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Comparison of ELISA and Microscopy for Detection of *Cryptosporidium* Oocysts in Animals

Wafa A.I. AL-Megrin
Department of Biology, Princess Nourah Bint Abdul Rahman University, Saudi Arabia

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Corresponding Author:
Wafa A.I. AL-Megrin
Department of Biology,
Princess Nourah Bint Abdul Rahman University, Saudi Arabia

ABSTRACT
Cryptosporidium parasite has gained an attention as an emerging pathogen in the last few decades. It is a zoonotic and is transmitted via the fecal-oral route; this has been implicated as one of the more important opportunistic infections in patients with Acquired immune deficiency syndrome. The results showed the presence of Cryptosporidium oocyst in fecal of sheep (11.1 and 22.2%), goats (15.5 and 10.3%) and camels (18.4 and 22.4%) by using microscopic Ziehl-Neelsen and a serological Enzyme-linked immunosorbent assay, respectively. Since there is no a gold standard technique for detection of Cryptosporidium oocysts; this study aimed to compare the efficacy of microscopic tests in the detection of oocysts in feces with ELISA assay. Results showed that positive samples (22.4 and 22.2%) were detected by ELISA in camels and sheep respectively. Contrary, Ziehl-Neelsen exceeded ELISA in the detection of oocysts in goats samples (15.5 and 10.3%) respectively. Statistical analysis clarified a significant correlation between Ziehl-Neelsen and ELISA for the detection of Cryptosporidium oocysts.

Key words: Detection, cryptosporidium, ELISA, Ziehl-Neelsen, animals, oocyst

INTRODUCTION

Cryptosporidium is a coccidian, intracellular protozoan parasite pathogen, which may lead to diarrheal illness and other severe diseases of animals and humans (Caccio and Pozio, 2006; Xiao and Feng, 2008).

A lot of techniques have been used to detect Cryptosporidium infection in humans and animals. These include examination of feces for the presence of oocysts and detection of Cryptosporidium antigens or DNA. Moreover, histology and ultra-structural examination of biopsy materials for life-cycle stages (Smith, 2008).

Detection of infection with Cryptosporidium usually requires the observation of the infective stage of oocysts, which are mostly 4-6 μm in size. Due to the tiny size of these oocysts, differential staining using the modified Ziehl-Neelsen (ZN) technique and wet mount preparation methods have a limited value for the detection of Cryptosporidium in fecal samples where oocysts can easily be confused with other substances present in the fecal (Connelly *et al*., 2008). The use of conventional methods such as flotation concentration and modified acid-fast stains may be inadequate to proof the presence of the parasites. Cryptosporidium are generally not observed during direct examination of specimens in the absence of special stains however, Enzyme-linked immunosorbent assay (ELISA) is used as standard assays in clinical laboratories (Weber *et al*., 1991).

Even though, the fewer cases being detected by acid-fast staining than by immunosorbent assay. The ELISA have been recorded to be up to 10 times more sensitive than acid-fast staining (Katanik *et al*., 2001). However, most laboratory diagnosis of Cryptosporidiosis is achieved by microscopic detection of Cryptosporidium oocysts in stool samples. The coproantigen assays are less time-consuming and easier to perform but are less sensitive than conventional microscopic methods. Thus, these tests might be a useful in addition to but not a substitute for microscopic methods in the diagnosis of cryptosporidiosis (Weitzel *et al*., 2006).

There has been no a direct evaluation of all assays used generally in Saudi for testing Cryptosporidiosis in animals. Moreover, that the specificity and sensitivity of different techniques has not been applied for testing animal species such as sheep, goats and camels specifically in veterinary research. The aim of this study was to compare microscopic tests (ZN) with commercially available coproantigen assays (ELISA) to detect Cryptosporidium infections in animals farm such as sheep, goats and camels since there is no a gold standard technique for detection of Cryptosporidium oocysts (Smith, 2008).

MATERIALS AND METHODS

Study area: This study was carried out in three farms including mixed sheep, goats and camels, located in Riyadh region, Central of Saudi Arabia.

Sample collection: A total of 179 fecal samples (72 sheep, 58 goats and 49 camels) were randomly collected from September, 2014 to March, 2015. In each farm, a single fecal sample was collected directly from rectum for each animal using sterile gloves and labeled in a sterile plastic container, transported to the laboratory and stored refrigeration at 4°C. The feces were classified according to their consistency as diarrheic and non diarrheic.

Experimental design

Comparison of conventional and immunological methods: The sensitivity and specificity of microscopic examination and the detection of *Cryptosporidium* spp. antigen in the fecal samples from different animals were compared due to the absence of a standard method for the detection of Cryptosporidium oocysts in fecal samples.

