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Overview on the diagnostic methods of Cryptosporidiosis in bovine calves

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ABSTRACT

Cryptosporidiosis is one of the most important causes of neonatal enteritis and diarrhea in calves leading to significant morbidity and mortality rates globally. This study was conducted to highlight the diagnostic methods of cryptosporidiosis in pre-weaned cattle calves. For this purpose, a total of 88 diarrheic faecal samples of pre-waned calves at Giza and El-Fayoum governorates, Egypt, were microscopically examined using Modified Ziehl-Neelsen staining (MZN), Immunochromatographic test (ICT) and Enzyme linked Immunosorbent Assay (ELISA). The prevalence of infection was 71.59% (63/88), 73.86% (65/88), and 75.00% (66/88) for MZN, ICT, and ELISA respectively. The Sensitivity was 96.9%, 100%, and 100% whereas; Specificity was 100%, 100%, and 95.7% for MZN, ICT, and ELISA, respectively.

INTRODUCTION

The zoonotic protozoan parasites of the genus Cryptosporidium are obligate, intracellular parasites that infect the epithelial cells lining the luminal surfaces of the digestive and respiratory tracts of a wide variety of vertebrates, including humans, livestock, wild animals, and birds (**Fayer et al. 2000).** *Cryptosporidium parvum* was first described in 1907 by Tyzzer in the small intestine of mice **(Chalmers and Katzer, 2013).** Cryptosporidiosis was first reported in cattle in early 1970 **(Panciera et al. 1971),** but the observed clinical disease could not be solely attributed to Cryptosporidium as there was evidence of confection with other viral bacterial pathogens. Neonatal diarrhoea in experimentally infected calves with *Cryptosporidium* species was reported as the single infective agent (**Castro-Hermida et al. 2002).** Four species of Cryptosporidium are commonly found in cattle: *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni*, but only *C. parvum* is associated with clinical disease in neonatal calves (**Thomson et al. 2017**). *C. parvum* predominates in pre-weaned calves, *C. bovis* and *C. ryanae* in post-weaned calves, and *C. andersoni* in older calves and adult cattle **(Robinson et al. 2006).** Cryptosporidium infection in dairy calves can lead to villous atrophy in the small intestine mucosa and

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increase intestinal permeability (Thomson et al., 2017). Consequently, these pathologies can lead to diarrhoea and an increased risk of mortality from dehydration **(Delafosse et al. 2015**).

Cryptosporidium parvum infections in calves present as profuse watery diarrhoea with acute onset, and can be accompanied by depression, weakness, and anorexia **(Santín, 2013).** Some infected cattle exhibit reduced weight gain compared with uninfected controls, and another study had found that infection may interfere with milk production in dairy cows **(Robinson et al. 2006).**

Cryptosporidium is a monoxenous parasite, undergoing all life cycle stages, including sexual and asexual reproduction, within a single host and producing thick-walled, environmentally hardy oocysts **(Brown, 2014).** Naturally infected calves can shed in excess of 3.89×10^{10} infective oocysts over a 6-day period which leads to widespread contamination of grazing lands, water sources, and the general environment **(Nydam et al. 2001).** *Cryptosporidium* oocysts are transmitted between hosts via the faecal-oral route, either directly via contact with faeces from infected hosts, or indirectly through environmental contamination or ingesting of contaminated food or water **(Niine et al. 2018; Thomson et al. 2017).** Autoinfection with Cryptosporidium was reported due to thin-walled oocyst production **(Leitch and He, 2011).** Importantly, cryptosporidiosis is not only a hazard for animal health and production but also its zoonotic characteristics represents a life-threatening disease **(Elmahallawy et al. 2020).**

Cryptosporidium can be diagnosed by a number of techniques including microscopic examination, either by the wet mount preparation or staining the smears with modified acid-fast stain, or by fluorescent stains **(Khurana and Chaudhary, 2018).** Immunological diagnosis using either antibody or antigen detection in faecal samples is available. Examples of these tests are Enzyme-Linked Immunosorbent Assays (ELISA), Immunochromatographic tests (ICT), and Immunofluorescence assays (IFA). Various molecular methods for the detection of DNA are also available **(Aboelsoued and Abdel Megeed, 2022).** The modified acid-fast staining is broadly applied for clinical diagnosis owing to its simplicity and cost-effectiveness (**Jafari et al. 2015; Mahmoudi et al. 2021).**

The incidence rate of Cryptosporidium in calves, all over the world ranged from 3.4 to 96.6% (**Thomson et al. 2017).** In Egypt, the highest prevalence rate of Cryptosporidium in calves was 56.32% **(Essa et al. 2014),** while, the lowest one was 9.2% **(Mahfouz et al. 2014).**

The objectives of this study were to investigate the rate of Cryptosporidium infection in pre-weaned cattle calves in the Giza and El Fayoum governorates, Egypt. Additionally, the diagnostic methods by acid-fast staining, immunochromatographic, and ELISA were compared in terms of statistical factors, duration of the laboratory experiment, and the cost-effectiveness of testing, to determine the superior method for the detection of Cryptosporidium in the infected calves.

