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Comparative evaluation of microscopy and PCR for detection of *Cryptosporidium* SP. oocysts in bovines

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Abstract
Many coprological and serological techniques have been described for detection of the parasites with the limitations of sensitivity and specificity. Molecular techniques are being increasingly employed for detection of *Cryptosporidium* during the last two decades mainly because of the inability to differentiate *Cryptosporidium* species by conventional microscopy. PCR technology offers an effective alternative to conventional diagnosis of *Cryptosporidium* for both clinical and environmental samples. We compared microscopic examination by a conventional modified Ziehl-Neelsen (mZN) acid-fast staining procedure with a nested PCR test directed against the 18S SSU rRNA gene as standard reference test for the diagnosis of cryptosporidiosis in bovines. Out of 103 random faecal samples collected from bovines, specific PCR amplification was achieved in 44 samples (42.72%), whereas, only 19 samples (18.45%) turned positive by acid-fast staining. Microscopy therefore exhibited 63.77% sensitivity as compared to PCR. PCR was more sensitive and easier to interpret but required more hands-on time to perform and was more expensive than microscopy. PCR, however, was very adaptable to batch analysis, reducing the costs considerably. An important advantage of the PCR test, its ability to directly differentiate between different *Cryptosporidium* genotypes, will assist in determining the source of cryptosporidial outbreaks. Sensitivity, specificity, ability to genotype, ease of use, and adaptability to batch testing make PCR a useful tool for future diagnosis and studies on the molecular epidemiology of *Cryptosporidium* sp. infections. However, conventional modified ZN method provided the required sensitivity and specificity along with nominal cost for diagnosis on per sample basis, and may be considered as a viable diagnostic alternative when PCR is not an option for a particular laboratory setting, especially in developing countries.

Keywords: Bovines, *Cryptosporidium*, molecular diagnosis, PCR

Introduction
Cryptosporidium, a parasitic protist dwelling in the small intestine of wide spectrum of vertebrate hosts including humans derives its name from cryptic intracellular but extra-cytoplasmic location inside the cell. Although *Cryptosporidium* may infect all animals, but more attention has been paid to the infections in cattle due to the high prevalence, its economic importance and the potential of the parasite as a reservoir for human infections, in this livestock species. The precise economic losses associated with bovine cryptosporidiosis have not thus far been examined in detail but include the cost of treatment and management of enteritis, reduced feed conversion and production efficiency and losses due to animal death with mortality reaching up to 35.2% (Singh *et al.*, 2006) [1]. Cryptosporidiosis accounts for up to 20% of all cases of childhood diarrhea in developing countries, and is a potentially fatal complication of AIDS (Mosier and Oberst, 2000) [2] and often, in early childhood, is associated with poor cognitive function and failure to thrive. *Cryptosporidium* poses a substantial health threat due to characteristics of pathogen such as lifecycle completion within an individual host, zoonotic transmission, prolonged survival in environment and transmission through water and food (Lal *et al.*, 2013) [3]. In recent years, cryptosporidiosis has been attracting increased interest owing to the life-threatening nature of infection in immuno-compromised patients, several recent waterborne outbreaks, and the economic losses incurred by the dairy sector as a consequence of cryptosporidiosis (Egyed *et al.*, 2003) [4].
An array of staining techniques have been established of which modified Ziehl–Neelsen (Henricksen and Pohlenz, 1981) [5] and Kinyoun (Soave, 1983) [6] staining techniques are considered superior (Fayer, 2004) with diagnostic (Morgan *et al.*, 1998) [8].

The detection limit of acid-fast staining techniques was reported to be 50,000 oocyst per gram of faeces, whereas, the detection limit was 500,000 oocysts per gram faeces when flurochrome dyes were used (Balatbat *et al.*, 1996) [9]. Detection limit of modified Kinyoun technique was reported to be 1–5 x 10^4 oocyst per gram of faeces (Weber *et al.*, 1991). In order to increase the sensitivity of faecal examination-based diagnosis, a number of concentration techniques have been devised, of which sucrose floatation method was reported to be having 93% sensitivity and 100% specificity when a sample containing 1.5 x 10^3 oocysts was tested (Barwick *et al.*, 2000) [11].

