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Sateesh AG
Ph.D Scholar, Department of Veterinary Medicine, Veterinary College, Bidar, Karnataka, India

Patil NA
Director of Extension, KVAFSU, Bidar, India

Vivek R Kasaralikar
Prof and Head, Department of VCC, Veterinary College, Bidar, Karnataka, India

Dilipkumar D
Dean, Veterinary College, Bidar, Karnataka, India

Basavaraj Awati
Prof and Head, Department of Microbiology, Veterinary College, Bidar, Karnataka, India

Ravindra BG
Associate Prof, Department of Veterinary Medicine, Veterinary College, Shimoga, Karnataka, India

Shrikant Kulkarni
Prof and Head, Department of Veterinary Physiology and Biochemistry, Veterinary College, Bidar, Karnataka, India

Corresponding Author
Sateesh AG
Ph.D scholar, Department of Veterinary Medicine, Veterinary College, Bidar, Karnataka, India

Study on diagnosis of bovine cryptosporidiosis

Sateesh AG, Patil NA, Vivek R Kasaralikar, Dilipkumar D, Basavaraj Awati, Ravindra BG and Shrikant Kulkarni

Abstract
Cryptosporidium protozoal infection had major impact on human and animal health implications causing severe gastroenteritis with episodes of diarrhea. A total of 50 diarrhoic calves were screened for the cryptosporidiosis at various farms in and around Bidar. The calves were aged 6 days to 6 months. The fecal samples collected were subjected to direct microscopical examinations, mZN staining and safranin staining technique and molecular diagnosis for confirmation. Direct microscopic examination revealed prevalence of 4.00 per cent under (1000x) oil immersion. Prevalence of 18.00 per cent and 16.00 per cent was noticed in mZN and safranin staining techniques respectively. The nested PCR study stated prevalence of 43.33 per cent positivity in 30 fecal samples. Out of 13 samples tested 7 were tested positive for *Cryptosporidium parvum* by immuno-chromatoghapy test based kits (DipFit^{TM}, BioX Diagnostics, Belgium). Haematological study reveled that there was no significant difference observed in values of total erythrocyte count and haemoglobin concentration when compared to healthy control calves. However, there was marginal significant (P<0.05) increase in the haematocrit values in study group when compared with healthy control groups indicating moderate dehydration. Total leucocyte count was significantly (P<0.05) increased in the affected calves when compared with healthy control calves due to secondary bacterial infections. In erythrocyte indices, the values of MCH and MCHC were significantly (P<0.05) decreased in study groups when compared to healthy control calves. Adoption of standardized techniques to identify the organism where possible, for diagnosis and typing would allow better correlations between animal, human, and environmental data at national and international levels as it has zoonotic implications.

Keywords: Cryptosporidiosis, Calves, Molecular techniques, mZN staining, Haematology

Introduction
The cryptosporidiosis is the major protozoal disease of public and animal health concern. Therefore, now it is being classified under the emerging diseases by OIE. Cryptosporidiosis is associated with diarrhoeal diseases in calves and leads to significant morbidity and mortality in animal husbandry and is of major concern in calf rearing and its management. The cryptosporidium protozoa belongs to phylum Apicomplexa, class Sporozoacida, sub Class Coccidia, order Eucoccidiorida, sub order Eimeriidae, and family Cryptosporidiae. It infects multiple species *Viz.*, humans, birds, cattle, sheep, goats, horses, dogs and cats *etc*. Some species are host specific and some affects multiple hosts (Nguyen *et al*., 2007) [8]. Cryptosporidiosis in cattle and humans is mainly caused by *C. parvum* whereas other species that only infect the cattle are *C. bovis*, *C. andersoni*, and *C. ryanae* (Diaz-lee *et al.*, 2011) [4]. Cryptosporidiosis drew attention in veterinary practice due to severe economic loss to farmer in terms of retarded growth, reduced production and difficult to control disease (Maurya *et al*., 2013) [7]. In immunocompetent animals the infection is self limiting whereas in immunocompromised animals the infection can be life threatening therefore younger calves have higher prevalence than adult animals (Xiao and Feng, 2008) [16]. The diagnostic trend of cryptosporidiosis was primarily done by microscopic examinations, followed by enzyme immunoassays and then confirmed by molecular techniques. The microscopic examination include acid-fast staining methods with or without concentration methods are commonly used then followed by immunofluorescence microscopy (Checkley *et al*., 2015) [2].

Materials and methods
A total of 50 diarrhoic calves were screened for the cryptosporidiosis at various farms in and around Bidar. The calves were aged 6 days to 6 months. The fecal samples were collected directly from the rectum in a sterile plastic screw capped sample collecting vials. The collected samples were packed properly with details of age, sex, place and date of collection.

