



Comparative evaluation of conventional diagnostic methods for detection of bovine cryptosporidiosis*

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Received: 17 December 2011; Accepted: 12 June 2012

ABSTRACT

Cryptosporidium is an important enteropathogen that causes diarrhoea in dairy calves. Keeping in view of the importance, there is a need for simple and inexpensive diagnostic and screening tests for the detection of *Cryptosporidium* infection in dairy calves. In the present study, 4 conventional coprological detection methods were compared and evaluated in detection of *Cryptosporidium* oocysts in dung samples of dairy calves. Direct smear (DS) examination detected 21 positives (3.15%) for *Cryptosporidium* oocysts, out of 667 samples screened. In faecal concentration techniques, sugar floatation (SF) and formal-ethyl acetate (FEA) methods detected 39 (5.85%) and 41 (6.15%) positives, respectively. A total of 74 positives were detected by modified Ziehl-Neelsen (mZN) staining technique with a prevalence of 11.09%, showing that mZN technique as the most sensitive method of detecting *Cryptosporidium* oocysts. Statistical analysis revealed that highly significant difference was observed between DS and SF, FEA and mZN techniques. Inclusive and exclusive detection of *Cryptosporidium* oocysts by various conventional microscopic methods were recorded. In conclusion, among the conventional microscopic methods followed in the present study, mZN staining technique was more sensitive for detection of *Cryptosporidium* infection. Further, mZN staining technique is more appropriate and recommended to its use for developing countries like India for diagnosis of *Cryptosporidium* infection in bovine calves.

Key words: Cattle, *Cryptosporidium*, Diagnostics, Microscopy

Cryptosporidium, a coccidian parasite, can infect a wide range of the mammals, including humans. The oocyst stage is important for spreading of infection (Fayer *et al.* 1997, Fayer *et al.* 2000). *Cryptosporidium* spp. have recently attracted increasing interest due to several serious water-borne outbreaks, the life-threatening nature of infection in immunocompromised patients, and the realization of economic losses caused by these pathogens in livestock (Egyed *et al.* 2003) particularly in dairy cattle. Cryptosporidiosis, a disease of neonates, is characterized by acute gastro-intestinal disturbances associated with mucoid or haemorrhagic diarrhoea, fever, lethargy, anorexia leading to loss of condition (Navin and Juranek 1984). The infection

associated mortality was as high as 35% (Singh *et al.* 2006). Diagnosis is based primarily on demonstration of oocyst in the dung samples. Numerous techniques, conventional microscopic methods to molecular techniques, are available for detection of *Cryptosporidium* infection. In India, these techniques are widely in use for detection of the parasite in animals (Roy *et al.* 2006, Chhabra and Pathak 2012). Some of the detection techniques are more sensitive and accurate than others in identifying *Cryptosporidium*, thereby influencing the prevalence data (Enemark *et al.* 2002). Concentration and staining techniques were developed to facilitate the reliable detection of oocysts even when present in small numbers (O'Donoghue 1995).

The direct demonstration of the organisms is considered as the 'gold standard' of diagnosis. The prevalence of the *Cryptosporidium* parasite varies widely depending on the sensitivity and specificity of screening methods used. Hence, the present study was undertaken to compare and evaluate the various conventional diagnostic procedures, viz. direct smear (DS), sugar floatation (SF), formal-ethyl acetate (FEA), and modified cold strong Ziehl-Neelsen (mZN) staining methods in detection of *Cryptosporidium* in dairy calves.

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MATERIALS AND METHODS

Dung samples were collected from dairy calves of age ranging from 3 days to 6 months from various dairy farms/ small holdings in Andhra Pradesh, Karnataka, Kerala, Tamil Nadu and Puducherry during the period from February 2009 to June 2010. Samples (667) were collected directly from the rectum of 667 dairy calves by wearing a disposable hand glove and transferred immediately into a 50 ml sterile plastic screw capped sample container. All the dung samples were screened by conventional coprological techniques such as DS, SF and FEA concentration techniques for detection of oocysts of *Cryptosporidium*. Direct smears of unconcentrated faecal samples were stained by mZN method. A minimum of 300 microscopic fields of the stained smear was first observed under a compound microscope at a magnification of 400 $\times$ and then 1000 $\times$. Samples were considered to be positive even when a single oocyst was observed. Results of the study were analyzed by Vetstat statistical programme using Chi-square analysis. The procedures followed in the present study are briefly mentioned below.

Direct smear examination: A drop of distilled water was placed on a clean, non-greasy microscopic glass slide and match-stick head sized dung sample was placed on the drop of water with the help of tooth pick and mixed well to form a uniform suspension. All the coarse particles were removed and a cover slip was placed over the smear. Care was taken to prepare a thin smear through which newsprint could be read with difficulty. The condenser was kept lowered while examining the smear under oil immersion objective lens to reduce the back ground light to visualize the oocysts of *Cryptosporidium* clearly as they were quite transparent and small in size.