Molecular techniques are being increasingly employed for detection of *Cryptosporidium* during the last two decades mainly because of the inability to differentiate *Cryptosporidium* species by conventional microscopy. As far as detection of the *Cryptosporidium* at species level is concerned, both the microscopic and immunological techniques suffer from limitations, whereas, molecular diagnostic techniques, viz., PCR has detection limit up to single oocyst per sample and can ensure specific diagnosis up to species level coupled with 100% diagnostic sensitivity and specificity (Xiao *et al.*, 1999; Coupe *et al.*, 2005, Paul *et al.*, 2009) [12, 13, 14].

Nevertheless, it is essential to assess the potential of a diagnostic test for routine laboratory use in terms of its sensitivity and specificity compared with a standard test. Therefore, the present study was aimed to compare the diagnostic accuracies of conventional coprological modified Ziehl-Neelsen staining technique with that of a nested PCR assay which can detect up to single oocyst per sample and thereby assess the diagnostic efficiency of the techniques for routine laboratory use, especially for developing countries like India where the facilities for molecular detection are limited.

Materials and Methods

Collection of samples

Faecal samples were collected from the rectum of 103 bovine animals (cattle & buffaloes) from five agroclimatic zones of north western Rajasthan and placed in clean, sterile plastic vials. In the laboratory, each faecal specimen was portioned in to two halves immediately on the sampling day, the first portion was processed by the modified Ziehl-Neelsen acid-fast staining method and the remaining portion was preserved in 2.5% potassium dichromate (w/v) solution in 1:1 ratio and kept at 4^0C for molecular studies.

Coprological examination

Faecal concentration

The concentration methods namely sucrose flotation and formal-ethyl acetate methods were used for screening of dung samples for *Cryptosporidium* oocysts in the present study.

Sugar flotation

Sugar flotation protocol was followed as described by Fujino *et al.* (2006) [15]. Sugar solution of specific gravity 1.266 (128g sucrose, 100 ml distilled water) was prepared which usually applies for detection of coccidian oocysts. One gram of faecal sample was mixed with 14 ml of sucrose solution and transferred into a 15 ml centrifuge tube and centrifuged at 2000 rpm for 15 min. Supernatant was washed with distilled water and pellet was examined.

Formol- ether centrifugal sedimentation: (OIE, 2008)

About 1 gm (1 ml in case of diarrhoeic) of faecal sample was taken in a screw caped centrifuge tube of 15 ml capacity containing 7 ml of 10% formalin. It was thoroughly emulsified with the help of a glass rod. The suspension was filtered through a sieve of 400 µ pore size in to a beaker. Then the filtrate was poured back to the centrifuge tube to which 3 ml of diethyl ether was added and after applying the cap the whole suspension was vigorously shaken for 30 seconds. Then this was centrifuged at 1100 x g for 2 minutes which resulted in four distinct layers. The fatty plug was loosened with the help of an applicator stick and the fluid below and above the fatty plug was discarded by inverting the tube. Care was taken to retain the pellet at the bottom along with few drops of formalin. Then the pellet was re-suspended in the residual formalin by agitating the tube and the whole amount was transferred to microscope slides with the help of pipette to prepare smears of concentrated material. Formalin was allowed to evaporate from the smears and then the dried smears were stained by mZN technique.

Microscopy of faecal specimens

Oocysts were stained with Modified Ziehl-Neelsen (MZN) technique as described by Fayer and Xiao, (2008) [17] for detection and identification of *Cryptosporidium* sp. under the 100× magnification of compound microscope. Sample was considered positive if an oocyst of correct morphology, optical properties, internal structure, with size and shape (4–6 µm, refract pink, spherical round to oval with a residuum and sporozoites) was detected (Fayer and Xiao, 2008) [17].

Isolation of genomic DNA

Genomic DNA was extracted from 17 microscopically positive faecal specimens using HiPurA® Stool DNA Purification Kit (Himedia) following manufacturer's protocol and was stored at −20 °C until further analysis.

