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Comparative evaluation of various diagnostic techniques for detection of *Cryptosporidium* infection from the faecal samples of diarrhoeic bovine calves

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Abstract

Background: *Cryptosporidium*, an opportunistic, zoonotic, apicomplexan parasite, is one of the most common causes of diarrhea in neonatal bovine calves around the globe. Bovine calves act as a major source of infection by excreting huge numbers of highly resistant oocysts in faeces, which can survive for a long time in extreme environmental conditions. As low as ten oocysts can cause disease and mortality, leading to the requirement of an early and accurate diagnosis for proper and favorable prognosis, management, and control. **Aims:** The current study was conducted with the objective to evaluate various diagnostic techniques (acid fast staining, negative staining, fluorescent, ELISA, PCR, nested PCR, and qPCR) for the detection of *Cryptosporidium* in the faecal samples of diarrheic bovine calves. **Methods:** Two hundred diarrheic faecal samples from bovine calves were collected and subjected to these techniques for *Cryptosporidium* diagnosis. Results of these were evaluated for diagnostic comparison. **Results:** Out of 200 faecal samples evaluated, 24% (48/200) were detected positive for *Cryptosporidium* using a combination of two techniques as gold standard criteria. Cohen's kappa value indicated moderate to almost perfect agreement (0.616 to 0.986) among all the techniques used in the present study. Leishman staining showed the lowest sensitivity (54.17%), while nested PCR and qPCR showed the highest sensitivity (97.92%). Diagnostic specificity of all these tests ranged from 98.68 to 100%. **Conclusion:** Auramine stain was used for the first time in the bovine calves in India for the detection and diagnostic comparison of *Cryptosporidium*. It showed strong agreement with the molecular as well as classical diagnostic techniques, and can be used for primary screening for better diagnosis.

Key words: *Cryptosporidium*, Diagnostic accuracy, ELISA, Fluorescence, PCR

Introduction

Cryptosporidium is an intracellular, extracytoplasmic, zoonotic coccidian protozoan parasite belonging to the phylum apicomplexa. It causes water and food borne gastrointestinal infections. The parasite is not only a problem in developing world including India, but also Europe, USA, and Canada tries to prevalent it (Guy *et al*., 2021). Large scale, worldwide, outbreaks in human beings as well as bovines have been reported in recent years with high morbidity and mortality (Brar *et al*., 2017b; Ouakli *et al*., 2018; Ryan *et al*., 2018). Adult cattle are an important reservoir of this pathogen and act as a source of infection to a variety of susceptible animals and human beings (Brankston *et al*., 2018; Diaz *et al*., 2018; Santin, 2020). Livestock manure adds 3.2 ×

10^{23} oocysts per year which are very resistant to common water disinfectants and remain infective for a long time in a high humid environment (Vermeulen *et al*., 2017; Shrivastava *et al*., 2017). Interspecies transmission has been reported between cattle, humans, and avian species due to sharing of common habitats and contamination of water sources (Wells *et al*., 2019). There is no available vaccine, and drugs have limited efficacy (Thomson *et al*., 2017). Previous surveys indicated *Cryptosporidium* as the second most common cause of neonatal calf diarrhea after Rotavirus (Yimer *et al*., 2015).

There are few reports that have used the diagnostic comparison techniques in human medicine, but none have analysed the combination of classical, immunological, and molecular techniques, which is

included in our study. The present study is designed to evaluate the diagnostic efficiency of a combination of seven techniques to detect *Cryptosporidium* from faecal samples of diarrheic bovine calves.

Materials and Methods

Faecal samples were collected directly from rectum of diarrhoeic bovine calves (n=200) of age less than two month from Ludhiana district (30.9°N, 75.8°E) of Punjab in three aliquots, first for smear preparation to perform staining, second for ELISA, and third for molecular detection (PCR, nested PCR, and real-time PCR). Faecal smears were made immediately after collection and fixed in absolute methanol for 3 min, for cases that immediate staining was not possible. Second and third aliquots were stored at -20°C without any preservative until further use.