The fecal samples were scored based on consistency and colour. The collected samples were taken to laboratory for further analysis. Direct smear examination was done by taking small amount of fecal sample on clean non greasy glass slide and a drop of distilled water was added to it and mixed well. All the coarse material and debris were taken out and then cover slip was placed on it. Slides were primarily observed under higher (400x) magnification and then observed under oil immersion (1000x) in a microscope.

Then the samples were subjected to modified ZN staining method as suggested by Fayer and Xiao (2008) [5] and safranin staining method. The half gram of dung sample was taken in a 2ml capped tube and 1.5 ml of distilled water was added to it. The samples were thoroughly mixed by vortexing and a thin smear was prepared on a glass slide. Further the staining procedure was carried out after fixing it with methanol. Then the slides were observed under 400x and 1000x under microscope for minimum of 200 microscopic fields. Samples were considered positive even if single oocyst was observed. Further the samples were subjected to molecular diagnosis by nested PCR. The genomic DNA samples were extracted from the suspected dung samples by using QIAamp® DNA stool mini kit according to the manufacturer's instructions. A two-step nested PCR protocol was followed to amplify at 834bp fragment of 18S rRNA gene of cryptosporidium *spp.* as described by Xiao *et al*. (2001) [18]. The suspected fecal samples were subjected to immuno-chromatography based test DipFit™, BioX Diagnostics, Belgium. Blood samples were collected from all the affected calves in two ml EDTA coated vials for the haematological study.

Table 1: Prevalence of cryptosporidiosis by different diagnostic methods

Prevalence	DS	mZN	SF	nPCR
Numbers screened	50	50	50	30
Positive	2	9	8	13
Per cent positive (%)	4	18	16	43.33

DS - Direct smear
mZN - Modified ZN staining method
SF - Safranin staining method
nPCR - Nested PCR

Results

The overall prevalence of cryptosporidiosis in Bidar area was 26.00 per cent in diarrhoic calves (Table-1). Direct microscopic examination revealed prevalence of 4.00 per cent under (1000x) oil immersion (Table-1). The microscopic examination revealed red/ magenta coloured spherical bodies with green background of malachite green in modified ZN staning method (Plate-1) whereas orange coloured round bodies were observed in safranin staining method (Plate-2) with the prevalence of 18.00 per cent and 16.00 per cent respectively. The nested PCR study stated prevalence of 43.33 per cent positivity in 30 samples (Table-1). The amplified products were subjected to gel electrophoresis and secondary amplicons were observed at 834bp for 13 samples against 100bp DNA ruler (Plate-3).

The suspected fecal samples were subjected to immuno-chromatography based test kits for detection of *Cryptosporidium parvum*. Out of 13 samples tested, 7 were positive for *Cryptosporidium parvum* (Plate-4). Haematological study reveled that there was no significant difference observed in values of total erythrocyte count and haemoglobin concentration when compared to healthy control calves (Table-2). However, there was significant ($P<0.05$) increase in the haematocrit values in study group when compared with healthy control groups. Total leucocyte count was significantly ($P<0.05$) increased in the affected calves when compared with healthy control calves (Table-2). In erythrocyte indices, the values of MCH and MCHC were significantly ($P<0.05$) decreased in study groups when compared to healthy control calves whereas MCV did not differ significantly (Table-2).

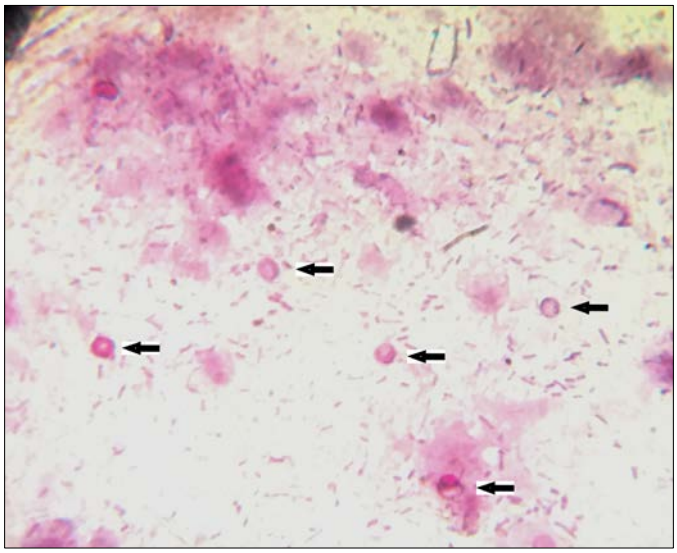


Plate 1: Megenta/red coloured round shaped cryptosporidium oocysts in mZN staining