MATERIALS AND METHODS

For this study, 88 Fresh faecal samples were collected directly from the rectum of diarrheic pre-weaned cattle calves in Giza (n=54) and El Fayoum governorates (n-34), Egypt between 2021 and 2022. The age of sampled calves ranged from two days to two months old. Each sample was kept in a labeled clean container and transferred in ice boxes to the Parasitology Laboratory at Animal Health Research Institute Dokki, Giza on the same collection day.

Laboratory Diagnosis

The collected samples were concentrated using faecal flotation with a Sheather's sugar solution **(Singh et al. 2006).**

Detection of oocysts using Modified Ziehl-Neelsen staining method (MZN)

A Floated material of concentrated faecal smear from each sample was transferred to a glass slide and allowed to dry at room temperature. Following fixing by methanol (2 min), the slides were flooded with basic carbol-fuchsin for 5 min. After a brief rinse with tap

water, the slide was decolorized with acid alcohol until the disappearance of red color (45–60 s) and rinsed again. Malachite green 0.5% or methylene blue 1.4% solution (1 min) was used as the counterstain **(El Kelesh et al. 2009; Smith et al. 2008).** The smear slides were washed with tap water and air-dried. Finally, all slides were examined using the oil immersion lens (100×), and Cryptosporidium oocytes were identified (4 - 6 microns, pink to red cells in the green or blue background). The duration of the MZN stain was 20 min (including procedure and examination).

Immunochromatographic test (ICT):

An immunochromatography test strips kit was used following the manufacturer's instructions to discover *Cryptosporidium parvum* antigens in faeces (BIO K 387, Belgium). A spoonful of liquid faecal samples was diluted with the liquid contained in the bottle, and homogenized well, taking care to prevent foam formation. A device was plunged into the diluted samples of the bottle for 10 minutes. Positive sample for Cryptosporidium appears as 2 lines while negative one appesrs as one line. The duration of ICT method was 20 min for detection of Cryptosporidium antigens.

ELISA Method

A commercial sandwich, double wells ELISA kit (BIO-X Diagnostics, Belgium) was used to detect Cryptosporidium antigens following the manufacturer's instructions. The test was performed on 88 maintained faecal samples at 4 °C without preservative. The plate is coated with monoclonal antibodies. Faecal samples were diluted and added to each coated well. After 1 hour incubation at 37 °C the conjugated monoclonal antibody was added. Following the incubation, the reaction was visualized by tetramethylbenzidine (TMB) and the results were read at 450 nm using a Microplate ELISA reader. The duration of ELISA method was 2 hours for detection of Cryptosporidium antigens.

Statistical Methods:

Diagnostic accuracy was assessed via two methods. The first is the Composite Reference Standard (CRS), where the sensitivity and specificity were calculated for each of the three tests, considering the combined results from at least TWO individual tests as the diagnostic 'GOLD' standard. The second is Latent class analysis (LCA), which is used to identify a set of discrete, mutually exclusive latent classes (diseased and not diseased) based on the observed results of the samples to a set of categorical variables (positive and negative). The model with the best fit was chosen to estimate the conditional probability Pr (positive) to represent the sensitivity of the test and the conditional probability Pr (negative) to represent the specificity of the test. Agreement between each pair of tests was assessed using the k statistic. CRS and agreement were done using IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA). LCA was done using RStudio 2022.12.0+353 for Windows Mozilla/5.0, with the po LCA package.

RESULTS

Microscopical examination

Microscopic examination of fecal stained smears using MZN was confirmed to be infected with Cryptosporidium oocysts which appeared as pink spherical bodies 4-6µ against a green or blue background, Figure (1). 63 out of 88 calves were found to be infected with Cryptosporidium spp., with an overall prevalence of 71.59% in Egypt; at Giza governorate was 70.37% (38/54) and at El Fayoum was 73.52 (25/34).