Out of three smears, the first was stained with modified Ziehl-Neelsen staining (mZN) as per standard protocol (Henriksen and Pohlenz, 1981), the second smear was processed for negative staining (Leishman stain) as performed by Brar *et al*. (2017a). The samples found positive by both methods (mZN and Leishman) were included for comparative micrometry to evaluate the agreement between these two staining techniques by Olympus BX53 microscope (cellSens software). The third smear of the same sample was stained with phenolic auramine stain (Dzodanu *et al*., 2019) and viewed under a fluorescent microscope excited by blue (450-500 nm) light. Enzyme linked immunosorbent assay (ELISA) was performed by using antigen detection, sandwich ELISA kit (Bio K 346/2, Bio-X Diagnostics, Belgium) as per manufacturer's protocol.

DNA was extracted from fecal samples using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany) as per the manufacturer's instructions with minor modification in initial step which included five cycles of freeze-thaw (5 min in liquid nitrogen and 1 min in boiling water). The purity and concentration of isolated DNA was determined by spectrophotometer (NanoDrop 2000, Thermo Scientific) by measuring absorbance at 260 nm and 280 nm. PCR was performed to amplify *Cryptosporidium* outer wall protein (COWP) gene due to its high sensitivity (Yu *et al*., 2009) with previously published primers (Table 1). PCR reaction mixture consisted of 8 μL eluted DNA, 1 μL (10 pmol/μL) each forward and reverse primers, 12.5 μL GoTaq Green master mix (Promega, USA) and 2.5 μL nuclease free water. Product of primary PCR (1 μL) was used as a DNA template for nested PCR. Forty cycle amplification was carried out in primary PCR and 35 cycle in nested PCR (nPCR). The PCR products were subjected to electrophoresis using 1.5% agarose and subsequently viewed under UV transilluminator.

Table 2: Results of paired t-test for comparison of morphometric analysis

Sr. No.	Particular	Mean	SE	Estimated t	P-value
1	Leishman	4.795	0.0640	1.142	0.264
2	Modified ZN	4.721	0.0704		

Real-time PCR (qPCR) was performed in CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) in 10 μL reaction mixture containing 5 μL KAPA SYBR FAST qPCR Master Mix 2X (Merck, Germany), 0.2 μL of each forward and reverse primers of *Cryptosporidium* β-tubulin gene (Table 1). 0.5 μL (1 ng/μL) of DNA template and nuclease free water was added to make the final volume 10 μL. The thermal cycling includes an initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 50 s and annealing at 52°C for 1 min. Melting curve was analyzed at the end of PCR amplification.

Statistical analysis

To compare the size of oocysts by mZN and Leishman stain, the average of two perpendicular diameters of oocyst was calculated for both smears, and paired t-test was applied. Sensitivity, specificity, positive predictive value (PPV), and negative predictive values (NPV) were calculated by using standard formula. The agreement between each test and true positive samples were evaluated by Cohen's Kappa (k) coefficient by using SPSS software version 20.0. The results of Cohen's Kappa (κ) coefficient were categorized as none (0-0.20), minimal (0.21-0.39), weak (0.40-0.59), moderate (0.60-0.79), strong (0.80-0.90), and above 0.90 as almost perfect agreement (McHugh, 2012).

Results

In the present study, modified Ziehl-Neelsen (mZN) stained *Cryptosporidium* oocyst appeared pink to bright red in color with clear refractile hallow around it against greenish background (Fig. 1). Heterogeneous intracellular staining was a characteristic differentiating feature used to distinguish from other carbol fuchsin stained objects such as fungal spores etc.

