0 (0.00)	3 (7.50)
Malawi	24	0 (0.00)	2 (8.30)
Nigeria	58	1 (1.70)	1 (1.70)
UK	72	24 (33.30)	67 (93.00)
UK (Calves)*	52	27 (51.90)	16 (30.80)
Vietnam	56	0 (0.00)	0 (0.00)
Total	518	58 (11.20)	97 (18.73)

This data is presented in refrence[22].

Cryptosporidium oocysts were identified in 49 out of 518 (9.5%) samples by AP and 56 out of 518 (10.8%) by ZN. Forty-five samples were positive by both ZN and AP methods. Eleven samples were positive by ZN alone and 4 samples were positive by AP only. Among 61 old samples, 4 (6.6%) and 13 (21.3%) were positive by AP and ZN methods, respectively. This finding showed that storage of samples clearly affected the stability of the oocysts and they were no longer visible. The number of positive samples which were detected by the ZN staining method was greater than the number of positive samples detected by the AP technique. The difference between these methods was not statistically significant (P=0.460 0) (Table 2).

Table 2

Distribution of *Cryptosporidium* infection obtained by four techniques.

Methods	Positive [*n* (%)]	Negative [*n* (%)]	Total [*n* (%)]	*P* value
AP	49 (9.5)	469 (90.5)	518 (100)	0.460 0
ZN	56 (10.8)	462 (89.2)	518 (100)	
ZN	56 (10.8)	462 (89.2)	518 (100)	0.000 1
ELISA	97 (18.7)	421 (81.3)	518 (100)	
ZN	56 (47.1)	63 (52.9)	119 (100)	0.020 0
PCR	79 (66.4)	40 (33.7)	119 (100)	
ELISA	97 (81.5)	22 (17.5)	119 (100)	0.000 9
PCR	79 (66.4)	40 (33.7)	119 (100)	

3.2. ELISA

The ELISA method detected 97/518 (18.7%) positive samples. These include 56 positive samples obtained by ELISA only and 41 positive specimens gained by ELISA plus one or both of microscopy method. This test was negative for 19 positive samples obtained by microscopy (Table 2). Also, the ELISA detected CSA in 56/61 (91.8%) old samples which had been stored for about 20 years in cold room (4 °C). All fresh samples were positive for CSA antigen of cryptosporidium.The absorbance rate ranged between 0.154-2.445 which indicated the antigen and was identified by the ELISA test was extremely stable. Moreover, CSA was also detected in 18 (33.3%) samples out of 54 calves stool samples using ELISA test.

3.3. PCR

PCR of the 18S rRNA gene was amplified in 79 out of 119 (66.4%) positive samples obtained by microscopy or ELISA methods (Table 2). The positive rate of the parasite were 100% (11 samples) and 80.3% (49 samples) of fresh and old samples, respectively.

3.4. Comparison of detection methods for Cryptosporidium spp.

The relationship between microscopy and ELISA, microscopy and PCR and ELISA and PCR are shown in Table 3. McNemar's test showed higher number of positive samples was diagnosed by ELISA method in comparison with microscopy (P<0.000 1). Also, Liddell test shos that a positive result by ELISA is at least 1.7 times more than by microscopy. Furthermore, the value of Kappa shows that there is only fair agreement between microscopy and ELISA.

Comparison of results obtained by PCR of 18S rRNA gene for *Cryptosporidium* and microscopy showed that PCR could detect higher numbers of positive samples (P=0.020 0). Liddell test shows that a positive result obtained by PCR is at least 1.1 times as likely as by microscopy and the value of Kappa shows that there is only poor agreement between the two methods.

Table 3

The agreement between microscopy and ELISA, PCR and microscopy, and ELISA and PCR for detection of *Cryptosporidium* infection.

ELISA	Microscopy		Total	Kappa
	Positive	Negative	518	0.800
Positive	41	56		
Negative	19	402		
PCR	Microscopy		119	0.006
	Positive	Negative		
Positive	40	39		
Negative	20	20		
PCR	ELISA		119	0.550
	Positive	Negative		
Positive	75	4		
Negative	22	18		

The relationship between ELISA and PCR of 18S rRNA gene for *Cryptosporidium* showed that there was only moderate strength agreement between the two methods (Kappa=0.55). While McNemar's test shows that there is a significant higher number diagnosed by ELISA (P=0.000 9).

