Periurban outbreaks of bovine calf scours in Northern India caused by *Cryptosporidium* in association with other enteropathogens

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SUMMARY

Bovine calf scours reported to be caused by multiple aetiologies resulting in heavy mortality in unweaned calves and huge economic loss to the dairy farmers. Among these, cryptosporidiosis is an emerging waterborne zoonoses and one of the important causes of neonatal calf diarrhoea. Poor immune response coupled with primary cryptosporidial infections predispose neonatal calves to multiple secondary infections resulting in their deaths. In the present study, faecal samples from 100 diarrhoeic calves randomly picked up out of 17 outbreaks of bovine calf diarrhoea in periurban Ludhiana, Punjab in Northern India were subjected to conventional (microscopy, modified Zeihl–Neelsen (mZN) staining) and immunological and molecular techniques (faecal antigen capture ELISA and PCR) for detection of primary *Cryptosporidium parvum* infection as well as other frequently reported concurrent pathogens, *viz.* rotavirus and coronavirus, *Salmonella* spp., *Escherichia coli*, *Clostridium perfringens* and *Eimeria* spp. The faecal antigen capture ELISA and PCR revealed 35% prevalence of *C. parvum* in contrast to 25% by mZN staining with a relatively higher prevalence (66·7%) in younger (8–14-day-old) calves. The detection rate of the other enteropathogens associated with *C. parvum* was 45·71% for *C. perfringens* followed by *Salmonella* spp (40·0%), rotavirus (36·0%), coronavirus (16·0%), *E. coli* (12·0%) and *Eimeria* spp (4·0%) The sensitivity for detection of *C. parvum* by ELISA and mZN staining in comparison to PCR was 97·14% and 72·72%, respectively. An important finding of the study was that *C. parvum* alone was found in only 10% of the diarrhoeic faecal samples, whereas, majority of the samples (90%) showed mixed infections ranging from a combination of two to five agents. This is the first documentary proof of *C. parvum* and associated pathogens responsible for severe periurban outbreaks of bovine calf diarrhoea culminating in heavy mortality from Northern India.

Key words: Bovine calf diarrhoea, concurrent infections, *Cryptosporidium parvum*, ELISA, histopathology, PCR, periurban outbreaks.

INTRODUCTION

Diarrhoea, a common clinical presentation in bovine calves has a multifactorial aetiology [1], including *Cryptosporidium* and *Eimeria* spp., rotavirus, coronavirus, enteropathogenic *Escherichia coli* and

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Salmonella spp. [2]. The neonatal diarrhoeic syndrome caused by *Cryptosporidium parvum* is usually observed in 5–35-day-old claves with maximum incidence in the second week of life [3], resulting in enormous direct and indirect economic loss to dairy industry [4–6]. In addition to bovine calves, it is known to infect humans, domestic and wild animals, and birds [7–11]. The cryptosporidia, highly resistant to harsh environmental conditions, transmitted by faeco-oral route, have attained an increased zoonotic importance posing a great risk to dairy workers, milk handlers and general public through water contamination. The disease has particularly generated a great public health interest after a large human waterborne outbreak in Milwaukee in 1993 [11, 12].

Difficulties in the clinical diagnosis of infectious diarrhoea arise from frequent non-specific clinical signs and lesions, the presence of asymptomatic infections, the involvement of multiple agents, and the interplay of intrinsic and extrinsic factors that predispose the host to enteric dysfuction and pathology [13]. Traditionally, the infectious agents of diarrhoea have been identified using conventional techniques [14, 15]. However, these techniques are labour intensive, time consuming and less sensitive; therefore, advanced diagnostic methods may prove more efficacious in determining the precise aetiology of calf diarrhoea complex and thereby helpful in planning strategies for better herd management, disease surveillance and control programmes.