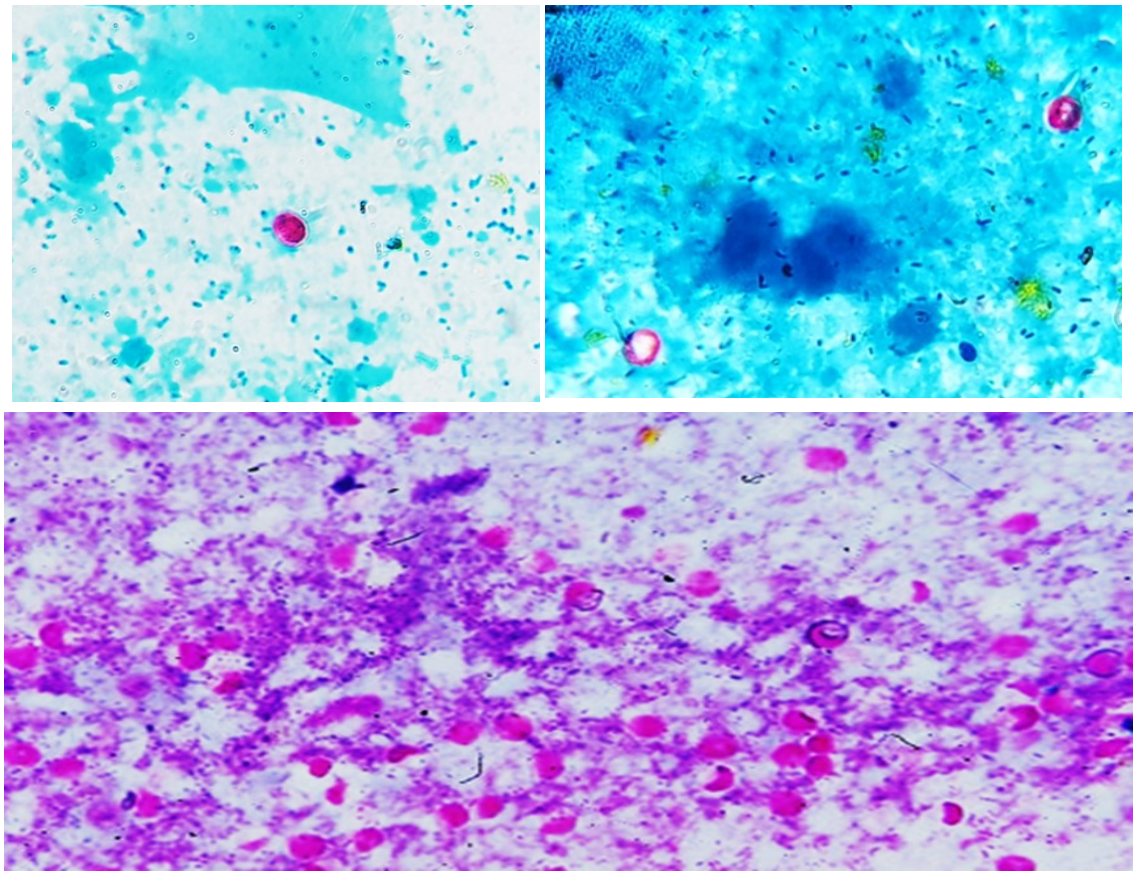

Fig. (1): Cryptosporidium spp. oocysts in fecal smear stained by Modified Ziehl-Neelsen (1000x).

Immunochromatographic test (ICT)

Results of ICT examination of 88 faecal samples from pre-weaned calves revealed that 65 samples were positive (73.86%) (table 1 & Fig. 2). The intensity of infection in positive samples was correlated with the clear appearance of the second line (Fig.2), as (B) represents positive result while, (A) is weak positive.