Table 1: Primer sets used for amplification of *Cryptosporidium* genes

Target gene	Sequences	Expected product size	Anealing temperature	References
COWP	GTAGATAATGGAAGAGATTGTG GGACTGAAATACAGGCATTATCTTG	550 bp	52°C	Yu *et al*. (2009)
COWP (nested)	TGTGTTCAATCAGACACAGC TCTGTATATCCTGGTGGGC	311 bp	60°C	
β-tubulin gene	ATGCTGTAATGGATGTAGTTAGACA GTCTGCAAAATACGATCTGG	160 bp	52°C	Tanriverdi *et al*. (2002)

Table 3: Results of *Cryptosporidium* positivity by various diagnostic techniques on fecal samples of diarrhoeic bovine calves

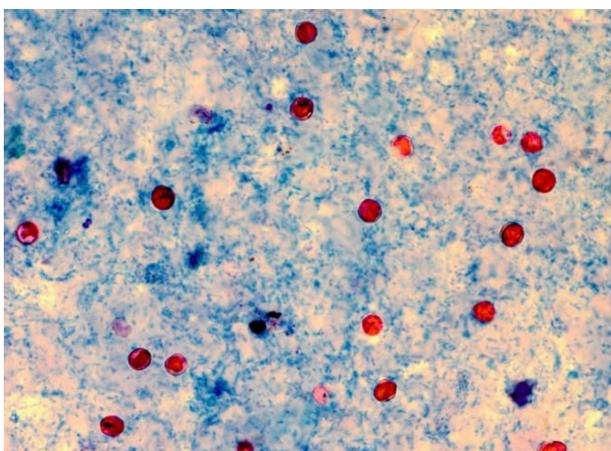| Leishman	mZN	Auramine	PCR	NPCR	ELISA	qPCR	Total
+	+	+	+	+	+	+	26
-	+	+	+	+	+	+	4
-	-	+	+	+	+	+	6
-	-	-	+	+	+	+	7
-	-	-	-	+	+	+	1
-	+	+	-	-	+	-	1
-	+	+	-	+	-	+	3
-	+	+	-	-	-	-	2
+	-	-	-	-	-	-	2
-	-	-	-	-	+	-	1
-	-	-	-	-	-	+	1
-	-	-	-	-	-	-	146
Total							200

Fig. 1: Modified ZN stained faecal smear that shows red colored *Cryptosporidium* spp. oocyst (×1000)
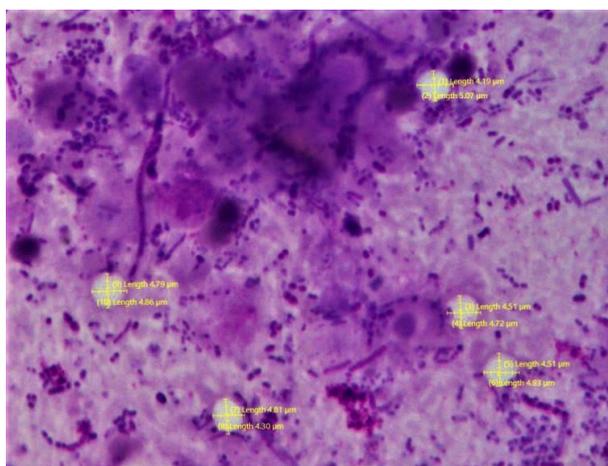

Fig. 2: Leishman stained faecal smear that shows *Cryptosporidium* spp. oocysts as blank spaces (×1000)

Cryptosporidium was detected in 36 samples (18%, 95% Probability Interval (PI): 0.13-0.24) by mZN (Table 3). By Leishman staining, only 28 samples were found positive (14% positivity with 95% PI: 0.10-0.20). Leishman stain was not taken up by cryptosporidial oocysts (negative staining) and oocysts appeared round unstained bodies with bluish background (Fig. 2). Twenty-six samples (13%) were found positive by both