In India, extensive research work has been conducted on bovine calf diarrhoea with majority of infectious aetiological agents incriminated using conventional methods. Studies on *Cryptosporidium* in the country have mainly focused on the morphological identification of oocysts in faeces [5, 7, 8, 11, 16–20] with little emphasis on immunomolecular methods [21–24]. These previous investigations were concentrated on isolated reports of cryptosporidia alone without an insight into multiple aetiologies. Even globally, very few studies have been conducted on *Cryptosporidium*-associated complex aetiology of bovine calf diarrhoea outbreaks [6, 25]. In fact very few comprehensive investigations ellucidating the most recognised agents, *viz.* protozoa (*C. parvum*, *Eimeria* spp.) viruses (rota and corona) and bacteria (*E. coli*, *Salmonella* spp., *Clostridium perfringens*) have been attempted [26–30]. Moreover, a comparative evaluation of multiple aetiological factors by traditional, immunological and molecular methods with a pursuit to determine their relative sensitivity using faecal samples is lacking. The present study deals with 17 periurban outbreaks of bovine calf diarrhoea investigated thoroughly comparing faecal examination, faecal ELISA and faecal PCR (most trusted methods) from 100 cases with 43% mortality.

MATERIALS AND METHODS

Study area and sample collection

The study was conducted in Ludhiana, located between *north latitude* 30°34′ and 31°01′ and *east longitude* 75° 18′ and 76°20′. It is most populated and centrally located district of Punjab (India) with a huge periurban dairy animal population. The representative faecal samples were collected in sterile plastic bags directly from the rectum of 100 calves covering 17 outbreaks (four to six animals each) of diarrhoea from the organised dairy farms located in the periurban areas consisting of about 300 organised dairy farms in a radius of 25 km. To determine the exact age-wise distribution of aetiological factors of neonatal bovine diarrhoea, the animals were divided into five subgroups, i.e. 1–7 days (group I), 8–14 days (group II), 15–21 days (group III), 22–30 days (group IV) and 31–60 days (group V) of age. Faecal samples were stored at −20 °C for ELISA and PCR.

The study was conducted after approval of the Institutional Animal Ethical Committee and consent of the dairy farmers was taken for collection of samples.

Detection of *Cryptosporidium*

Microscopic examination of faecal smears was conducted for bacteria and cryptosporidia by employing Leishman's and modified Zeihl–Neelsen's (mZN) stains [31].

Detection of infectious agents by ELISA

All the faecal samples collected from 100 neonatal bovine calves during scour outbreaks were subjected to faecal ELISA by using the protocol of commercial ELISA kits for *C. parvum*, rotavirus, coronavirus and *E. coli* (K99) (Bio-X Easy-Digest, Bio K 151; Bio-X Diagnostics, Belgique) and *C. perfringens* (type C) (BIO K 269; Bio-X Diagnostics, Belgique).

DNA extraction

DNA of target agents *C. parvum*, *E. coli*, *Salmonella*, *Clostridium* were extracted from faecal specimens

using Nucleo-pore[R] Stool DNA Mini kit (HiMedia, Mumbai, India) as per the manufacturer's instructions.

RNA extraction

RNA from all the faecal samples for detection of coronaviruses was extracted using RNASure® Mini kit (Genetix, India; Table 1).

Confirmation of infectious agent by PCR

PCR was employed for *Cryptosporidium*, coronavirus, *Salmonella*, *E coli* and *C. perfringens* individually. The details of primers used and amplification conditions are given in Table 1. PCR reaction mixture 25 µl was constituted by adding 2·5 µl of 10× PCR buffer (HiMedia, Mumbai, India), 0·75 µl of 50 mM MgCl$_2$, 2 µl of template DNA, 1 µl of 20 pmol/µl of forward and reverse primer each, 0·2 µl of Taq DNA polymerase 5 U/µl and rest with nuclease-free water. Amplified PCR products were separated by gel electrophoresis using 1% agarose and visualisation of the product was carried out using UV transilluminator (Alpha Imager, San Jose, California, USA).

First strand cDNA synthesis

For the first strand cDNA synthesis from the RNA sample, the kit (Fermantas, Thermo Fisher Scientific, Waltham, MA, USA) was used, and the reaction was set up as per the manufacturer's instructions. In brief, the following reagents were added into a sterile nuclease-free tube on ice total RNA (0·1–5 µg), random hexamer primer (1 µl), nuclease-free water to make volume 11 µl. Subsequently, 5× reaction buffer (4 µl), Ribolock RNAase inhibitor (20 units/µl) 1 µl, 10 mM dNTP mix 2 µl, M-MuLV Reverse Transcriptase (20 units/µl), 2 µl was added and volume was made upto 20 µl with nuclease-free water. The above contents were mixed gently and centrifuged and incubated for 5 min at 25 °C, followed by 60 min at 37 °C, and finally the reaction was terminated by heating at 70 °C for 5 min. The reverse transcription reaction product was directly used in PCR.

