The application of Immunochromatographic test for diagnosis of *Cryptosporidium parvum* in faecal samples in calves.

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Abstract

Cryptosporidium infection is one of the important causes of diarrhea in calves and lambs. Investigations were carried out on 127 animals (58 cow calves,23 buffalo calves and 46 lambs) from different farms suffering from diarrhea at Cairo Governorates for detection of Cryptosporidial infection and evaluation of the sensitivity, specificity and accuracy of immunochromatographic test (IC) using RIDA Quick *Cryptosporidium* copro-antigen detection kit with other microscopical identification. *Cryptosporidium* spp. were detected in 5/58 cow calves (8.6%), 1/23 buffalo calves(4.3%) and 3/46 lambs (6.5%) using sheather's flotation and7/58(12%), 3/23 (13%) and 6/46 (13%) in cow calves, buffalo calves and lambs using MZN technique respectively. Immunchromatographic test using RIDA Quick *Cryptosporidium* kit revealed that 18/58 cow calves (31%), 5/23 buffalo calves (21.7 %) and 13/46 lambs (28.2%) were positive for *Cryptosporidium* spp. oocysts. The Sensitivity, specificity and accuracy were done comparing between MZN and IC method. Although the MZN gave preferable result than Sheather's flotation diagnosis, yet the IC antigen detection method showed higher detection levels. Moreover, it also gave a good sensitivity, specificity and accuracy percentage. Thus the RIDA Quick antigen detection test proved to be a useful field test for diagnosis of *Cryptosporidium* infection in fresh faecal samples.

Key words: *Cryptosporidium, Immunochromatographic, MZN technique, Calves* .

Introduction

The enteric apicomplexan parasite of genus *Cryptosporidium* causes diarrhea in multiple hosts such as calves, lambs, foals, piglets and humans **(Xiao *et al*.,2004)** It is a primary enteropathogen of cattle and has been incriminated as an important cause of neonatal diarrhea in calves, causing substantial economic loss to the animal husbandry **(Xiao *et al*., 1999)**. The *Cryptosporidium spp.* oocysts , which is eliminated with faeces and is capable of survival during long periods in the environment **(Hunter and Thompson, 2005).** This stage is relevant for the detection and identification of *cryptosporidium spp*. oocysts. **(Fayer *et al.,* 2000).** The most prominent clinical sign of Cryptosporidiosis is diarrhea lasting 2to 12 days and this is sometimes accompanied by anorexia, dehydration, reduced milk intake, growth retardation and stiffness **(Sevinc *et al.,* 2005)**. Transmission of *Cryptosporidium* spp. oocysts between host species including humans, is by faecal oral route of the environmentally resistant oocysts **(Abebe *et al.,* 2008).** The infection is diagnosed by identifying the oocyst stage of the parasite in host faeces or in histological section taken during necropsy **(Casemore *et al*., 1985).** Detection of parasites in faecal specimens is enhanced by the concentration procedures **(OIE, 2005).**

It is possible to identify *Cryptosporidium spp.* oocysts by means of flotation technique (Sheather's Solution) or staining techniques (Modified Ziehl Neelsen).The most widely used have been the modified acid-fast stain, which differentiate red-stained oocysts from

similarly sized and shaped green-stained yeast forms **(Fayer *et al.*,2000).** Although acid-fast staining is a reference method for the detection of *Cryptosporidium* spp. Oocysts. It is suggested that an alternative test is also needed because microscopic examination is time consuming, user- dependent which needs an experienced microscopist and difficult to detect oocysts in subclinical infection **(Unger 1990).** However,these techniques are labor intensive and unpractical **(Garcia *et al*., 2000).** Further, it must be performed on three stool samples to increase sensitivity leading to delay in the final diagnosis **(Weitzel *et al*., 2006).**

In an attempt to establish sensitive and cost-effective field methods to diagnose intestinal infections with *Cryptosporidium*, a number of copro-antigen tests have been developed based on the detection of parasite antigens. Chromatography lateral flow immunoassay is becoming the gold standard for gastroenteritis diagnosis because of its simplicity, rapidity, sensitivity and specificity **(El-Helaly *et al.,* 2012).**

The aim of the present study was to detect *Cryptosporidium spp.* infection in the faecal samples of cow calves, buffalo calves and lambs using two microscopical techniques and evaluates these methods with a commercially available rapid copro-antigen assay.

