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Study on prevalence and risk factors of bovine cryptosporidiosis

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Abstract
The present study was undertaken to evaluate the prevalence, and assessment of risk factors associated with cryptosporidiosis in 205 calves. The selected calves were aged between 0-12 months and signalment data and clinical signs of calves were recorded along with epidemiological factors. Fecal samples examined by mZN staining method, revealed a overall prevalence of 14.63 per cent. Age-wise point prevalence of 60.00 per cent indicated that highest susceptible calves were younger age group however, there was decline in the positivity percentage as age advances. Seasonally, highest point prevalence was noticed in the rainy (50.00%) season than winter and summer. Sex-wise prevalence revealed, female calves had higher prevalence than male calves however the difference was statistically nonsignificant. Most of the diarrhoeic calves had watery consistency (Grade-3, 70.00%) among the affected calves. The organised dairy calves had higher point prevalence (70.00%) when compared unorganised dairy farms (30.00%). Diagnosis of cryptosporidiosis was done by microscopic examinations in present study. Direct smear examination revealed 5.37 per cent positivity (11/205) for *Cryptosporidium* oocysts. Thirty (14.63%) fecal samples were positive for cryptosporidiosis by modified Ziehl-Neelsen (mZN) staining method whereas, safranin methylene blue staining method detected 29 (13.66%) positive samples. The most reliable and cost-effective method for detection of *Cryptosporidium* oocysts was modified ZN staining method than any other staining method.

Keywords: Prevalence, risk factors, bovine cryptosporidiosis

Introduction
Cryptosporidiosis in calves is an alarming disease which causes diarrhoea, retarded growth and mortality. 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) [21]. 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) [7]. The cryptosporidium parasite is very difficult to eliminate from the micro and macro climate of the animal due to thicker outer shell of oocysts, higher resistance environmental temperatures and commonly used disinfectants (Casemore, 1990). The ability of cryptosporidium oocyts to break through multiple water treatment barriers and cause large scale out breaks in different parts of world therefore WHO recommended it as a reference pathogen to drinking water quality (Medema, 2009). The prevalence of cryptosporidiosis in India had been documented by many authors along with the prevailing factors associated with the disease such as age, sex, type of housing and climatic changes. The diagnosis of cryptosporidiosis poses a challenge to clinician due to smaller size and involvement of contaminants in fecal samples. The microscopic examinations are reliable diagnostic tool with less sensitivity however they are highly cost effective in diagnosis of cryptosporidiosis.

Materials and Methods
In the present study 205 calves were randomly selected from various organized and unorganized dairy farms. The selected calves were aged between 0-12 months and signalment data of calves were recorded along with epidemiological factors *Viz.*, age, sex, season, organized and unorganized dairy farms. The fecal samples were collected in plastic screw capped vials directly from the rectum in diarrhoeic calves. Then the samples were labeled properly with details animals, place and date of collection. The samples were subjected to direct microscopic examinations *viz.*, direct fecal smear, modified ZN staining technique and safranin methylene blue staining technique.

Half gram or 0.50 ml of the fecal sample was transferred into a 2ml microcentrifuge tube and 1.5ml of distilled water added to it and vortexed. To prepare a thin fecal smear for direct smear examination, around 2-3 drops of prepared fecal sample mixture was taken on clean non-greasy glass slide and coarse particle were removed with the help of a tooth pick. A cover slip was placed on the glass slide to make a thin smear. Then smear was observed under high power (400x) initially and then in oil immersion (1000x) in 200 different fields under microscope to identify the *Cryptosporidium* organisms.

The modified ZN staining method was done by making thin smear from vortexed sample and air dried. Then the prepared slides were stained by modified ZN method as per the descriptions of Venu *et al*. (2013) [32] to identify the *Cryptosporidium* spp. the smear was fixed primarily by methanol for 3 minutes then flooded with cold strong carbol-fuschin stain for 15 minutes. The slide was then washed with running water and decolouriser was added for 10-15 seconds to remove excess stain. Then the slide was counter stained with malachite green for 1 minute. Then the slide was washed, air dried and observed under microscope for minimum 200 different fields at high power (400x) and on oil immersion (1000x). Similarly the safranin methylene blue staining method was done as per stated by Baxby *et al*. (1984) on the thin smeared slides. The slide was fixed with acid alcohol for 3-5 minutes then washed and flooded with 1.00 per cent safranin solution for 60 seconds with gentle heat. Then the smear was counter stained with methylene blue for 30 seconds and dried. The slides were observed under microscope at high power (400x) and on oil immersion (1000x) to identify the *cryptosporidium* oocysts.

Ethical statement

The present study was undertaken with the approval of IAEC Reg. No: 13/20-21/VCB/VMD for the conduction of experiment on the calves affected with cryptosporidiosis.