Sucrose flotation: Sucrose flotation protocol was followed as described by Scott *et al.* (1995).

Formal-ethyl acetate method: This method was followed as per Nichols *et al.* (2006).

Modified cold strong Ziehl-Neelsen staining method: The protocol followed was as per the Fayer and Xiao (2008) with a slight modification in the smear preparation i.e., 0.5 g dung sample was transferred into a 2 ml microcentrifuge. Distilled water (1.4 ml) was added and vortexed thoroughly till a homogenous material was obtained. Faecal smear was prepared by placing a small drop of dung suspension on to a microscopic glass slide. Smear thickness was adjusted such a way that print on a page could just be seen through the preparation.

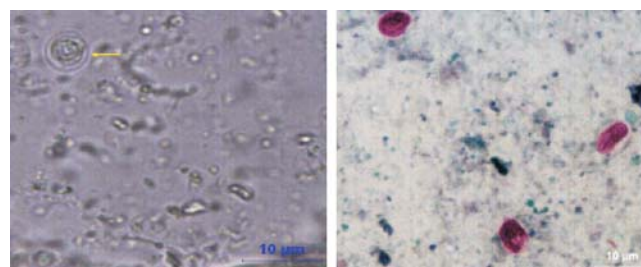
RESULTS AND DISCUSSION

Dung samples (667) were screened for *Cryptosporidium* infection using conventional microscopic methods. Direct smear examination 21 (3.15%) dung samples were positive for *Cryptosporidium* oocysts, whereas in mZN staining, SF and FEA technique the numbers positive were 74 (11.09%), 39 (5.85%) and 41 (6.15%), respectively (Table 1).

Table 1. Detection of *Cryptosporidium* infection by conventional microscopic methods

Dung samples	Conventional microscopic methods			
	DS	mZN	SF	FEA
Number screened	667	667	667	667
Number positive	21	74	39	41
Per cent positive	3.15 ^a	11.09 ^c	5.85 ^b	6.15 ^b

DS: Direct smear; mZN: modified Ziehl-Neelsen staining; SF: sugar flotation; FEA: formal-ethyl acetate sedimentation; Chi-Square test using vetstat statistical programme; percentages with different superscripts are significantly different (P<0.05).



Figs. 1–2. 1. *Cryptosporidium* oocyst – wet film (1000 $\times$). 2. Modified Ziehl-Neelsen stained *Cryptosporidium* oocysts (1000 $\times$).

Unstained wet faecal preparations were examined and *Cryptosporidium* oocysts were observed under compound microscope as small in size and were transparent in nature (Fig. 1). In mZN method the *Cryptosporidium* oocysts appeared red spherules on pale green back ground, but the degree and proportion of staining varied with individual oocysts (Fig.2). Apart from *Cryptosporidium* oocysts, some fungal spores also stained red. They could however be differentiated from *Cryptosporidium* oocysts by their homogenous appearance and absence of granular or faint crescent shaped sporozoites inside.

In the present study, direct smear examination detected 3.15% of *Cryptosporidium* infection, which is low when compared to the results of concentration methods. This proved that concentration techniques are more sensitive in detecting *Cryptosporidium* oocysts. This observation is in agreement with other findings (Scott *et al.* 1995, Uga *et al.* 2000), clearly highlighting the lack of sensitivity of direct smear examination in detecting *Cryptosporidium* oocysts.

Sugar floatation method detected 5.85% of the samples to be positive for *Cryptosporidium* oocysts. This finding is lower when compared with other investigations in India (Chattopadhyay *et al.* 2000, Kumar *et al.* 2004, Singh *et al.* 2006, Shobhamani and Singari 2006, Sahu and Maiti 2009) and other countries (Casemore *et al.* 1997, Pena *et al.* 1997, Enemark *et al.* 2002, Hamnes *et al.* 2006). Similar results of detection have also been reported by others (Scott *et al.* 1995, Bukhari and Smith 1996, Wade *et al.* 2000, Santin *et al.* 2004, Starkey *et al.* 2005). Several studies have shown that there

could be substantial differences in the infection rate influenced by a range of factors including analytical method and design of study, geographical differences, age, composition of sampled animals, number of samples screened, sampling season and intermittent oocyst shedding (Hamnes *et al.* 2006).

In the present study 6.15% of *Cryptosporidium* infection was recorded using FEA method. A similar finding was reported by Kaminjolo *et al.* (1993) in calves in Trinidad and Tobago. Studies carried out in other countries like Argentina (Coco *et al.* 2008), Brazil (Pena *et al.* 1997), Spain (Quilez *et al.* 1996) and United Kingdom (Sturdee *et al.* 2003) reported higher infection rates. High levels of environment contamination, high output of oocysts, and extensive husbandry systems may have accounted for the apparently high rate of infection (Kaminjolo *et al.* 1993). Slightly more prevalence was noted in the present study using FEA when compared to sugar flotation. This might be due to the advantage of FEA technique to extract lipids from faecal specimens, thus dispersing oocysts in an aqueous phase and also in the present study the collected samples were from young dairy calves, which are rich in fat as they are fed on milk. The present finding was similar to the observations of Casemore *et al.* (1985) and Mtambo *et al.* (1992).