Material and methods

Animals and collected samples:

A total number of 127 animals (58 cow calves, 23 buffalo calves and 46 lambs) from different farms at Cairo governorates suffering from diarrhea were examined by microscopic techniques (sheather's flotation and MZN stain) and copro-antigen detection method by RIDA® Quick *Cryptosporidium* kit (R-Biopharm AG, Landwehrstr.54,D- 64293 Darmstadt, Germany). The samples were collected in sterile disposable plastic bags that were closed tightly and labeled by their age, place, date of collection and the clinical status of the animals and sent to the laboratory as quick as possible for investigations.

Microsopical examination :

All collected faecal samples were examined by centrifugation flotation technique using sheather's sugar solution with specific gravity of 1.27 to detect the oocysts of *Cryptosporidium spp.* **(Hendrix, 1998)** under a 40 x objective lens. The microscopic examination of Cryptosporidium spp. was performed with MZN **(Henriksen and Pohlens 1981)** for all collected faecal samples. As; thin faecal smears were dried at room temperature and fixed in methanol for 2 minutes and then flooded with basic fuchsine solution for 10 minutes. Then the slides decolourised in 1% sulphuric acid for 2 minutes. Following washing, the slides were pooled in malachite green stain for 5 minutes, washed , air dried, and examined under a 100 x objective lens.

Immunochromatographic examination (RIDA® Quick *Cryptosporidium* Kit): All the collected faecal samples were subjected to copro antigen detection for *Cryptosporidium* using a commercially qualitative immunochromatographic (IC) assay by RIDA Quick *Cryptosporidium* kit from R-Biopharm AG, Landwehrstr. 54,D-64293 Darmstadt, Germany. According to **Liorente *et al*.,(2002).**

The procedure consists of dissolving approximately 50 mg of faecal samples in a tube containing 0.5 ml of a diluting buffer provided with the kit . After mixing and waiting for 5 minutes, one test strip was dipped into the faecal suspension for 5-10 minutes at room temperature.

A negative strip yield one blue line, while a positive one shows two lines (blue and red lines).

Statistical analysis:

Sensitivity, specificity and accuracy of the commercial RIDA Quick *Cryptosporidium* kit as mentioned in the instruction manual: According to **Liorente *et al*., (2002).**

Sensitivity: It is the probability by which infected animals were identified by the test as positive. Calculated by the following equation:

$$\textbf{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}}$$

Specificity: It is the probability by which non infected animals were identified by the test as

negative. Calculated by the following equation:

$$\textbf{Specificity} = \frac{\text{True negative}}{\text{True negative + False positive}}$$

$$\textbf{Accuracy} = \frac{\text{True posiive + True negative}}{\text{True positive + True negative + False positive + False negative}}$$

Results

Microscopical examination:

The present study showed that the microscopical examination of the faecal samples collected from 58 cow calves, 23 buffalo calves and 46 lambs revealed that the prevalence of *Cryptosporidial* infection were 5/56, 1/23and 3/46 by sheather's flotation and 7/58, 3/23 and 6/46 using MZN stain respectively (Table 1). The oocysts of *Cryptosporidium* were found spherical to ovoid in shape with smooth wall and appeared as acid fast (red-pink) on green back. The average measurements of about 50 oocysts in cattle were varied from 4.1-3.8x4.3-4.2 um of mean (4.3x4.6) and in buffalo calves and sheep were varied from 4.2- 4.5 x 4.1 – 4.3 um of mean (4.4 x3.9). The measurements tend to be *Cryptosporidium parvum* as described by **(Potters,I.and Van Esbroeck, M. 2010** (Fig1).