methods (Leishman and mZN) (26) and were included for comparative micrometry. Paired t-test results (Table 2) revealed no significant difference between the two techniques (t-value 1.142 and P-value 0.264). The *Cryptosporidium* oocysts were detected in 42 faecal samples (21% with 95% PI: 0.16-0.27) and appeared apple green fluorescent spherical structures (Fig. 3) with a diameter of 4 to 6 µm using phenolic auramine staining technique.
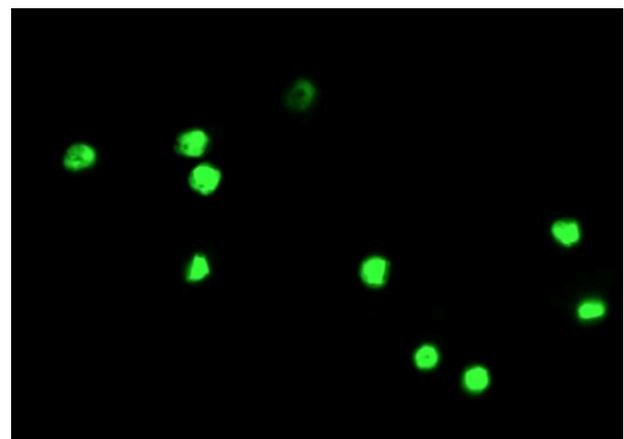

Fig. 3: Phenolic auramine stained faecal smear that shows green fluorescent *Cryptosporidium* spp. oocyst (×1000)

Forty-six (23% with PI: 0.17-0.29) samples were found positive by ELISA. Out of these, only one sample was positive using ELISA and negative using all methods. Primary PCR has shown DNA band at 550 bp (Fig. 4) in 43 samples (21.5%, PI: 0.16-0.28) while nested PCR was positive in 47 samples (23.5% with 95% PI: 0.18-0.30) showing 311 pb band in gel electrophoresis (Fig. 5). Real-time PCR (qPCR) was positive in 48 samples (24% with 95% PI: 0.18-0.30) (Fig. 6). CT value of qPCR ranged between 21.54 to 31.74 with mean 26.90 and standard deviation 2.57. Out of 200 samples 26 (13%) were positive by all the methods.

The sensitivity, specificity, PPV, and NPV of all diagnostic tests is summarized in Table 4. Leishman staining showed the lowest (54.17%), while nested PCR and qPCR showed the highest sensitivity (97.92%). PCR
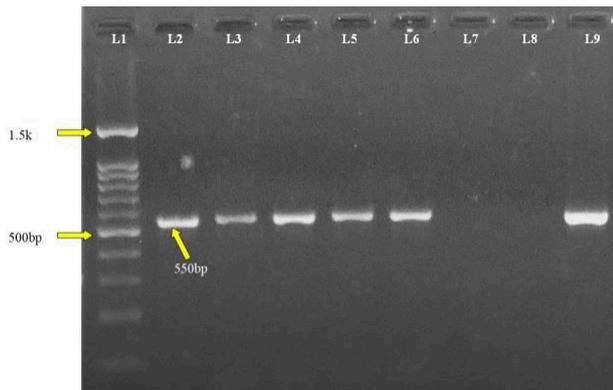
Fig. 4: Primary PCR amplification of *Cryptosporidium* COWP gene. Lane L1: 100 bp DNA ladder, Lanes L2 to L6: Positive samples, Lane L7: Negative sample, Lane L8: Negative control, and Lane L9: Positive control
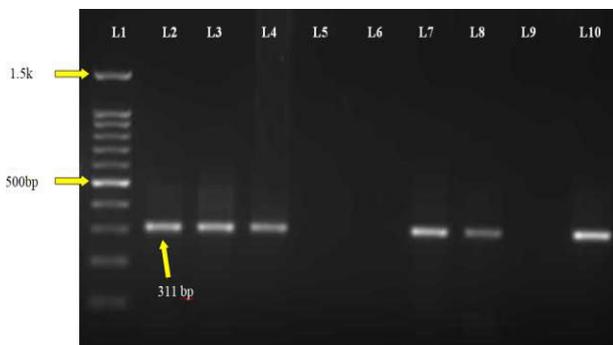