Mortality analysis

During the investigation of 17 outbreaks in question, 43 claves died within 1 weak after sample collection, and the tissues from representative animals were collected for histopathology.

Table 1. *Primers used for PCR*

Sr. no.	Agent	Primer	Sequence (5′–3′)	PCR conditions	Product size (base pair)	Reference
1	*Bovine coronavirus (N)*	BCoVF BCoVR	CCGATCAGTCCGACCAATC AGAATGTCAGCCGGGGTAT	Initial denaturation 95 °C (5 min), 40 cycles of denaturation 94 °C (30 s), annealing 55 °C (1 min), elongation 72 °C (1 min) and final elongation at 72 °C (7 min)	406	Tsunemitsu *et al.* (1999) [60]
2	*Escherichia coli K99+ (K99)*	K99F K99R	GCGACTACCAATGCTTCTGCGAATAC GAACCAGACCAGTCAATACGAGCA		230	Cho *et al.* (2010) [25]
3	*Salmonella (16SrDNA)*	16 SF 16SR	TGTTGTGGTTAATAACCGCA CACAAATCCATCTCTGGA		575	Lin *et al.* (1996) [61]
4	*Cryptosporidium (COWP)*	BB-3F BB-4R	GCGAAGATGACCTTTTGATTTG AGGATTTCTTCTTCTGAGGTTCC		194	Balatbat *et al.* (1996) [48]
5	*Clostridium perfringens*	Clostri. F Clostri. R	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	Initial denaturation 94 °C (5 min), 35 cycles of denaturation 94 °C (1 min), annealing 53 °C (1 min), elongation 72 °C (1 min) and final elongation at 72 °C (7 min)	279	Yoo *et al.* (1997) [62]

Statistical analysis

The data were statistically analysed by χ^2 test using SPSS 16.0 software.

Sensitivity of PCR in relation to ELISA, mZ–N stain and Leishman stain for detection of *Cryptosporidium* was done following the formulae of Perrin and Sureau [32].

RESULTS AND DISCUSSION

Detection of *Cryptosporidium* by conventional and immunological and molecular methods

Microscopic examination of the faecal smears stained with Leishman's stain showed cryptosporidial and eimerial oocysts, besides other faecal inclusions, *viz.* cocci, thick and thin bacilli, stumpy bacilli/coccobacilli, squamous epithelial cells, intestinal epithelial cells, cell debris, undigested plant fibres(s), red blood cells (RBCs), fungus/yeast, bile pigment, leucocytes and undigested (Table 3). The Leishman-stained smears proved highly useful in preliminary screening of faecal smears for the presence or absence of coocidia, particularly cryptosporidia. Morphometric analysis of oocysts of *Cryptosporidium* ($n = 10$; 10 field each sample) in Leishman ($4\cdot24 \pm 0\cdot28$ μm) and mZN-stained ($4\cdot26 \pm 0\cdot29$ μm) faecal smears revealed non-significant ($0\cdot16$ NS) variation in the size. In the Romanowsky-stained faecal smears, the cryptosporidial oocysts were seen as hollow round bodies and with a size compatible with that recorded in this study and documented earlier by mZN staining [33] and the coocidian oocysts were larger hollows and oval (Fig.1a, b); but on mZN staining, cryptosporidial oocysts were seen as bright red, oval to spherical bodies in 25% cases (Fig. 1c; Table 2). Both the faecal antigen-ELISA and PCR showed an improved 35% prevalence of *Cryptosporidium* in diarrhoeic calves. In fact, direct visualisation by light microscopy of cryptosporidia in faeces or intestinal contents as well as the detection of its antigens (e.g. Ag-ELISA) or nucleic acids (e.g. PCR) in specimens have been widely accepted as alternative diagnostic methods [9, 34].