Immunochromatographic examination (RIDA-Quick Cryptosporidium Kit):

Cryptosporidium spp. oocysts was detected in 18/58 (31 %) cow calves, 5/23 (21.7 %) buffalo calves and 13/46 (28.3%) lambs using RIDA Quick *Cryptosporidium* spp. copro-antigen detection IC test (Table 1& Fig 2).

The comparison between the results of microscopical techniques (MZN) and immunochromatographic (IC) method using RIDA Quick Kit for *Cryptosporidium* spp. infection revealed that 16 faecal samples were positive by IC method and MZN stain (True positive). Besides, Ninety-one faecal samples were negative by IC method and MZN stain (True negative).

Twenty samples were positive by IC method and negative by MZN stain (False positive). Moreover, no samples which gave negative results in IC test, gave positive results in MZN stain (False negative) Table 2. The sensitivity, specificity and accuracy were 100 %, 81.9 % and 84.3 % respectively (Table 3)

Table (1). Incidence *Cryptosporidium* spp. Infection in cow calves, buffalo calves and lambs using microscopical techniques and immunochromatographic (IC) test.

Animals	No.of examined samples	Microscopical techniques		IC test
		Sheather's flotation Positive (%)	MZN Positive (%)	RIDA Quick Kit Positive (%)
cow calves	58	5	7	18
Buffalo calves	23	1	3	5
Lambs	46	3	6	13
Total	127	9(7 %)	16 (12.6%)	36(28.3 %)

Table (2). Comparison between results of MZN stain and immunochromatographic (IC) test for diagnosis of *Cryptosporidium* spp. infection in different animals

Animals	MZN stain		IC test (RIDA Quick Kit)	
	Positive	Negative	Positive	Negative
Cow calves	7	51	18	40
Buffalo calves	3	20	5	18
Lambs	6	40	13	33
Total	16(12.6%)	111(87.4%)	36(28.3%)	91(71.7%)

Table (3). Sensitivity, specificity and accuracy of the commercial RIDA Quick *Cryptosporidium* Kit .

IC Test → / MZN Stain ↓	Positive	Negative	Total (%)
Positive	16	0	16
Negative	20	91	111
Total	36(28.3 %)	91(71.6 %)	127

True Positives (TP) =16 **True Negatives (TN) =91**

False Positives (FP) = 20 **False Negatives (FN)=0**

Sensitivity = 100 % **Specificity = 81.9 %** **Accuracy = 84.3 %**

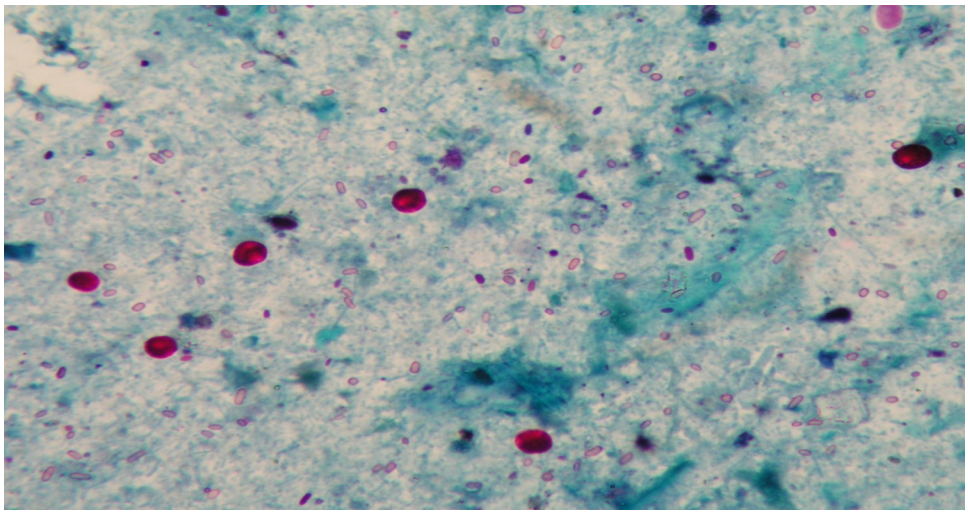

Figure 1. *Cryptosporidium spp*. Oocysts in calve faecal smears stained with Modified Zeihl Neel sen technique (MZN) X1000