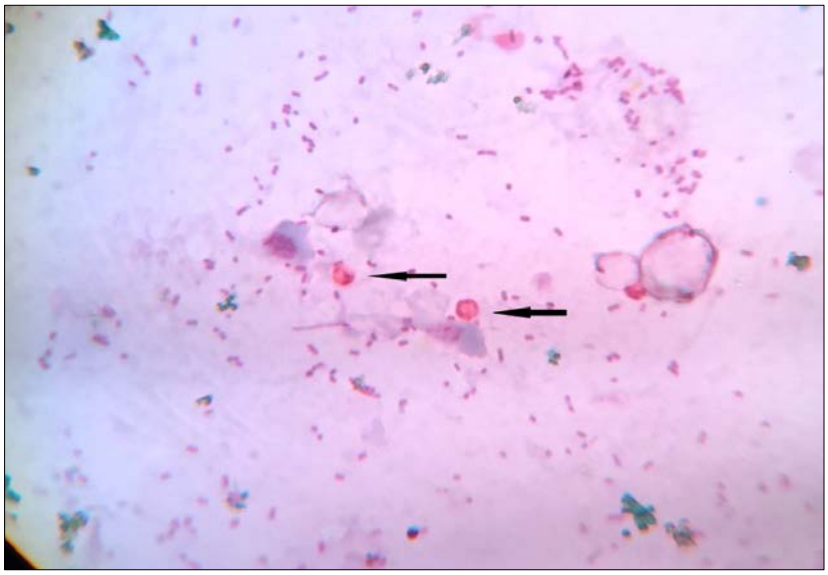
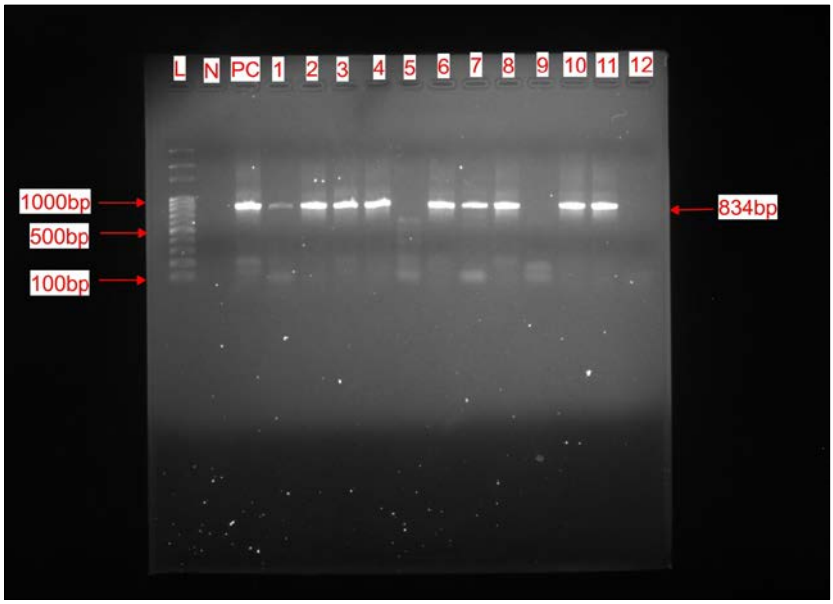


Plate 2: Red coloured cryptosporidial oocysts in safranin staining



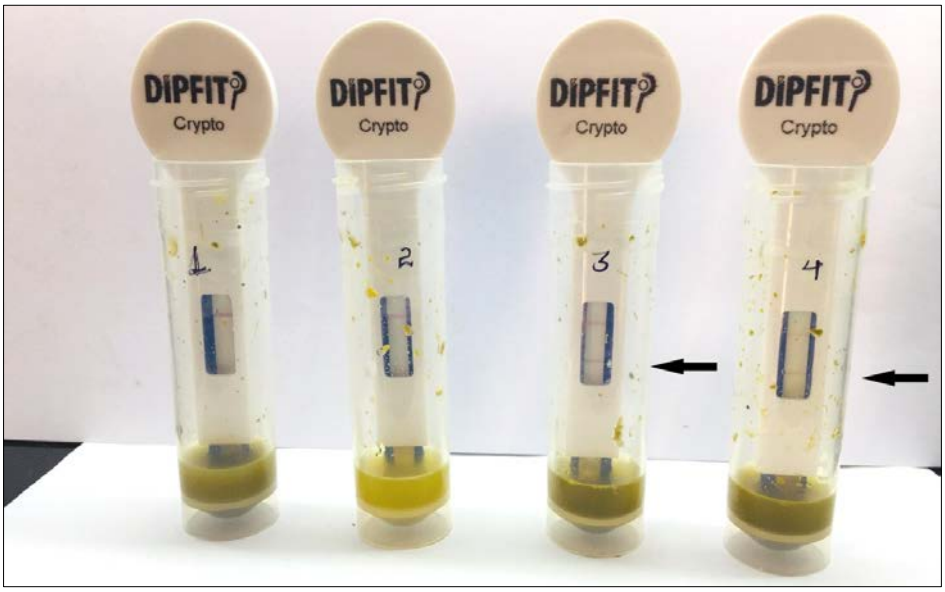
L: 100bp DNA Ladder
N: Negative control
PC: Positive control
Positive samples: 1, 2, 3, 4, 6, 7, 8, 10 & 11.
Negative samples: 5, 9 & 12.