Sample preparation, staining and microscopic examination: One hundred and seventy nine fecal samples were processed and examined microscopically.

Four grams of each fecal sample were concentrated by formalin-ether sedimentation (Garcia *et al*., 1983). Then, using a wet mount preparation by the following techniques modified ZN staining technique (Henriksen and Pohlenz, 1981). Then the slides were examined microscopically for the presence of *Cryptosporidium* spp. oocysts by using a light optical microscope (40x and 100x) magnification was used.

Coproantigen diagnosis: Each fecal samples were subjected into coproantigen detection for Cryptosporidium using a commercial ELISA kit for stool samples (BIO-X Diagnostics, Belgium) according to manufacturer's instructions.

Statistical analysis: Pearson's chi square and Cohen's kappa tests were performed to measure the agreement between the ZN staining technique and the ELISA. Chi-squared test is significant when $p<0.05$ and Cohen's kappa test is significant if kappa values are close to 1.

Sensitivity, specificity, positive predictive values and negative predictive values were calculated using the total positive samples by ELISA techniques as a reference or standard.

RESULTS

All the 179 fecal samples were tested for Cryptosporidium oocysts by the microscope ZN and ELISA. The most positive samples (22.4 and 22.2%) were detected by ELISA in camels and sheep, respectively. Contrary; ZN exceeded ELISA in the detection of oocysts in goats samples (15.5%) (Table 1 and Fig. 1).

Table 1: Detection of Cryptosporidium in animal feces by the microscopic and immunological methods

Examination method	Statistical analysis	No. of sheep (%)	No. of goats (%)	No. of camels (%)
ZN	χ^2 =1.024, p>0.05	8/72 (11.1)	9/58 (15.5)	9/49 (18.4)
ELISA	χ^2 =3.78, p>0.05	16/72 (22.2)	6/58 (10.3)	11/49 (22.4)

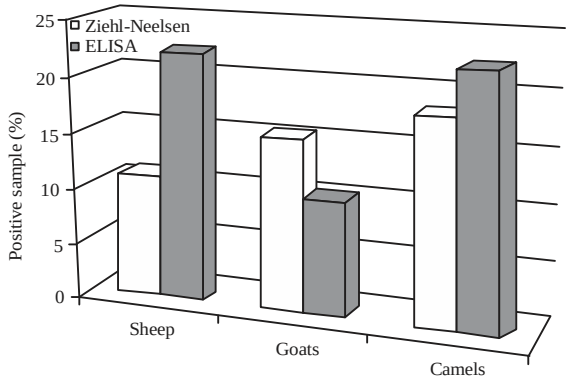

Fig. 1: Efficacy of detection of Cryptosporidium oocysts using ELISA and ZN

Table 2: Correlation between ZN and ELISA for the detection of Cryptosporidium oocysts

	ELISA		
ZN	Positive	Negative	Total
Positive	25	1	26
Negative	8	145	153
Total	33	146	179
Statistical analysis	χ^2 = 122.01 *p<0.001 **Kappa test = 0.833		

*Significant relationship between the test, **Values close to 1 show there is a significant relationship between the tests

Table 3: Efficacy of ZN in the detection of Cryptosporidium oocysts

ZN	Percentage
Sensitivity	75.8
Specificity	99.3
Positive predictive value	96.15
Negative predictive value	09.77

Statistical analysis clarified the significant correlation between ZN and ELISA for the detection of Cryptosporidium oocysts: Kappa test value 0.833 (Table 2).

Sensitivity and specificity of ZN technique in the detection of Cryptosporidium oocysts were determined. The technique proved 75.8% sensitivity and 99.3% specificity (Table 3).

DISCUSSION

With the increase in the interest in gastrointestinal diseases potentially caused by the waterborne outbreak, Cryptosporidium (coccidian protozoan) parasite has gained an attention as an emerging pathogen in the last few decades (Bouzid *et al*., 2013). It is zoonotic and is transmitted via the fecal-oral route. This has been implicated as one of the more important opportunistic infections in patients with AIDS (2-5%). Symptomatic intestinal and respiratory cryptosporidiosis has been seen in both immune competent and immune compromised patients of all ages (Current *et al*., 1983).

Now, various methods are available for the detection of Cryptosporidiosis in different clinical specimens but the method which can be used for routine screening purpose in stool samples from the cases of gastroenteritis should be acceptable in terms of sensitivity and specificity and provide clinically relevant, cost effective, rapid results, particularly in a potential waterborne diseases prone regions (Mittal *et al*., 2014).