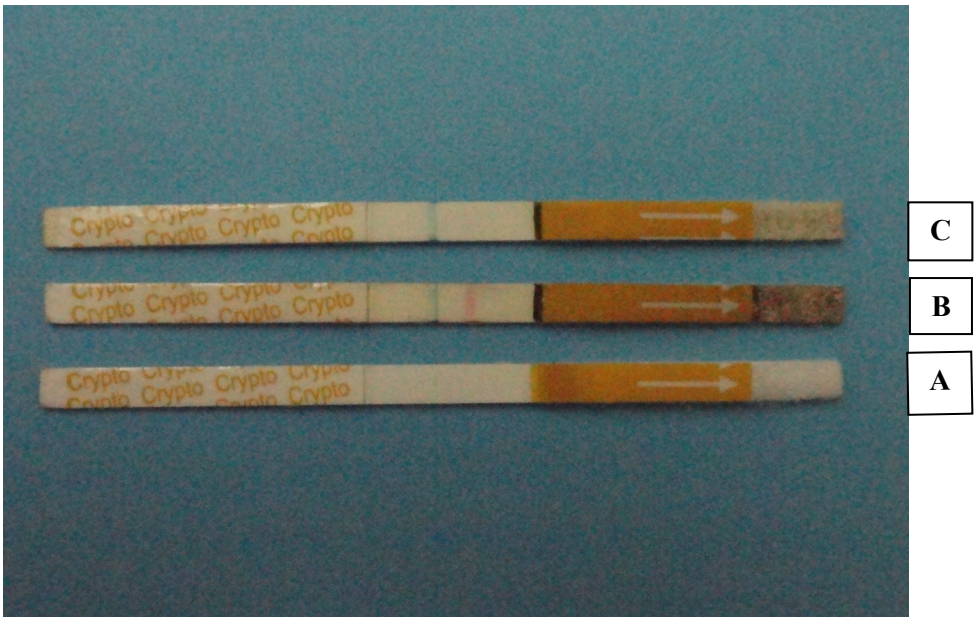

Figure 2. Immunochromatographic (IC) test for antigen detection using RIDA Quick *Cryptosporidium* Kit in faecal samples of cow calves.
A: Unused strip.
B: Positive result: shows two lines (blue and red lines).
C: Negative result: shows one line (blue line only).

Discussion

In the present study, it was found that the prevalence of C*ryptosporidium* infections in cow calves, buffalo calves and lambs using microscopical techniques (sheather's flotation) were 5 (8.6%), 1 (4.3 %) and 3 (6.5 %) respectively. Using MZN stain technique revealed that the prevalence of infection were 7 (12%), 3 (13%) and 6 (13 %) in cow calves, buffalo calves and lambs respectively. The rate of infections in buffalo calves was approximatley similar to that obtained by **Wahba (1994) and Aboulkhir (1996)** in Egypt using MZN technique, who showed that 9.9% and 10.2% of examined buffalo calves were positive for C*ryptosporidium* spp. Also**, El Khodery and Osman (2008) and El Kelesh *et al.,* (2009)** who found that 12.2 % and 10% of examined buffalo calves were positive for C*ryptosporidium* spp. infection. On the other hand, **Fayer *et al.*, 2000 and Geurden *et al.,* (2006)** who recorded a higher rate of infection with C*ryptosporidium* spp. were 21.5% and 16% respectively.

The rate of infection in cattle was similar to that obtained by **Atwill *et al.,* (1999)** who found 3.9% of examined cattle were positive for *Cryptosporidium* spp. by using MZN stain. Higher incidence (62.4%) was reported by **Scott *et al.,* 1995** in cattle. Comparable results were reported in other countries, **Olson *et al.,* (1997), Lise *et al.,* (2005) and Wang *et al.,* (2011)** the prevalence of infection for C*ryptosporidium* spp. were 27.8% in Canada, 40.5% in Canada and 24.8% in Spain respectively. Concerning lambs, 6(13 %) out of 46 of examined animals by MZN technique were found infected with C*ryptosporidium* spp. Higher incidence (40%) , (63.3%) was reported by **Abd – Elwahed (1999) and El Kelesh (2009)** in sheep respectively by using MZN technique . In contrary, lower incidence (4.4%) of C*ryptosporidium* spp. infection was recorded by **Wahba 1994.** Such variations between the rates of C*ryptosporidium* spp. infection in sheep might be due to age difference, resistance of examined animals and method of diagnosis.