Fig. 5: Nested PCR amplification of *Cryptosporidium* COWP gene. Lane L1: 100 bp DNA ladder, Lanes L2-4, L7, L8: Positive samples, Lanes L5 and L6: Negative sample, Lane L9: Negative control, and Lane L10: Positive control
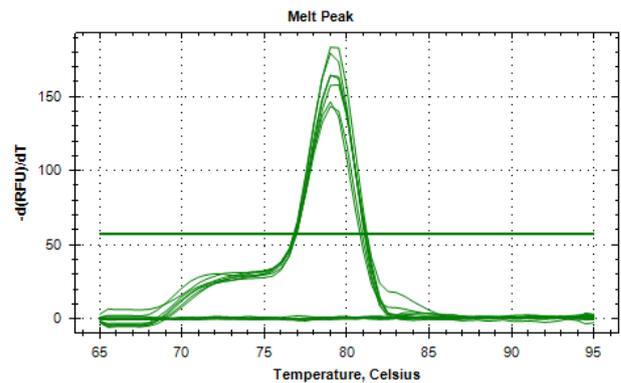

Fig. 6: Real-time PCR amplification melt curve of β-tubulin gene specific *for Cryptosporidium* spp.

and nested PCR revealed the highest (100%) specificity for detection of *cryptosporidium*. Depending on criteria used for true positivity in the present study (*i.e.* positivity by two or more techniques among acid fast, immunological, and molecular tests), 24% prevalence of *Cryptosporidium* was detected.

Discussion

Only few reports, involving 2-3 diagnostic techniques, are available from India for comparative detection of bovine cryptosporidiosis (Bhat *et al*., 2012; Rekha *et al*., 2016; Brar *et al*., 2017a). There is a lack of a universal gold standard reference test for the diagnosis of *Cryptosporidium* (Papini *et al*., 2018). In this study, we defined the "true positive" for samples that showed positive results by at least any of the two different

Table 4: Comparative analysis of Leishman, mZN, Auramine, PCR, nested PCR, ELISA, and qPCR for the diagnosis of *Cryptosporidium* spp.

TEST		True[*] positive	True negative	Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Cohen's kappa value (κ)
Leishman	Positive	26	2	28	54.17	98.68	92.86	87.21	0.616
	Negative	22	150	172					
	Total	48	152	200					
mZN	Positive	34	2	36	70.83	98.68	94.44	91.46	0.760
	Negative	14	150	164					
	Total	48	152	200					
Auramine	Positive	40	2	42	83.33	98.68	95.24	94.94	0.857
	Negative	8	150	158					
	Total	48	152	200					
Primary PCR	Positive	43	0	43	89.58	100	100	96.82	0.929
	Negative	5	152	157					
	Total	48	152	200					
Nested PCR	Positive	47	0	47	97.92	100	100	99.35	0.986
	Negative	1	152	153					
	Total	48	152	200					
ELISA	Positive	45	1	46	93.75	99.34	97.83	98.05	0.944
	Negative	3	151	154					
	Total	48	152	200					
qPCR	Positive	47	1	48	97.92	99.34	97.92	99.34	0.973
	Negative	1	151	152					
	Total	48	152	200					

Samples positive by any ≥2 different techniques (acid fast, ELISA, and PCR) were considered "true positive" (n=48) for calculation of sensitivity, and samples negative by all techniques were taken as "true negative". [*] $P<0.0001$

techniques out of acid fast staining (mZN, Auramine), antigen detection (ELISA), and polymerase chain reaction (PCR, nested PCR, qPCR). There were 54 cases diagnosed positive by at least one technique but only 48 cases were found fit with our true positivity criteria.