Nested PCR

A two-step nested PCR protocol was used to amplify a ~840 bp fragment of the 18SSU rRNA gene of *Cryptosporidium* species as described by Fayer and Xiao, (2008) [17]. In the primary PCR, a PCR product of 1317 bp was amplified using the forward and reverse primers CRPDIAG1F: 5'-TTCTAGAGCTAATACATGCG-3' and CRPDIAG1R: 5'-CATTTCCTTCGAAACAGGA-3'. The following PCR conditions were used: after an initial denaturation of 1 min at 95 °C, a set of 35 cycles were run, each consisting of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C. In the secondary PCR, a product size of ~840 bp was amplified using the forward and reverse primers CRPDIAG2F:5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and CRPDIAG2R: 5'- AAGGAGTAAGGAACAACCTCCA-3' (Xiao *et al.*, 1999 [12], 2001; Fayer and Xiao, 2008 [17]. The secondary PCR reactions followed the earlier defined PCR conditions with an annealing temperature of 56°C instead of 52 °C.

Sensitivity and specificity of diagnostic techniques

The sensitivity and specificity of the coprological diagnostic techniques were calculated following the formulae given below (Morgan *et al.*, 1998) [8] considering PCR as a standard reference test:

The samples which detected as positive by PCR were considered as true positive. The false negative sample for mZN was calculated by subtracting the samples detected negative by that mZN technique and the samples detected positive by PCR. The faecal samples which showed the presence of oocysts like bodies in stained/unstained smears during microscopic examination but subsequently did not yield positive PCR amplification were considered as false positive.

Results and Discussion

On microscopical examination, the oocysts of *Cryptosporidium* appeared as pink spherical bodies ranging between 4-6 µm in size against a green background of malachite green in smears stained by modified Ziehl–Neelsen (mZN) technique (Figure 1).

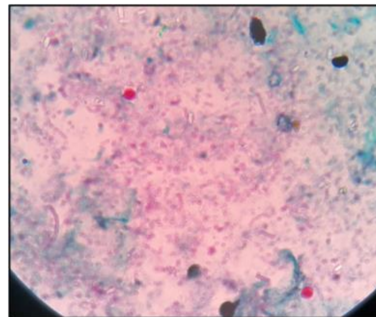

Fig 1: Modified Ziehl–Neelsen *stained Cryptosporidium* oocysts

PCR analysis revealed a 1317 bp primary product amplified out of the 1,750 bp 18S rRNA gene of *Cryptosporidium* sp., however non-specific banding had also been observed in primary round of PCR which was further resolved by a highly specific and sensitive nested amplicon of 834 bp in the secondary PCR, thus validating the MZN positive faecal samples as positive for *Cryptosporidium* sp. (Figure 2). Out of additional 103 samples, subjected to nested PCR, 44 samples (42.72%) were found positive including the 19 microscopically known positive samples. Nested PCR detected additional 25 samples (18.45%) positive for *Cryptosporidium* sp., which were tested negative in microscopy. However, 4 samples which were found positive by microscopy were tested negative by nested PCR which were considered as false positive. Microscopy therefore showed 63.77% sensitivity and 93.65% specificity compared to 100% sensitivity and specificity for PCR (Table 2).

Fig 2: Nested PCR product of amplified 18 SSU rRNA gene of *Cryptosporidium* species (834 bp)

Table 1: Comparison of mZN and Nested PCR for detection of *Cryptosporidium* sp. infection in bovines

Methods	Examined	Infected	Prevalence (%)
mZN Stain	103	19	18.45%
Nested PCR	103	44	42.72%
$\chi2$ Value	-	14.291**	-

Table 2: Sensitivity and specificity of mZN as compared to Nested PCR for detection of *Cryptosporidium* sp. Infection

Method	No. of samples examined	No. of samples infected	Sensitivity (%)	Specificity (%)
mZN Stain	103	19	63.77%	93.65%
Nested PCR	103	44	100%	100%

The economic assessment of the various methods revealed that the conventional mZN technique was much cheaper than the nested PCR (Table 3). The faecal concentration followed by preparation of each slide and the performance of the acid-fast stain procedure required about 90-100 min time. The reading of the slide required an additional 5 min. Interpretation of the acid-fast stain requires considerable expertise on the part of the operator. However, the cost of reagents per test for the acid-fast stain was very low (Rs. 21.5) though it is not amenable to bulk processing. The extraction of total DNA, PCR amplification, and subsequent gel analysis required a total of 5.5 (5-6) hours for a single sample plus controls; however, only about 1 hour of this time was hands-on time for the expert. PCR analysis is particularly amenable to bulk processing, and 103 samples can easily be processed in 1 to 2 days. The cost per test, including gel electrophoresis for the PCR procedure, was calculated Rs. 375.93. However, Interpretation of the PCR test was easy, as it was based simply on the presence or absence of a band and the size of the band denoted the genotype of the isolate detected. A period of approximately two days was required to

process 103 samples, with approximately 11 to 12 h of expert time, although this time could be greatly reduced with improvements to the technique.