The age-wise prevalence of *Cryptosporidium* by PCR and antigen detection ELISA showed non-significant variation with highest rate of 66·6% in 8–14 days group calves (Table 2). The maximal prevalence of *Cryptosporidium* was recoded in the 2-week-old diarrhoeic bovine calves. Similar prevalence of cryptosporidial infection in neonatal diarrhoeic calves with a gradual reduction with age has been reported

previously from various regions of India [5, 21, 22, 35] and across the globe [27, 36–39]. In the present study, all calves positive by faecal ELISA were also positive for the *Cryptosporidium* by PCR, except one. The sensitivity of ELISA and mZN staining with respect to PCR was 97·14% and 72·72%, respectively (Table 4). Comparable results regarding sensitivity between ELISA and PCR, matching those of ours, were previously recorded by other workers [25, 40] irrespective of the variations in the type of reagents and kits used. Settawy and Fathy [41] observed high detection rate by PCR (24·4%) and low by microscopy (18·6%), while it was 20·9% by ELISA. Their data showing higher sensitivity of PCR compared with ELISA are contradictory to our results showing equivalent positivity. In the differentiation of the aetiology of the diarrhoea in neonatal calves, PCR is being increasingly employed for the detection of *Cryptosporidium* during the last two decades mainly because of its accuracy, specificity and sensitivity and it has made significant contributions in understanding the epidemiology of *Cryptosporidium* infection [42–46]. Moreover PCR has added advantages such as ease of use, ability to analyse large numbers of samples simultaneously, relatively low cost and ability to speciate (eliminating false positives and cross-reactions of antibodies to non-pathogenic species) [45]. Though nested PCR involving a second round of amplifications has been applied to *Cryptosporidium* to increase specificity and sensitivity [35, 47, 48], yet it has not been generally recommended for use in diagnostic laboratories owing to a major risk of contamination with PCR products [45, 49].

Detection of *Cryptosporidium*-associated enteropathogens

The progression of diarrhoea with multifactorial aetiology is rapid. Hence, a quick diagnosis is critical for not only rapidly confirming the causes but also helping clinicians and cattle producers to implement appropriate interventions in a timely manner. Antigen capture ELISAs are well known for rapid turnaround, high-throughput testing and portability [50]; whereas, PCR is especially useful for detecting viruses that are difficult to isolate in cell culture or bacteria that require a long time to grow [51]. Therefore, all the samples tested positive for other enteropathogens either/or by faecal antigen capture
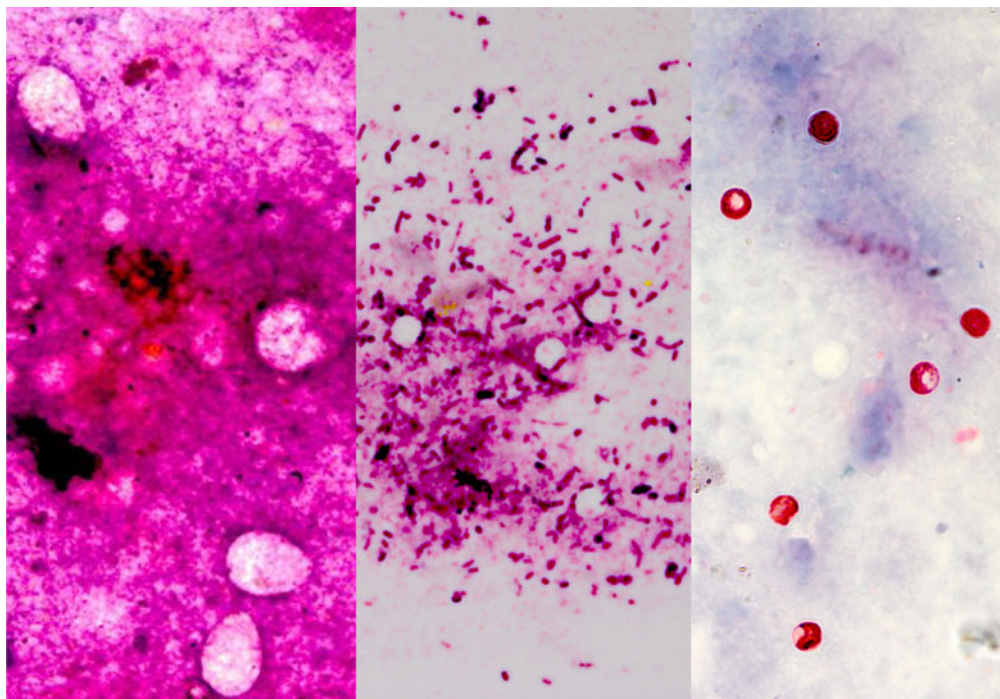
Fig. 1. Leishman-stained faecal smears showing large oval bodies resembling *Eimeria* spp. oocysts (left frame; a), small hollow round bodies resembling cryptosporidial oocysts (middle frame; b) and modified Zeihl–Neelson-stained faecal smear conforming the presence of cryptosporidial oocysts (right frame; c) besides several other faecal inclusions (original magnification × 1000×).