Plate 3: Amplification of 834bp fragment of 18S rRNA gene of Cryptosporidium *spp*

Table 2: Haematoloical changes in calves affected with cryptosporidiosis when compared with healthy control calves

Parameter	Control group	Calves affected with cryptosporidiosis
RBC ($x10^6$)	9.25 ± 0.45^a	10.46 ± 0.50^a
Hb (g/dL)	11.37 ± 0.43^a	10.44 ± 0.51^a
PCV (%)	34.19 ± 1.17^a	39.31 ± 1.44^b
TLC ($x\ 10^3$)	9.62 ± 0.41^a	11.18 ± 0.56^b
MCV (fL)	39.11 ± 1.92^a	36.72 ± 1.66^a
MCH (pg)	13.08 ± 0.62^a	9.58 ± 0.65^b
MCHC (g/dL)	33.49 ± 0.59^a	26.02 ± 1.16^b
PLT ($x10^3$)	129.20 ± 5.17^a	140.62 ± 10.64^a

*Means bearing different superscript differ significantly



Positive samples: 3 & 4
Negative samples: 1 & 2

Plate 4: Immuno-chromatography based test DipFit™ to identify *Cryptosporidium parvum*

Discussion

In the present study, direct smear examination revealed 4.00 per cent infection and similar results were observed by Venu *et al.* (2012) [15]. However staining techniques had better sensitivity than direct smear examination *viz.*, modified Zeihl-Neelson technique and safranin methylene blue technique. Similar results were obtained by Rekha *et al.* (2011) [12], Chattopadhyay *et al*. (2000) [1]. Unlike conventional techniques for identification of oocysts, molecular methods have greater sensitivity and specificity. Therefore application of nested PCR was employed for the detection of cryptosporidium *spp*. by amplifying 18S rRNA gene. Many authors had used similar gene for amplification to identify the cryptosporidium *spp.* (Xiao *et al*., 1999 and Santin *et al*., 2008) [19, 13]. The cryptosporidium DNA was detected in 43.33 per cent (13/30) of fecal samples and results were in accordance with Paul *et al*. (2009) and Venu *et al*. (2012) [10, 15]. The ELISA based chromatography test kits had 100 per cent sensitivity and 94 per cent specificity to identify C*ryptosporidium parvum* (Fleece *et al*., 2016) [6]. However in the present study the test kits detected 7 positive samples that specify the *C.parvum* infection. Haematology revealed marginal increase in concentration of haematocrit values in study group when compared to healthy control calves. The haemoglobin and total erythrocyte count were insignificant in study group however mild fluctuations were observed. The results were in accordance with Darabus *et al.* (2009) [3], Rekha *et al*. (2011) [12] and Randhawa *et al.* (2012) [11]. The increase in the haematocrit values are suggestive of mild to moderate dehydration in affected calves (Darabus *et al*., 2009) [3]. There was significant increase in total leucocytes count in study group and can be attributed to immunogenic response to secondary bacterial infection due to damage to microvilli (Rekha *et al*., 2011) [12]. The decrease in the MCH and MCHC values might be due to reduced haemoglobin concentrations and were in accordance with O'Donoghue (1995) [9], Rekha *et al*. (2011) [12] and Thakre *et al.* (2015) [14].

Conclusion

Cryptosporidiosis currently pose a challenge to buiatricians to indentify the infection at optimal and economical way. By implementation of different diagnostic methods to identify the Cryptosporidium *spp* was carried out. However, mZN staining technique was most effective and economical technique at field level diagnosis. The immuno-chromatography based diagnosis by use of lateral flow tests are also a better modality. The nPCR technique was having highest sensitivity and specificity among all other aids for used diagnosis. Direct application of next-generation sequencing to faecal samples would allow the detection of multiple pathogens and their variants based directly on the presence of their DNA. Adoption of standardized techniques to identify the organism where possible, for diagnosis and typing would allow better correlations between animal, human, and environmental data at national and international levels as it has zoonotic implications.

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