Table 3: Time and cost required for conventional and molecular techniques for detection of *Cryptosporidium* sp. Infection

Method	Time taken for diagnosis (per sample)	Cost of diagnosis (per sample)	
		INR	US Dollars
mZN Stain	90-100 min	21.5	0.27
Nested PCR	5-6 h	375.93	4.74

Discussion

We compared conventional modified Ziehl-Neelsen acid-fast staining (Fayer and Xiao, 2008) [17] with a nested PCR test (Xiao *et al*., 1999, 2001; Fayer and Xiao, 2008) [12, 17] for the detection of *Cryptosporidium* and found microscopy to be considerably less sensitive and less specific than PCR analysis. Despite of the fact that the specific microscopical detection of the oocysts in the faecal samples provides unambiguous testimony of the infection, the method suffers from low sensitivity. Since, the PCR relies on the specific template dependent primer directed amplification of nucleotide sequence under standardized condition, it was used as standard test for calculation of percent specificity and sensitivity of the common coprological diagnostic test mZN in the present study.

Beginning with the development of the first PCR assay for the diagnosis of *Cryptosporidium* in stool specimens (Laxer *et al.*, 1991) [24], many PCR techniques have been described for detection of *Cryptosporidium* oocysts in clinical and environmental samples (Webster *et al.*, 1993; Egyed *et al.*, 2003 and Smith *et al.*, 2006) [22, 4, 23] and proven more specific and sensitive than conventional microscopy techniques.

Present study revealed that molecular method for detecting *Cryptosporidium* in clinical specimens have been shown to be more sensitive than conventional microscopy which is in congruence with the findings of several authors (Venu *et al.*, 2012, Chalmers and Katzer 2013; Yang *et al.*, 2013) [19, 20, 21].

The present study revealed that the diagnosis of *Cryptosporidium* oocysts could be considerably enhanced by staining the material obtained after concentrating the oocysts by sucrose flotation (Fujino *et al*., 2006) [15]. On assessment of time and cost involved for detection of cryptosporidiosis in faecal samples by conventional and molecular (nPCR) technique, it was found that the conventional mZN technique was far less time and money consuming than PCR which is in accordance to the findings of Morgan *et al.* (1998) [8] and Paul *et al.* (2009) [14]. Therefore, use of the fecal concentration followed by mZN staining technique is strongly advocated either as a supplement to PCR technique or as routine diagnostic technique in the absence of facilities for PCR assay. Although PCR was found to be time consuming and slightly expensive, it provided better diagnostic sensitivity and specificity in comparison to the conventional microscopic tests and also have the added advantage of differentiating among the different species of the organism. However, when large number of samples is processed, the cost and time involvement may be justified by the fact that on a single run all the samples could be diagnosed and per sample diagnosis cost could also get minimized. Nevertheless, in developing countries where the availability of PCR machines are restricted to research institutions, modified Ziehl-Neelsen technique is recommended to be used as a screening test for the diagnosis of bovine cryptosporidiosis in field conditions.

Conclusion

The present study describes the comparative sensitivity and specificity of conventional modified Ziehl-Neelsen technique and nested PCR vis a vis associated time and cost involvement on individual basis. This study may help in formulating a cost and time efficient standardized protocol for diagnosis of bovine cryptosporidiosis.

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Conflict of interest

We declare that we have no conflict of interest.