Variable sensitivity (ranging from 37% to 100%) and specificity (78% to 100%) of mZN staining had been reported by previous researchers (Kaushik *et al*., 2008; Aghamolaie *et al*., 2014; Elsafi *et al*., 2014; Shams *et al*., 2016). The acid fast technique in our study concluded lower sensitivity and specificity as compared to molecular techniques and ELISA that is in agreement with most of the studies (Kaushik *et al*., 2008; Khurana *et al*., 2012; Mahmoudi *et al*., 2021). However, Newman *et al*. (1993) reported higher sensitivity of mZN than ELISA. Kabir *et al*. (2020) documented equal sensitivity of mZN and PCR for the detection of *Cryptosporidium*. The reduced sensitivity observed in our study may be due to the low level of oocysts present in the samples and undetectable by microscopy. The mZN failed to detect 14 true positive cases, which were most likely derived from samples with a low concentration of oocysts. Another reason for the lower sensitivity was variable staining characteristic of this parasite and low grade infection of some of the samples. Relatively lower specificity may be due to the presence of other acid fast organisms just like yeasts and fungal spores. In spite of the lower sensitivity of mZN, this technique was considered gold standard by some of the earlier workers due to the direct demonstration of the organism, providing unambiguous testimony of infection (Paul *et al*., 2009; Ghoshal *et al*., 2018).

The micrometry results of Leishman and mZN methods showed no significant difference that confirms the presence of same organism by both techniques. The low sensitivity of Leishman test could be due to the presence of other hollow structures of similar size in the faecal smear (fungal spores, fat globules, and other organisms etc.), causing confusion and leading to false positives. However, experience, skill, and knowledge of observer are critical in screening of oocysts especially in cases of Leishman and mZN staining, improvement of which can increase the sensitivity, specificity and reduces subjective error up to some extent, which may be the probable cause of variation of result among different studies. The average diameter of *Cryptosporidium* oocysts observed in our study was 4.72 ± 0.36 and 4.80 ± 0.33 µm by mZn and Leishman techniques, respectively. This is in accordance with that reported by Brar *et al*. (2017a). Cryptosporidial micrometery using single stain has been performed by various researchers and they reported the size between 4-6 µm (Xiao *et al*., 2004; Jain *et al*., 2009; Rekha *et al*., 2016). On the other hand, phenolic auramine stain has shown considerably higher sensitivity and specificity as compared to mZN. Though it has lower sensitivity compared to ELISA and PCR, it has shown specificity of 98.68%. The present results showed relatively lower sensitivity of phenolic auramine compared to previous researchers who reported sensitivity as high as 97 to 100% (Khurana *et al*., 2012;

Chhina *et al*., 2017; Ninama, 2018). In contrast to our finding, Ghaffari *et al*. (2014) reported lower sensitivity of auramine compared to mZN. The stain provides a rapid and cheaper way of screening the samples, and the only constraint is availability of fluorescent microscopes in few laboratories.

Three important test available for cryptosporidial copro-antigen detection are fluorescent antibody test (FAT), immunochromatographic tests (ICT), and ELISA. FAT is less commonly used as it requires more time, and is a costly fluorescent microscope. ICTs are easily available, cost effective, gives result within 15-20 min, and do not require technical expertise, but most studies have reported their poor sensitivity and high false positives (Danišová *et al*., 2018; Manouana *et al*., 2020). ELISA is the most widely used method in research since it does not require faecal sample concentration and has a detection limit of 10^3-10^4 oocysts/ml. Since spectrophotometer is used in ELISA, it reduces the chances of manual error (Ghoshal *et al*., 2018). In the present study, we observed high sensitivity (93.75%) and specificity (99.34) of ELISA but some previous scientists reported lower sensitivity (Kumar *et al*., 2014; Ouakli *et al*., 2018); however varying sensitivity ranging from 58.82% to 100% and specificity from 93% to 100% have been reported globally (Ghoshal *et al*., 2018). The lower sensitivity may be due to depletion of antigens during freeze-thaw, long storage, and use of different kits, which causes false negative results (Jayalakshmi *et al*., 2008). Reduced specificity could be attributed to the use of poorly-defined antigens (Paul *et al*., 2009). We recorded one false positive and three false negative cases in our study by ELISA. False positive might be due to sample collection during the recovery phase when the oocysts shedding may not be continued but antigen shedding is persisted (Jayalakshmi *et al*., 2008). Nonspecific binding by use of secondary antibodies in ELISA kits can also cause a false positive reaction (Hassan *et al*., 2021).