Results

The overall prevalence of calves affected with cryptosporidiosis in diarrhoeic calves in present study was 14.63 per cent (30/205). Among the positive calves affected with cryptosporidiosis, the point prevalence was 60.00 per cent (18/30), 23.33 per cent (7/30), 13.33 per cent (4/30) and 3.33 per cent (1/30) in <1 month old calves, 1-2 month old, 2-3 month old and 3-6 months old calves respectively (Table-1). There was decline in per cent positivity was noticed as the age advances and no cases found to be positive in 6-12 month age group. the point prevalence of cryptosporidiosis in different seasons were found to be 50.00 per cent (15/30), 30.00 per cent (09/30) and 20.00 per cent (06/30) in rainy, winter and summer season respectively (Table-2). The prevalence of cryptosporidiosis was highest in rainy season when compared to winter and summer season.

The point prevalence of cryptosporidiosis in male calves were 36.67 per cent (11/30) whereas point prevalence in female calves were 63.33 per cent (19/30) (Table-3). There was no significant difference observed between either sexes when subjected to chi square analysis. The fecal samples were graded based on consistency *viz*., grade-1, grade-2 and grade-3 and their point prevalence were 3.33 per cent (01/30), 26.67 per cent (08/30) and 70.00 per cent (21/30) respectively (Table-4). Statistically there was no significant difference noticed between fecal scoring grades. However, highest numbers of fecal samples were having watery consistency (severe diarrhoea) and least were having patty consistency (mild diarrhoea). The farm-wise point prevalence of cryptosporidiosis in organised dairy sector was 70.00 per cent (21/30) whereas in unorganised dairy sector was 30.00 per cent (9/30) (Table-5). The chi-square analysis of farm-wise prevalence did not show significant association between organised and unorganised dairy farms in propagating crytposporidiosis however, the prevalence was higher in organised dairy farms.

Direct smear examination revealed 5.37 per cent positivity (11/205) for *Cryptosporidium* oocysts which were round refrectile bodies (Plate-1). Amongst 205 fecal samples 30 (14.63%) fecal samples were found to be positive for cryptosporidiosis by modified Ziehl-Neelsen (mZN) staining method whereas, safranin methylene blue staining method detected 28 (13.66%) positive samples of 205 fecal samples. In mZN staining method, the *Cryptosporidium* oocysts were red/megenta coloured spherical shaped with light green background (Plate-2) whereas safranin methyline blue staining method in which *Cryptosporidium* oocysts were red coloured spherical shaped bodies with blue background (Plate-3).

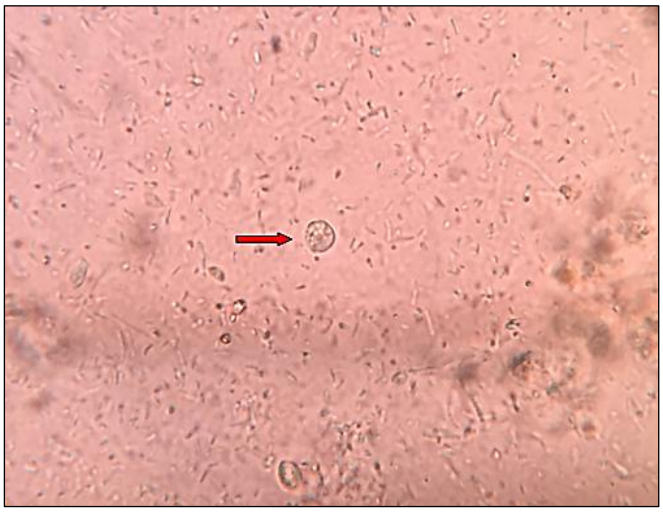

Plate 1: Refrectile round shaped *Cryptosporidium* oocysts in direct smear at 1000x

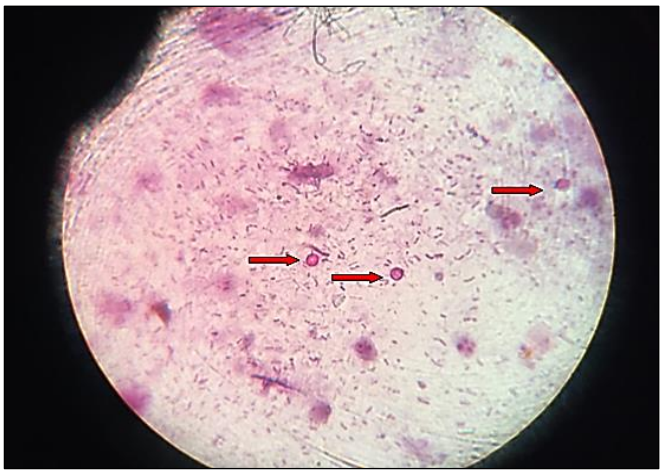

Plate 2: Megenta/red coloured round shaped *Cryptosporidium* oocysts against blue background in mZN staining in 1000x