4. Discussion

Diagnosis of cryptosporidiosis is regularly established by the detection of oocysts in fecal specimens based on a variety of laboratory techniques[4]. In the current study, the percentage of positive samples which were detected by the ZN staining method (10.8%) was slightly more than the percentage of positive samples detected by the AP technique (9.5%). Although, the difference between these methods was not statistically significant (P=0.460 0), regarding to clear structural details provide by ZN staining method, the results obtained by the AP method should be confirmed with this technique. However, this finding is in a good agreement with other studies which indicated no qualitative differences in the results obtained with these staining methods[7,12]. The results obtained from the present study demonstrated that the higher percentage of *Cryptosporidium* infection was detected by the ELISA technique in comparison with the AP and ZN techniques. These results are in agreement with findings obtained from several studies which have indicated that immunodiagnostic methods such as ELISA are more sensitive than microscopy methods[3,5,8,13]. However, there are some studies reporting that ELISA method was less sensitive than microscopy, either ZN or AP[7,12]. On the other hand, the present study showed that application of preservative had no effect on the outcome of the ELISA test. This finding is in line with other repots which indicate that ELISA is a suitable technique even for preserved specimens. In addition, the ELISA is a simple method, easy to perform in a short time, applicable for large number of samples, very easy to read and interpret the results[5,14]. Additionally, the ELISA can detect antigen of different species of *Cryptosporidium* as shown in the current study and reviewed elsewhere[15]. However, the ELISA test was negative for 19 samples which were positive by microscopy methods. There are some possible explanations which explained as follow: i) false positive samples were detected by microscopy particularly for AP staining due to presence of yeasts and debris in stool; ii) false negative results obtained by the ELISA test due to condition of the samples. For instance, the test is less sensitive for formed specimens, antigenic variability within clinical isolates of *Cryptosporidium*, low or very high density of parasite, and low parasite densities due to the late stage of infection or asymptomatic persons, owing to difficulty in homogenizing of semi-solid or solid samples[13,14].

An interesting result obtained in this work was detection of CSA in 56/61 (91.8%) old samples which had been stored for about 20 years in cold room (4 °C) while oocysts of the parasite was detected only 6.5% and 21.3% by AP and ZN staining, respectively. This result was not in agreement with the kit's instruction which recommended that storage time of feces specimens is 2- 60 d at 2-8 °C.

Furthermore, finding of CSA in 18/54 (33.3%) calves stool samples using ELISA test confirmed that the ELISA kit (ProSpecT, Alexon/Remel) is useful for screening of *Cryptosporidium* in animal stool samples. This result was in agreement with other studies which used this kit to screen animal fecal samples[7,16]. In the current study, comparison of the laboratory diagnostic methods for *Cryptosporidium* using AP, ZN, ELISA techniques among 518 samples showed that the positivity rates are 9.5%, 10.8% and 18.7%, respectively (Table 2). There was a fair strength of agreement between these tests with a significant higher number of positive samples diagnosed by ELISA (P<0.000 1) (Table 3). These results are in agreement with findings obtained from other studies[5,17], but are not in line with others[18,19]. In this study, we are not able to determine a sensitivity and specificity for the ELISA test because of absence of a gold standard test. This study also compared the results obtained from microscopy, ELISA and PCR. Poor agreement between microscopy methods and PCR of 18S rRNA gene indicated that PCR could detect higher number of positive samples in comparison with microscopy. There are some studies showing that PCR is recognized more significantly *Cryptosporidium* infection than microscopy[7,13,18,20]. But our finding was in contrast with results obtained from another study which showed that there was a good agreement between microscopy and PCR in our finding demonstrated by Goñi P *et al*[21].

Seventy-nine out of 97 positive samples by ELISA test amplified a PCR of 18S rRNA gene indicating a moderate strength of agreement between these methods. This finding is in agreement with results of some study which indicated that PCR and ELISA are the most sensitive methods[12,13].

In conclusion, this study demonstrated moderate agreement between ELISA and PCR techniques for diagnosis of *Cryptosporidium* infection. It also found that using microscopy method alone is not sufficient to deem a sample be negative particularly for non-fresh stool samples. Therefore, using PCR or ELISA methods with one of microscopy techniques should be used for screening of *Cryptosporidium* infection in stool samples collection.

Conflict of interest statement

We declare that we have no conflict of interest.

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