Table 2. *Detection of* Cryptosporidium parvum *by conventional and modern techniques in different age groups of diarrhoeic bovine calf faeces (*n = 100*)*

Age group (days)	Number of animals	mZn stain positive (%)	ELISA positive (%)	PCR positive (%)
1–7	9	3 (33·3)	3 (33·3)	3 (33·3)
8–14	3	1 (33·3)	2 (66·7)	2 (66·6)
15–21	30	9 (30·0)	10 (30·0)	10 (30·0)
22–30	19	5 (26·3)	7 (36·8)	7 (36·8)
31–60	39	7 (17·9)	13 (30·8)	13 (30·8)
Overall	100	25 (25·0)	35 (33·0)	35 (33·0)
χ^2				1·446

ELISA or PCR were considered positive as *Cryptosporidium*-associated enteropathogens.

Electrophoretic analysis of PCR products of *Salmonella* spp., *C. perfringens* and bovine corona-virus in association with *C. parvum* are shown in Figure 2. Majority of the cases, in this study, showed as many as 13 combinations of *Cryptosporidium*-associated infections (71·40%) with *Cryptosporidium* alone in 28·60% diarrhoeic calves only (Table 5). Moreover, the age-wise faecal prevalence of *Cryptosporidium* as a single agent and combination aetiologies with it, showed a decreasing and increasing trend (χ^2 13·28, $P < 0·01$), respectively (Table 5). This indicates that initial primary infection with *Cryptosporidium* led to significant enterocytic damage favouring flare up of other secondary infections with the passage of time. In addition, the findings of severe intestinal epithelial damage (Fig. 3), lymphoid depletion in mesenteric lymph nodes (Fig. 4) and demonstration of *Cryptosporidium* along with coccidiosis (eimeriosis) (Fig. 5) were amply augmented by histopathology of the representative tissue samples collected from calf mortality during the outbreaks. Histopathology is considered as a gold standard for demonstrating the intestinal involvement and cryptosporidia in one go in the animals dying of diarrhoeal disease [6]. Furthermore, the haematobiochemical findings of significant neutrophilic leucocytosis, lymphopaenia and anaemia along with significant increase in serum albumin and blood urea nitrogen with corresponding significant decrease in total globulins hinted at secondary suppurative infection, dehydration and immunosuppression, respectively, as have been reported by the authors earlier [52, 53]. Contrary to our findings, de la Fuente *et al.* [27] reported a significant