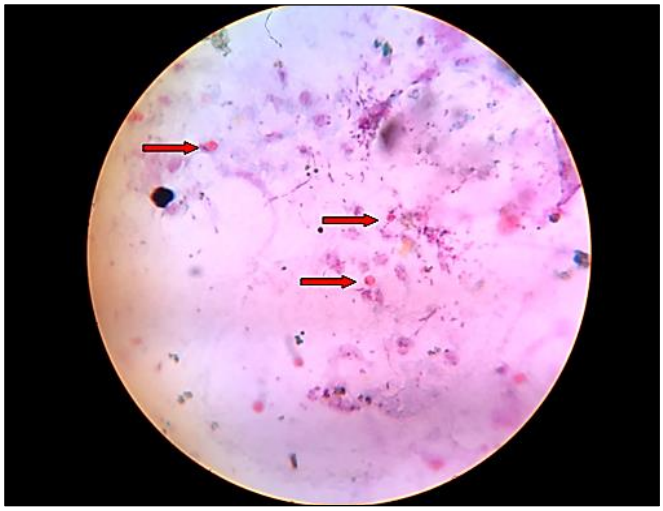

Plate 3: Megenta/red coloured round shaped *Cryptosporidium* oocysts against blue background in safranin methylene blue staining in 1000x

Table 1: Age-wise prevalence of cryptosporidiosis in calves

Age	Number of samples screened	Number of positive samples	Per cent positivity (%)	Point prevalence (%)
<1 month	71	18	25.35	60.00 (18/30)
1-2	42	7	16.67	23.33 (7/30)
2-3	26	4	15.38	13.33 (4/30)
3-6	47	1	2.13	3.33 (1/30)
6-12	19	0	0	0
Total	205	30	14.63	100.00

χ^2 value-10.983^{S}

Table 2: Season-wise prevalence of cryptosporidiosis in calves

Season	Number of samples screened	Number of positive samples	Per cent positivity (%)	Point prevalence (%)
Rainy	78	15	19.23	50.00 (15/30)
Winter	56	9	16.07	30.00 (9/30)
Summer	71	6	8.45	20.00 (6/30)
Total	205	30	14.63	100.00

χ^2 value-2.708^{NS}

Table 3: Sex-wise prevalence of cryptosporidiosis in calves

Sex	Number of samples screened	Number of positive samples	Per cent positivity (%)	Point prevalence (%)
Male	88	11	12.50	36.67 (11/30)
Female	117	19	16.24	63.33 (19/30)
Total	205	30	14.63	100.00

χ^2 value-0.421^{NS}

Table 4: Prevalence of cryptosporidiosis in calves based on fecal scoring

Fecal scoring	Number of samples screened	Number of positive samples	Per cent positivity (%)	Point prevalence (%)
Grade-1	29	1	3.45	3.33 (1/30)
Grade-2	68	8	11.76	26.67 (8/30)
Grade-3	108	21	19.44	70.00 (21/30)
Total	205	30	14.63	100.00

χ^2 value-4.17 ^{NS}

Table 5: Farm-wise prevalence of cryptosporidiosis in calves

Farm sector		Number of calves examined	Positive	Per cent positivity (%)	Point prevalence (%)
Organised	11	107	21	19.63	70.00 (21/30)
Unorganised	17	98	9	9.18	30.00 (9/30)
Total		205	30	14.63	100.00