Among all conventional methods used in the current study, mZN method showed highest detection rate, this might be due to the clear visibility of *Cryptosporidium* oocysts as red spherules on pale green back ground counter stain. The observations in the present study by using mZN method were in conformity to Kaminjolo *et al.* (1993) and Chattopadhyay *et al.* (2000). Higher rate of infection was reported by several workers (Quilez *et al.* 1996, Fuente *et al.* 1999, Enemark *et al.* 2002, Sturdee *et al.* 2003, Singh *et al.* 2006, Coco *et al.* 2008, Sahu and Maiti 2009). The variation in the infection rate could be attributed to a number of factors including overcrowding, seasonal stress, selection of study population, contamination of premises, animal husbandry practices, age of the animal, consistency of dung, concurrent infections and nutritional factors.

Based on the data analysis, the inclusive and exclusive detection of *Cryptosporidium* oocysts by various conventional techniques were analyzed (Table 2). All 21 samples positive by direct smear examination were also positive by other 3 methods i.e., mZN, SF and FEA. Out of 39 total positives by SF method, only 29 samples were positive by mZN, but all 39 positive samples were confirmed by FEA method. Out of 41 samples positive by FEA method, only 33 were confirmed by mZN method. Further, mZN method is unable to detect the oocysts in some of the positive dung samples by SF and FEA, the possible reason may be the concentration of oocysts in SF and FEA, and the smear prepared for mZN method was from unconcentrated faecal suspension. Some of the samples which were negative by the concentration methods (SF and FEA) were additionally

Table 2. Inclusive and exclusive detection of *Cryptosporidium* oocysts by various conventional microscopic methods

DS	mZN	SF	FEA
21	21	21	21
21	74	29	33
21	29	39	39
21	33	39	41

DS: Direct smear; mZN: modified Ziehl-Neelsen staining; SF: sugar flotation; FEA: formal-ethyl acetate sedimentation.

Figures shown in bold were the actual number of positives by their respective diagnostic method out of 667 dung samples screened.

found positive by mZN method. The reason may be the staining of the oocyst (red colour) and its clear visibility on counter stain back ground (pale green). Further, presence of a single oocyst in all the detection methods employed in the current study was considered as positive sample. Presence of few/low number of oocysts detection by SF and FEA i.e., wet film examination is difficult with ordinary compound microscope, because of small size and transparent nature of oocysts of *Cryptosporidium*. But at the same time in the staining methods like mZN, even presence of a single oocyst can easily be detected with the same microscope.

The economic assessment of all 4 conventional methods used in this study was analysed. All these 4 methods were much cheaper and affordable in Indian conditions. The variations in cost among these methods were very slight, except for direct smear examination, where there is no involvement of chemicals/reagents. In the present study, the cost of non-recurring items (equipment), electricity and technical/human resources was ignored, while assessing the cost of each method. Further, depending on the involvement of skill, usage of chemicals/reagents can be minimized per sample. It is well established that, the number of samples processed may also be considered because when large number of samples are processed, the diagnosis cost could also get minimized. Keeping in view all the factors in consideration and also the efficacy of various conventional methods used in the current study except for direct smear examination, it is roughly estimated that the cost involvement was around ₹ 10 per sample (Indian currency) during the year 2010. The cost variation is not much among the mZN, SF and FEA, but in view of the added advantage of mZN, it would be more appropriate and recommended to its use for developing countries like India for diagnosis of *Cryptosporidium* infection in bovine calves. In a study by Paul *et al.* (2009) in India, economic assessment of coprological diagnostic methods and PCR for detection of *Cryptosporidium* spp. in bovines was evolved and concluded the low cost involvement of conventional techniques over molecular techniques.

Among the conventional methods used in the current study for detection of *Cryptosporidium* oocysts in the collected

faecal samples, mZN staining method was comparatively good enough over wet film examinations, viz. DS, SF and FEA. Although, the materials for SF and FEA methods were inexpensive, similar to mZN staining technique, it was technically exacting to prepare the correct specific gravity sugar floatation solution. All these wet film procedures did not adapt well to the work flow in the laboratory and specimens could not be easily processed as a batch, which required reading of results within few minutes of preparation in contrast to mZN technique where the stained slides could be examined later and also kept as a permanent records (Mac Pherson and Mc Queen 1993). It is concluded that, among all the conventional methods followed in the current study, mZN is more convenient and sensitive over the wet faecal examinations in detection of *Cryptosporidium* infection in dairy calves in India.

ACKNOWLEDGMENT

The facilities provided by TANUVAS, Chennai is greatly acknowledged

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