The difference between the results of this study and those reported by other authors might be

regarded to some extend to the difference in the environmental conditions, nature of the soil (which affects the oocysts viability and the parasite distribution), hygienic measures and husbandry systems which probably affected the dissemination of the oocysts . Also, it might be attributed to the animal breed and its age.

Regarding to the immunochromatographic method for the coproantigen detection of *Cryptosporidium* spp. by RIDA Quick *Cryptosporidium* kit , the over all prevalence of Cryptosporidium spp. infection was 18 (31%) in cow calves, 5 (21.7%) in buffalo calves and 13 (28.3%) in sheep out of 127 collected samples. The sensitivity, specificity and accuracy were 100%, 81.9 % and 84.3 % respectively.

Liorente *et al.,* (2002) evaluate an immunochromatographic test (Crypto-strip) for the detection of *Cryptosporidium parvum* in stool samples. They found the sensitivity and specificity were 98% and 100% respectively. They added that the results obtained indicate that the Crypto-strip test can be a useful alternative system for the quick detection of *Cryptosporidium* in stools, giving results in less than 10 minutes , and easy to interpret, without the need for specialist person. **Iqbal *et al.,* (2001)** reported that shedding of *Cryptosporidium* oocysts may be intermittent, so that microscopical examination must be performed on 3 faecal samples to increase sensitivity which may lead to delays in the final diagnosis. **Luginbiihl *et al.,* (2005) and Regnath *et al* .,(2006)** observed that all faecal samples were positive by RIDA Quick assay, weak but visible bands were seen for samples with lower number of oocysts and reported that advantages of antigen detection assays include labor , time , and batching efficiencies that may lead to cost reduction . Also, **Lanz *et al.,* (2008)** used the fastest- strips for the diagnosis of *Cryptosporidium* in faecal samples of 147 calves with a percent 55%. **Diaz-Lee *et al.,*(2011)** stated that the combination of MZN and coproantigen detection with IC methods might give an excellent sensitivity level and confidence for *Cryptosporidium* detection in calves. **Agnamey *et al*.,(2011)** used Immunochromatographic assays for detection of *Cryptosporidium* antigens in stool samples (RIDA Quick Crypto. Kit) . They found the sensitivity and specificity were 87% and 100% respectively. **Goni, et al., (2012) e**valuate IC dip strip with microscopical methods for detection of Cryptosporidium spp. antigen in human faecal samples. They found the sensitivity and specificity were 72% and 93.6 %.Moreover, **Cicek *et al.,* (2008)** investigated the prevalence of C*ryptosporidium* spp. in slaughtered animals (sheep, cattle) and workers. They used acid – fast staining method to determine the oocysts of C*ryptosporidium* spp. in faecal samples of slaughtered animals and acid fast stain with RIDA Quick C*ryptosporidium* spp. kit for workers. They found the prevalence of C*ryptosporidium* spp. infection in faecal specimens were 13.17% in sheep and 8.13% in cattle. They detect the C*ryptosporidium* spp. in one worker (1.14%) by MZN and RIDA Quick strips.

Although the MZN gave preferable result than Sheather's flotation diagnosis, yet the IC antigen detection method showed higher detection levels. Moreover, it also gave a good sensitivity, specificity and accuracy percentage. Thus the RIDA Quick antigen detection test proved to be a useful mean for diagnosis of *Cryptosporidium spp.* infection in fresh faecal samples. We can conclude that the RIDA Quick antigen detection is a specific, sensitive and easy to be used as a field test for diagnosis of *Cryptosporidium* infection in fresh faecal samples.

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