Table 3. *Microscopic findings in Leishman's-stained faecal smears*

| Microscopic findings | Age | | | | | Total | Rank | *r*-value |
	1–7 days (*n* = 9)	8–14 days (*n* = 3)	15–21 days (*n* = 30)	22–30 days (*n* = 19)	31–60 days (*n* = 39)			
Cocci	6 (66·67)	2 (66·67)	23 (76·67)	15 (78·95)	26 (66·67)	72 (72·00)	1	−0·011
Thick bacilli	2 (22·22)	2 (66·67)	17 (56·67)	14 (73·68)	28 (71·79)	63 (63·00)	2	0·598
Thin bacilli	5 (55·56)	3 (100·00)	21 (70·00)	14 (73·68)	14 (35·90)	57 (57·00)	4	−0·669
Stumpy bacilli/coccobacilli	–	–	5 (16·67)	4 (21·05)	7 (17·95)	16 (16·00)	7	0·699
Squamous epithelial cells	–	1 (33·33)	4 (13·33)	–	8 (20·51)	13 (13·00)	8	0·092
Other cells and cell debris	4 (44·44)	3 (100·00)	17 (56·67)	13 (68·42)	25 (64·10)	62 (62·00)	3	−0·061
Undigested plant fibre(s)	2 (22·22)	1 (33·33)	4 (13·33)	1 (5·26)	3 (7·69)	11 (11·00)	10	−0·710
RBCs	2 (22·22)	3 (100·00)	13 (43·33)	6 (31·58)	14 (35·90)	38 (38·00)	5	−0·303
Fungus/yeast	1 (11·11)	–	8 (26·67)	5 (26·32)	4 (10·26)	18 (18·00)	6	0·108
Eimeria spp.	–	1 (33·33)	5 (16·67)	–	1 (2·56)	7 (7·00)	12	−0·431
Cryptosporidium spp.	1 (11·11)	1 (33·33)	1 (33·33)	5 (26·32)	2 (5·13)	10 (10·00)	11	−0·349
Thin filamentous granulated bacilli (Actinomycetes)	2 (22·22)	1 (33·33)	8 (26·67)	–	–	11 (11·00)	10	−0·824
Pigment	–	–	8 (26·67)	–	4 (10·26)	12 (12·00)	9	0·157
Leucocytes	–	–	1 (3·33)	–	2 (5·13)	3 (3·00)	13	0·744
Undigested fat	–	–	–	1 (5·26)	–	1 (1·00)	14	0·146

Table 4. *Sensitivity of ELISA, mZN and Leishman's staining of faecal smears with PCR as gold standard for determination of* Cryptosporidium parvum *in diarrhoeic bovine calf faeces (*n = 100*)*

| Tests | | PCR | | | Per cent sensitivity |
		Positive	Negative	Total	
ELISA	Positive	34	1	33	97·14
	Negative	1	64	67	
	Total	35	65	100	
mZN stain	Positive	24	1	25	72·72
	Negative	9	66	75	
	Total	33	67	100	

age-associated decrease in the detection rate of mixed infections. In fact, the epidemiological studies have proven that diarrhoea is more severe in mixed infection(s), especially in immunocompromised individuals [54]. In this study, the *Cryptosporidium*-associated pathogens responsible for bovine calf scour outbreaks and mortality were *C. perfringens* 45·71% (16/25), followed by *Salmonella* spp 40·0% (10/25), rotavirus 36·0% (9/25), coronavirus 16·0% (4/25), *E. coli* 12·0% (3/25) and *Eimeria* spp. 4·0% (1/4), respectively, which fell nearly in line with that for *C. perfringens* (54·0%) and in contrast to rotavirus (87%), coronavirus (11·1%), *E. coli* (27·8%) and *Salmonella* spp.
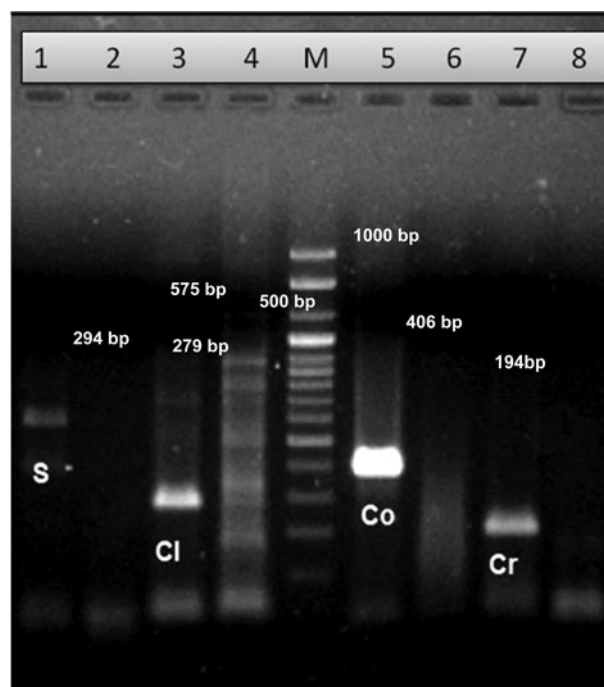