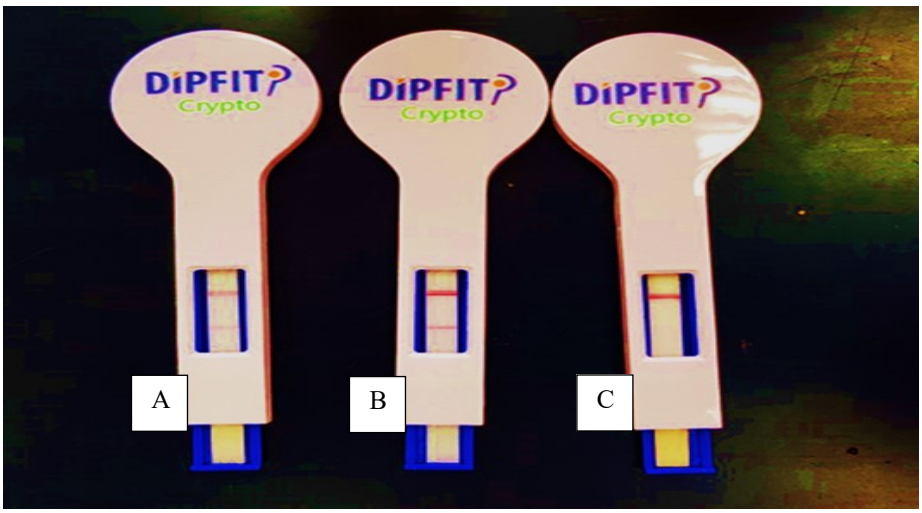

Fig. (2) A weak positive, B positive and c negative.

Results of ELISA

Examination of 88 fecal samples from pre-weaned calves using ELISA revealed 66 samples (75.00%) were positive (Table 1).

Table 1. Detection of *Cryptosporidium* spp. using MZN, ICT, and ELISA, in faecal samples of cattle calves (no = 88).

MZN	IC	ELISA	Observed frequency (n)	percentage (n/N)
+	+	+	63	71.59%
-	+	+	2	2.27%
-	-	+	1	1.13%
-	-	-	22	25.00%
Total number of positive results				
MZN			63	71.59%
ICT			65	73.86%
ELISA			66	75.0%

Assessment of MZN, ICT, and ELISA for the diagnosis of Cryptosporidium

The sensitivity (SE) of MZN, ICT, and ELISA to detect *Cryptosporidium* spp. on the same 88 faecal samples from pre-weaned diarrheic calves was 96.9%, 100%, and 100%, respectively. In contrast, their specificity (SP) was 100%, 100%, and 95.7%, respectively. Positive predictive values (PPV) of MZN, ICT, and ELISA were 100%, 100%, and 98.3% while, their negative predictive values (NPV) were 92%, 100%, and 100%, respectively (Table 2).

Table 2. Measures of the comparative performance of the three tests (MZN, ICT, and ELISA) used to detect *Cryptosporidium* in feces of pre-weaned cattle calves.

From	MZN	IC	ELISA
Sensitivity	96.9%	100%	100%
Specificity	100%	100%	95.7%
Positive predictive value % (PPV)	100%	100%	98.3%
Negative predictive value% (NPV)	92%	100%	100%
Accuracy	97.7%	100%	98.9%
Test time	20 min	20 min	2 hours

DISCUSSION

Among the opportunistic enteric parasites, *Cryptosporidium* species is considered the most common cause of neonatal enteritis and diarrhea in cattle calves leading to significant morbidity and mortality worldwide **(Schnyder et al. 2009).**

The detection of Cryptosporidium infection in faecal samples of humans and animals is accomplished by different tools and techniques **(Chalmers and Katzer, 2013).** A direct staining method of fecal samples by Modified Ziehl –Neelsen (MZN) was conducted in this study to screen positive samples for Cryptosporidium oocysts, this technique is the main tool that is recommended as a screening method in epidemiological studies **(Thompson and Ash, 2016).**

In the present study prevalence was 71.59% (63 out of 88) by using a Modified Ziehl–Neelsen stain (MZN). These results were lower than that reported in Algeria (84%) (**Benhouda et al. 2017),** but higher than those previously reported in Egypt; **(El-Khodery and Osman, 2008)** in Dakahlia and Kafr El-Sheik Governorates, recorded a prevalence of

14.2%, **Hassanain et al. (2011)** in Behira Governorate, found that infection rate of *Cryptosporidium parvum* in calves was (54.4%), **Helmy et al. (2013)** in Ismailia province observed that the prevalence rate was 30.3% in cattle calves aged 1 day to 3 months, **Essa et al. (2014)** reported that 56.32% of diarrheic calves of less than one month were infected with cryptosporidium oocysts, **Mahfouz et al. (2014)** in Kafr El-Sheikh Governorate showed a lower incidence of (9.2%), **Bessat (2019)** in Behira Governorate estimated a prevalence rate of 41.4 % in 1–4-week-old calves, **Abu El Ezz et al. (2020)** in Giza and Sharkia Governorates found 45.9% of the total investigated pre-weaned diarrheic calves were infected with cryptosporidiosis and **Elmahallawy et al. (2022)** In Assiut Governorate, stated that the rate of infection in diarrheic cattle calves was 45.16%.