The present study aimed to compare the efficacy of a microscopic (ZN) and a serological (ELISA) method in the diagnosis of Cryptosporidium oocysts. Feces samples have been collected from three animal cohorts, namely: sheep, goats and camels (n = 179).

The results show present of Cryptosporidium oocyst in fecal of Sheep (11.1 and 22.2%), goats (15.5 and 10.3%) and camels (18.4 and 22.4%) by using microscopic ZN and a serological ELISA, respectively, this results are in an agreement with Gharekhani *et al*. (2014) who found Cryptosporidium oocysts in 11.3% of sheep. In addition, it is compatible with who detected Cryptosporidium in camels (10 and 20.33%) by using modified Ziehl-Neelsen (Yakhchali and Moradi, 2012; Sazmand *et al*., 2012), respectively.

Contrary to ELISA; the microscopic method was proved to be less effective for the detection of Cryptosporidium oocysts. Statistical analysis showed that no significant difference between the animal for the high detection of Cryptosporidium oocysts (χ^2 = 1.024 and 3.78, p>0.05) by using ZN and ELISA, respectively. The analysis also revealed differences under fair significant correlation between the two methods (χ^2 = 122.01, p>0.001, Kappa test = 0.833). The ZN test showed a high specificity and sensitivity in the detection of oocysts (99.3 and 75.8%, respectively). The positive predictive value ZN was 96.15% while the negative predictive value was only 9.77%. This result is in an agreement with Khurana *et al*. (2012), who recorded sensitivity of ZN and ELISA were 79.06 and 95.35%, respectively. Also, it is acceptable with other studies which have been reported traditional staining techniques to be less specific and sensitive (Clark, 1999; Morgan *et al*., 1998; Quilez *et al*., 1996).

Also, these results coincide with the findings of Mittal *et al*. (2014) who found that stool microscopic detection was more sensitive method than ELISA for detection of Cryptosporidium in stool samples but the specificity of ELISA was more than microscopy. In a study conducted in South Africa; comparative diagnostic techniques for Cryptosporidium infection have been evaluated (Omoruyi *et al*., 2014). The ZN staining, sandwich antigen detection (sad)-ELISA and the direct Polymerase Chain Reaction (PCR) assay techniques were evaluated for diagnostic efficacy. Results of this study revealed that Cryptosporidium incidence following diagnostic techniques were 13 (37.1%; ZN staining), 26 (74.3%; sad-ELISA) and 23 (65.7%; PCR), respectively. These results coincide our finding where ZN staining showed the minimum diagnostic efficacy in both cases.

Another study used microscopic investigation of labeled antibodies, nested PCR and real-time PCR for the detection of *Cryptosporidium* sp. in stool samples (De Waele *et al*., 2011). Results of this study showed that quantitative real-time PCR was proved to be the most sensitive and specific test for detecting infection irrespective of the age of the calf. The microscopic techniques were the least sensitive and exhibited only moderate efficiency. This also matches our findings despite the difference in the investigated diagnostic techniques.

Results of this research also coincide with the findings of Uppal *et al*. (2014) who found that detection rates of Cryptosporidium were 29.4, 67.3 and 77.5% by modified ZN staining, antigen ELISA and nested PCR assay, respectively.

A North island study also investigated the efficacy of three techniques in the diagnosis of Cryptosporidium, namely: microscopic examination, nested-PCR and quantitative real-time PCR. Quantitative real-time PCR was proved to be the most sensitive and specific test for detecting the infection while the microscopic techniques were the least sensitive (De Waele *et al*., 2011). These findings match the results of the current study despite the difference in the used techniques. On the other hand, it disagreed with Hassan *et al*. (2002) who reported that ZN stain was able in diagnosed Cryptosporidium more than ELISA and other studies who found reduced capacity of ELISA to identify the presence of Cryptosporidium antigens in samples with low numbers of oocysts (Doing *et al*., 1999; Johnson *et al*., 2003; Mirhashemi *et al*., 2015).

Based on findings of the current study; ELISA tests is recommended for the diagnosis of Cryptosporidium. It's also recommended to investigate the efficacy of these techniques on humans taking into account their immune-competence situation. It is also recommended to investigate the efficacy of nested PCR, Real-Time PCR and flow cytometry in the diagnosis of Cryptosporidium.

CONCLUSION

ELISA has the potential for an accurate diagnosis of Cryptosporidium oocysts because of its high sensitivity, specificity and ease of use. All of that made ELISA a useful tool for future diagnosis and to be used in studies of the epidemiology of Cryptosporidium infections. In spite of these advantages, its wide use is still hindered by its relatively high cost and it remains till now confined to research purposes and epidemiological studies. However, there exists a valid explanation for this assay to be routinely used for Cryptosporidium diagnosis.

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