Fig. 2. Agarose gel electrophoretic analysis showing PCR amplified products of multiple agents of diarrhoea. Lane 1: *Salmonella* sp(s) positive (575 bp), lane 2: *Salmonella* sp negative, lane 3: *Clostridium perfringens* (Cl) positive (279 bp), lane 4: *C. perfringens* (Cl) negative, lane M: DNA marker (100 bp plus, SRL), lane 5: bovine coronavirus (Co) positive (406 bp), lane 6: bovine coronavirus (Co) negative, lane 7: *Cryptosporidium parvum* (Cr) positive (194 bp), lane 8: *C. parvum* (Cr) negative control.

Table 5. *Detection of* Cryptosporidium *and its combination with other enteropathogens in different age groups of diarrhoeic calves*

Enteropathogens detected	1–7 days (N = 9) (%)	8–14 days (N = 3)	15–21 days (N = 30)	22–30 days (N = 19)	31–60 days (N = 39)	Total (N = 100)
Cryptosporidium pavrum	–	2 (66·67)	6 (20·00)	1 (5·26)	1 (2·56)	10 (10·00)
C. pavrum + rotavirus	–	–	–	–	2 (5·13)	2 (2·00)
C. pavrum + *Clostridium perfringens*	1 (11·11)	–	1 (3·33)	2 (10·53)	1 (2·56)	5 (5·00)
C. pavrum + *Salmonella* spp.	–	–	1 (3·33)	1 (5·26)	2 (5·13)	4 (4·00)
C. pavrum + *Escherichia coli*	–	–	–	–	1 (2·56)	1 (1·00)
C. pavrum + rotavirus + *Salmonella* spp.	–	–	–	–	–	1 (1·00)
C. pavrum + *Eimeria* + *C. perfringens*	–	–	–	1 (5·26)	–	1 (1·00)
C. pavrum + rotavirus + *C. perfringens*	1 (11·11)	–	–	–	1 (2·56)	2 (2·00)
C. pavrum + coronavirus + *C. perfringens*	–	–	1 (3·33)	1 (5·26)	–	2 (2·00)
C. pavrum + *E. coli* + *Salmonella* spp.	–	–	–	–	1 (2·56)	1 (1·00)
C. pavrum + rotavirus + *C. perfringens* + *Salmonella* spp.	–	–	–	–	3 (7·69)	3 (7·69)
C. pavrum + rotavirus + coronavirus + *C. perfringens*	1 (11·11)	–			–	1 (1·00)
C. pavrum + coronavirus + *C. perfringens* + *Salmonella* spp.	–	–	–	1 (5·26)	–	1 (1·00)
C. pavrum + *E. coli* + *C. perfringens* + *Salmonella* spp.	–	–	–	–	1 (2·56)	1 (1·00)
Total	3 (33·33)	2 (66·67)	10 (33·33)	7 (36·8)	13 (33·3)	35 (35·00)
Per cent concurrent infection	33·33	–	40·00	85·71	92·30	71·40

Figures in parentheses indicate percentage.
All the agents shown in the table were detected by both ELISA and PCR except *Salmonella* spp., for which only PCR was performed and for rotavirus only ELISA was performed.
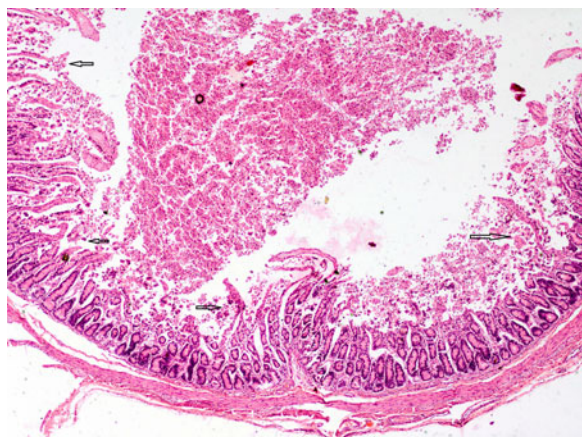