In accordance to our findings, most of the researchers documented high sensitivity and specificity of PCR (Kaushik *et al*., 2008; Bhat *et al*., 2012). In the present study, conventional PCR showed lower sensitivities than ELISA, but nested PCR showed higher sensitivity over ELISA. Presence of inhibitors in faecal matters and very low number of oocysts may influence the sensitivity of conventional PCR over nested PCR. Nucleic acid (DNA) is a comparatively more stable molecule than proteins so it is less likely to be degraded during storage as compared to protein antigens which are commonly targeted by ELISA kits (Blanchard, 2012; Izzo *et al*., 2012). Ability of PCR to detect the pathogen even in the presence of antigen-antibody complexes is another reason for higher sensitivity of PCR (Blanchard, 2012).

Highest number of positive cases were diagnosed by qPCR (48; 24%). There was only one case which was negative by all the techniques but was positive by qPCR with the highest Ct value (31.74). This indicates the presence of very low amount of cryptosporidial DNA in the sample. There was no significant difference between

the diagnostic specificity and sensitivity of ELISA and qPCR as compared to true positive. The main advantage of qPCR is that gel electrophoresis is not required. The result of qPCR here is close to other techniques (such as nested PCR or ELISA or even Phenolic auramine staining) but imposes a higher levels of cost, time, and labor. So, this method is not really the best.

Results of coefficient of Cohen's kappa have shown moderate agreement between true positive and Leishman ($\kappa=0.616$) and mZN ($\kappa=0.760$). Auramine staining showed strong agreement ($\kappa=0.857$) while PCR, nested PCR, ELISA, and qPCR were in almost perfect agreement ($\kappa>0.90$) with true positives. Ghaffari *et al.* (2014) applied kappa statistics and concluded poor agreement between PCR and microscopy (0.006) while moderate between PCR and ELISA (0.550) and the highest between ELISA and microscopy (0.800).

In the present study overall prevalence of true positive cases was 24% (48/200). Variable prevalence has been reported by earlier Indian researchers ranging from 11.7 to 50% (Singh *et al.*, 2006; khan *et al.*, 2010; Bhat *et al.*, 2012; Joute *et al.*, 2016; Rekha *et al.*, 2016; Das *et al.*, 2019). In our study, most of the samples were collected from a dairy farm where the workers were having very less literacy ate. They were having lesser awareness about good sanitary practices and were residing in low hygienic conditions which might be the reason for the higher prevalence of this parasite. Similarly, Santín *et al.* (2004), Trotz-Williams *et al.* (2005), Wang *et al.* (2017), Ouakli *et al.* (2018), Papini *et al.* (2018), and Yildirim *et al.* (2020) reported 35.5%, 40.6%, 14.5%, 52.2%, 62.12%, and 35.5% prevalence from USA, Canada, China, Algeria, Italy, and Turkey, respectively. Short life cycle, interaction of animal and human being, season, age of animal, time of collection of sample, self-limiting infection, and hygienic condition of farm may be the cause of variation in prevalence reported by various scientists from different part of world.

The mZN staining is satisfactory for primary screening, while phenolic auramine stain was found to be best as it has comparatively high sensitivity and specificity close to molecular techniques. Phenolic auramine is a rapid, cost effective technique and do not require skill to observe the oocyst under microscope. We do not recommend Leishman staining (negative staining) even for primary screening due to low sensitivity by which positive cases may be lost. Antigen detection technique (ELISA) is less time consuming, sensitive, and specific technique but the kits are quite costly. Staining techniques are a preliminary inevitable step of *Cryptosporidium* diagnosis, while genotyping molecular techniques are indispensable with highest sensitivity and specificity.

Acknowledgement

Conflict of interest

The authors of this research article declare that they have no conflict of interest.

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