Several factors may be responsible for the wide variation in the incidence of cryptosporidiosis including season of specimen collection, sanitary situation of the environment inside and outside the farms, source of water supply, differences in the age, and breed colostrum feeding of examined animals.

The high incidence of cryptosporidiosis in this study compared to previous studies may be due to the fact that all samples examined in this study were collected from pre-weaned cattle calves suffering from diarrhea.

Concerning the immunological diagnosis using ELISA and ICT techniques, the results of ICT revealed infection rate was 73.86% (65/88). This result is in agreement with other results in Egypt (**Helmy et al. 2013)** and France **(Agnamey et al. 2011).**

The results of ELISA revealed infection rate of 75.00% (66/88). This test has been previously used in similar veterinary epidemiological surveys conducted in Iran **(Badiei et al. 2011),** Iraq (**Al-Robaiee and Al-Farwachi, 2014),** and Algeria **(Ouakli et al. 2018).**

Regarding sample that was negative for Cryptosporidium using ICT but positive by ELISA, this could attributed to that ICT is licensed for the detection of *C. parvum*, but ELISA is for multispecies of cryptosporidium, Also, this indicates that the *Cryptosporidium* species in the sample was not *C. parvum* and could be one of the other species of Cryptosporidium.

Moreover, the three positive samples by ELISA, and negative in MZN might be due to these samples with low concentrations of cryptosporidium oocysts. This possibility is confirmed by the low positivity % of these samples by ELISA.

In the present study, the performance of the three tests (MZN, ICT, ELISA) used to detect Cryptosporidium in faeces of pre-weaned cattle calves revealed that ELISA and ICT had the highest sensitivity (100%), while MZN and ICT had the highest specificity (100%) and highest PPV (100%) using Cumulative positivity as the diagnostic gold standard but without significant (P=0.0). The sensitivity of the three tests was high and ranged between 96.9% in MZN and 100% in both ELISA and ICT. These results were in agreement with a previous study that evaluated different diagnostic methods used to detect Cryptosporidium in the stools of diarrheic children. MZN revealed higher specificity than ELISA and rapid strip, while ELISA showed the highest sensitivity **(Eassa et al. 2017).** The same results were detected in another study to evaluate immunological tests for diagnosis of Cryptosporidium in diarrheic animals (pigs, calves, and lambs), ELISA showed higher sensitivity (40.9%) than ICT (22.7%), while ICT showed higher specificity (100%) than ELISA (78.9%) **(Danišová et al. 2018)** Also, **Papini et al. (2018)** found that three ICT tests to detect *C. parvum* in diarrheic calves had high sensitivity and specificity (SE 100%, 100%, and 90.24%, SP 96%, 92%, and 100%, respectively). **Abdou et al. (2022)** confirmed the results of the present study as MZN had the highest specificity (98.29%) while, this result differed in the highest sensitivity as they found that IC had the highest sensitivity (74.07%), using a composite reference standard (CRS) as a gold standard.

In contrast, **Mirhashemi et al. (2015)** found that ELISA had higher specificity than standard microscopic assay (Kinyoun's carbol-fuchsin acid-fast staining) in cattle samples.

Different sensitivities and specificities of the diagnostic tests used to diagnose Cryptosporidium in faecal samples might be attributed to highly dependent on the difference of infecting species and the concentration of oocysts in the faecal sample.

In a comparison of the sensitivity and specificity between the three techniques MZN, ICT, and ELISA for the detection of Cryptosporidium, the results were very similar as Sensitivity was>96% and Specificity was>95% for all three techniques. So, rapid ICT was the best one to be proposed for examination of samples as it was quicker and the need for technical expertise or specialized laboratory equipment is virtually nil since the interpretation of results is non-ambiguous. On the other hand, a disadvantage of the rapid assays used in the present study is that they are unable to detect *Cryptosporidium* spp. other than *C. parvum* and deliver only qualitative results. Also, MZN is a simple way for diagnosis of Cryptosporidium, it is of low cost as a permanent stain can be used for screening numerous samples, but MZN is laborious and needs expert examiners to identify oocysts accurately. Simultaneously ELISA is costly and requires well-equipped laboratories and skilled technicians, but the advantage of ELISA is used for the diagnosis of multispecies of cryptosporidium.

In conclusion, accurate diagnosis is obtained by using ICT or MZN and confirmed with ELISA. Accurate and rapid detection of *Cryptosporidium* spp. during diarrhea epidemics in calves is crucial, as it is the key for preventing and controlling the spread of infection among animals and humans, so reducing economic losses, and improving animal welfare.

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