Fig. 3. Severe intestinal damage due to multiple aetiological agents characterised by massive superficial and deep necrosis extending into crypts with formation of marked debris in the lumen. H&E × 4× original magnification.
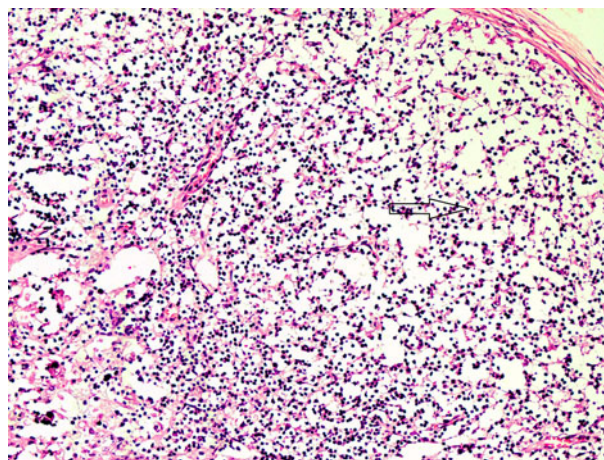

Fig. 4. Section of a mesenteric lymph node of the small intestine segment affected with severe diarrhoea showing massive lymphoid cell depletion. H&E × 10× original magnification.

(1·8%) in central Spain [55]. Almost similar infection rate of *Cryptosporidium* (21·28%) with *Blastocystis* 19·15%, *Giardia* (51·06%) and *Enterocytozoon* 36·17%, although in a higher 3–5-month age group

of diarrhoeic calves was reported [30]. The severity of *Cryptosporidium* in combination with other agents, or whether other agents extend the risk period for clinical cryptosporidiosis remains to be proved further
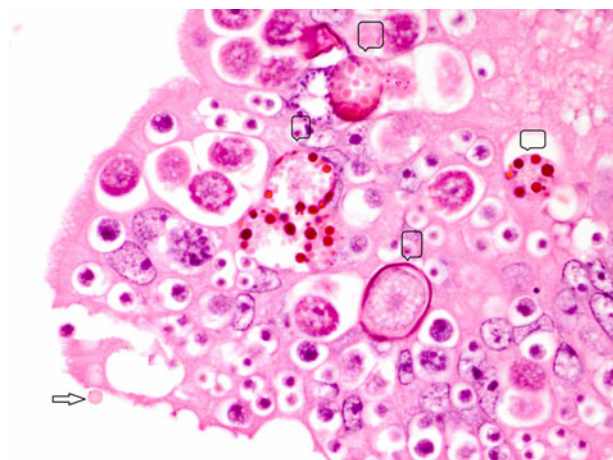
Fig. 5. Section of small intestine showing various developmental stages of coccidia and an oocyst (arrow) of *Cryptosporidium* present superficially. H&E × 100× original magnification.

experimentally [27]. The prevalence of each of pathogen and disease incidence can vary due to geographical location of the farms, farm managemental practices and herd size. A heavy mortality of 43% was recorded in the present study. A similar high mortality has been reported due to diarrhoea in the USA (57%) and Korea (53·4%) in unweaned dairy calves [56–57].

Among the various risk factors analysed during the study, poor hygiene, overcrowding, bad calf nutrition, including deprivation of colostrum and inadequate milk feeding, and sudden weather changes were found to be the major epidemiological factors. Poor managemental practices, including the method of cleaning, the type of flooring and the frequency of cleaning and deprivation of colostrum in suckling calves were also found to be the main risk factors associated with cryptosporium infections [58, 59].

From this first comprehensive report of 17 cryptosporidial outbreaks from Punjab State of North India, diagnosed based on a combination of techniques, we conclude that when *C. parvun* occurs in association with other concurrent enteropathogens, it may result into severe infection leading to outbreaks of neonatal bovine calf diarrhoea, resulting in huge mortality perpetuated by poor managemental practices and immunosuppression as augmented by histopathology of lymph nodes. In addition to *C. parvum*, the other aetiological agents, *viz. C. perfringens*, *Salmonella* spp and *E. coli* detected in the present study also carry marked zoonotic potential in children and immunocompromised people.

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