References

1. Singh BB, Sharma R, Kumar H, Banga HS, Aulakh RS, Gill JPS, *et al*. Prevalence of *Cryptosporidium parvum* infection in Punjab (India) and its association with diarrhoea in neonatal dairy calves. Veterinary Parasitology. 2006;140:162-165.
2. Mosier DA, Oberst RD. Cryptosporidiosis: a global challenge. Annals of the New York Academy of Sciences. 2000;916(1):102-111.
3. Lal A, Baker MG, Hales S, French NP. Potential effects of global environmental changes on cryptosporidiosis and giardiasis transmission. Trends in parasitology. 2013;29(2):83-90.
4. Egyed Z, Sreter T, Szell Z, Varga I. Characterization of *Cryptosporidium* spp.- recent development and future needs. Veterinary Parasitology. 2003;111:103-114.
5. Henricksen SA, Pohlenz JFL. Staining of cryptosporidia by a modified Ziehl-Neelsen technique. Acta Veterinaria Scandinavica. 1981;22:594-596.
6. Soave MPR. Three-step stool examination for cryptosporidiosis in homosexual men with protracted watery diarrhoea. J. Infect. Dis. 1983;147:824–828.
7. Fayer R. *Cryptosporidium*: A water-borne zoonotic parasite. Veterinary Parasitology. 2004;126:37–56.
8. Morgan UM, Pallant L, Dwyer D, Forbes DA, Rich G, Thompson RCA. Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal samples: clinical trial. Journal of Clinical Microbiology. 1998;36:995–998.
9. Balatbat AB, Jordan GW, Tang YJ, Silva Jr., J. Detection of *Cryptosporidium parvum* DNA in human feces by nested PCR. Journal of Clinical Microbiology. 1996;34:1769–1772.
10. Weber R, Bryan RT, Bishop HS. Wahlquist SP, Sullivan JJ, Juranek DD. Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: evidence of low sensitivity in current diagnostic methods. Journal of Clinical Microbiology, 1991, 1323–1327.
11. Barwick RS, Mohammed HO, White AB, Bryan RT. Detection of *Cryptosporidium parvum* and *Cryptosporidium muris* in soil samples. Biol. Fertile Soils. 2000;31:385–390.
12. Xiao L, Escalante L, Yang C, Sulaiman I, Escalante AA, Montali RJ, *et al*. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Applied and Environmental

Microbiology. 1999;65:1578–83.
13. Coupe S, Sarfati C, Hamane S, Derowin F. Detection of *Cryptosporidium* and identification to species level by nested PCR and restriction fragment length polymorphism. Journal of Clinical Microbiology. 2005;43:1017–1023.
14. Paul S, Chandra D, Tewari AK, Banerjee PS, Ray DD, Boral R, Rao JR. Comparative evaluation and economic assessment of coprological diagnostic methods and PCR for detection of *Cryptosporidium* spp. in bovines. Veterinary Parasitology. 2009;164:291–295.
15. Fujino T, Matsuo T, Okada M, Matsui T. Detection of a small number of *Cryptosporidium parvum* oocysts by sugar floatation and sugar centrifugation methods. Journal of veterinary medical science. 2006;68(11):1191-1193.
16. OIE. Cryptosporidiosis In: Terrestrial manual, 2008, pp:1192-1215.
17. Fayer R, Xiao L. *Cryptosporidium* and Cryptosporidiosis, second ed. CRC Press and IWA Publishing, Boca Raton, 2008.
18. Xiao L, Singh A, Limor J, Thaddeus K, Gradus S, Lal A. Molecular Characterization of *Cryptosporidium* Oocysts in Samples of Raw Surface Water and Wastewater, Applied and Environmental Microbiology. 2001;67:1097–1101.
19. Venu R, Latha BR, Basith SA, Raj GD, Sreekumar C. and Raman M. Molecular prevalence of *Cryptosporidium* spp. in dairy calves in Southern states of India. Veterinary Parasitology. 2012;188:19–24.
20. Chalmers RM, Katzer F. Looking for *Cryptosporidium*: the application of advances in detection and diagnosis. Trends in Parasitology. 2013;29:237–251.
21. Yang R, Murphy C, Song Y, Ng-Hublin J, Estcourt A, Hijjawi N, *et al*. Specific and quantitative detection and identification of *Cryptosporidium hominis* and *C. parvum* in clinical and environmental samples. Experimental Parasitology. 2013;135:142–147.
22. Webster KA, Pow JDE, Giles M, Catchpole J, Woodward MJ. Detection of *Cryptosporidium parvum* using a specific polymerase chain reaction. Veterinary Parasitology. 1993;50:35-44.
23. Smith HV, Caccio SM, Trait A, McLauchlin AJ, Thompson RC. Tools for investigating the environmental transmission of *Cryptosporidium* and *Giardia* infections in humans. Trends in Parasitoligy. 2006;22:160-167.
24. Laxer MA, Timblin BK, Patel RJ. DNA sequence for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. American Journal of Tropical Medicine and Hygiene. 1991;45:688-69.