χ^2 value-3.347^{NS}

Discussion

In the present study, majority of the cases detected in younger calves (<1 month old) followed by 1-2 month old calves and then there was decline in the positive cases as the age advances. However, no cases were detected in 6-12 month age calves. Similar results were obtained by Santin *et al.* (2004) [27], Shobhamani *et al.* (2005) [28], Kashyap *et al.* (2019) [15] and Dankwa *et al.* (2021) [6] and all the authors reported higher prevalence in younger calves. Age of the calves is an important factor that prevails for pathogenicity of cryptosporidiosis (Xiao and Ryan, 2004) [34]. The site of infection with *C. paruvm* is on the enterocyte where it results in cell damage, loss of brush borders enzymes and reduction in villous surface area. Immunologically compromised patients are more susceptible to clinical disease however low absorptive efficiency of IgG concentrations due to damage to villi and low serum IgG concentration had higher prevalence of *Cryptosporidium* oocysts shedding in neonatal calves (Lopez *et al*., 1988) [17]. *Cryptosporidium* induce both cell mediated and humoral antibody immunity therefore the adult animals are least susceptible and devoid of clinical disease. Seasonal prevalence of cryptosporidiosis in calves was highest in rainy season as compared to winter and summer season. The present observations were in accordance with Joute *et al.* (2016) [14], Singh *et al.* (2018) [29], Razakandrainibe *et al.* (2018) [23] and Kashyap *et al.* (2019) [15]. The prevalence of cryptosporidiosis was higher in rainy season in the present study and can be attributed to overcrowding of calves in small animal shelter (Swain *et al*., 2018) [31] and fecal contamination of water in the rainy season along with oocysts survival of cryptosporidiosis. On contrary to present study, Garber *et al.* (1994) [9] and Mohanty and Panda (2012) [20] reported higher prevalence in summer than in rainy season which can be attributed to hot and humid climates in turn propagating factors of cryptosporidiosis. Sex-wise prevalence of cryptosporidiosis in calves was highest in female than in males in the present study and the results were insignificant. The present observations were in agreement with Maurya *et al.* (2013) [19], Singh *et al.* (2018) [29] and Dankwa *et al.* (2021) [6] in which they found higher prevalence in females than in males. In the present study, higher number female calves were screened than male calves therefore the prevalence was lower in male calves. However, sex-wise prevalence did not indicate any influence on occurrence of cryptosporidiosis (Fayer and Santin, 2009) [8]. In the present study, fecal scoring was determined and concluded that most of the positive samples were having watery consistency and least calves had patty consistency in the affected calves. Prevalence was higher in diarrhoeic calves and most of them were having watery consistency was reported by Sivajothi *et al.* (2014) [30] and Swain *et al.* (2018) [31] and Singh *et al.* (2018) [29]. Cryptosporidiosis in young

calves leads to villous atrophy after infection and leads to reduction in the total surface area of small intestinal mucosa reducing absorptive ability and increase intestinal permeability therefore diarrhoea is most evident in cryptosporidiosis affected calves (Wyatt *et al*., 2010) [33] which substantiate the fecal scoring results in present study. On contrary to present study, Dankwa *et al*. (2021) [6] reported higher prevalence in non-diarrhoeic calves than diarrhoeic calves. Farm-wise study revealed highest prevalence in organized dairy sector than in unorganized dairy farms and were statistically insignificant. The findings of the present study were in accordance with Irshad *et al*. (2015) [13], Mallinath *et al*. (2009) [18] and Graef *et al*. (2018) [10]. Higher prevalence in organized dairy farms might be due to confinement crowding of calves at a place (Graef *et al*., 2018) [10] and also contamination through water and soil in a confined area can cause outbreaks of cryptosporidiosis very quickly (Caccio *et al*., 2001) [4]. On contrary, Almeida *et al*. (2010) [1] reported no correlation of housing system in assessing the risk for cryptosporidiosis.

Direct smear examination revealed 5.37 per cent prevalence of cryptosporidiosis in the present study, which states low prevalence when compared to other diagnostic techniques employed in the study. The present findings were in agreement with Venu *et al*. (2013) [32], Hingole *et al*. (2017) [12] and Rekha *et al*. (2016) [24]. Direct microscopic smear examination were having least sensitivity for cryptosporidiosis then concentration methods and staining techniques Venu *et al*. (2013) [32]. Due to least sensitivity of the test method led to low prevalence rate with this method. In the present study, modified Zeihl-neelsen staining technique was employed and detected a prevalence of 14.63 per cent whereas safranin methyline blue staining can be used and however, direct fecal smear examination in the present study revealed low prevalence. The present observations are in close confirmation with Randhawa *et al*. (2012) [22], Bhat *et al*. (2013a) [3], Rekha *et al*. (2016) [24] and Aydogdu *et al*. (2018) [2]. Higher prevalence of cryptosporidiosis by mZN staining method were reported by Nguyen *et al*. (2007) [21] and Sahu *et al*. (2010) [26] whereas lower prevalence was mentioned by Kumar *et al*. (2004) [16] and Swain *et al*. (2018) [31]. The mZN staining technique is a rapid, inexpensive and routine diagnostic tool in which oocysts stain red in colour against pale blue/green background (Henriksen and Pohlenz, 1981) [11]. Amongst staining methods modified Ziehl-Neelsen method and Kinyoun's staining method were equally effective in identifying *Cryptosporidium* oocysts (Rekha *et al*., 2016) [24]. The prevalence of 13.66 per cent was observed by safranin methylene blue staining method in the present study. The results were in concurrent with Reynolds *et al*. (1986) [25] and Rekha *et al*. (2016) [24] in which the oocysts were red coloured spherical shaped with light green background. Rekha *et al*. (2016) [24] stated that safranin methylene blue staining method was more reliable and it would differentiate yeast present in samples.

Conclusion

The overall prevalence of cryptosporidiosis in calves was found to be 14.63 per cent in the present study. Predisposing factors such as younger age group, rainy season and organized dairy sectors were concluded to influence on the propagation of cryptosporidiosis infection in calves. However, it did not show any sex-wise variation. Diagnosis by mZN staining method was most reliable and cost effective amongst all other microscopic examinations conducted in the study.

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