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Paper

Diagnostic evaluation of nested PCR and microscopy for Cryptosporidiosis in goats: can COWP gene based qRT-PCR be useful in assessment of active Cryptosporidial infections?

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Abstract

The present research delved into the transmission patterns, diagnostic methods, molecular traits, and phylogenetic analysis of *Cryptosporidium* species. The research was undertaken to enhance comprehension of the epidemiology and the potential for zoonotic transmission. A total of 80 goat-kid samples were tested, 7 were confirmed positive by mZN microscopy and 12 by nested-PCR. By PCR, 18SSUrRNA, HSP70, and GP60 amplicons were tested for *Cryptosporidium*. The restriction enzymes viz., *SspI*, *VspI* and *MboII* were used to genotype 12 *Cryptosporidium* positive samples by which *C. parvum* and *C. bovis* mixed infections were detected. Quantitative reverse transcription real-time PCR was used to transcriptionally screen the COWP-subunit genes to assess the severity of the infection in goat-kids, which showed upregulation of COWP6 and COWP4, while COWP9 and COWP3 genes were downregulated. A silent mutation was found at the codon CCA→CCC, which is being reported for the first time in goat field isolates. Phylogenetic and sequencing analyses confirmed the presence of the anthropozoonotic IIe subtype.

Keywords

Cryptosporidium, Goat-kids, PCR-RFLP, Zoonoses, COWP transcripts

Introduction

Cryptosporidiosis is a self-limiting, emerging zoonotic enteropathogenic apicomplexan protozoan disease of public health importance caused by various species of *Cryptosporidium*. *Cryptosporidium* is intracellular, extracytoplasmic and infects the microvillus epithelium of the gastrointestinal tract (Chen *et al.* 2002) causing acute gastrointestinal disturbances followed by diarrhoeic syndrome (Navin and Juranek 1984) mainly in immune-deficient individuals; it may lead to life-threatening chronic diarrhoea leading to an overall economic loss in the form of treatment of ailing animals (Paraud and Chartier 2012). Caprine cryptosporidiosis was first described in Australia from a 2-week-old kid with diarrhoea (Mason *et al.* 1981) and since then it has been reported worldwide (Santı´n and Trout 2008). Among the *Cryptosporidium spp.*,the species common infecting goats are *C. parvum*, *C. ubiquitum*, *C. hominis* and *C. xiaoi* (Quílez *et al.* 2008). However, *C. bovis*has also been reported in goats. Lately, cryptosporidiosis in goats has also been reported from India (Paul *et al.* 2013, Maurya *et al.* 2013, Utaaker *et al.* 2017, Dixit *et al.* 2018). There is neither any innate resistance to infection nor any passive protection that may be transmitted to newborns through colostrum (Viel *et al.* 2007, Paul *et al.* 2009). The situation is further complicated by the lack of any vaccine against the disease and suitable diagnostic procedures. Due to the lack of distinct morphological features of theoocysts, it is difficult to differentiate microscopically various species (Fall *et al.* 2003). In the present study, characterization of *Cryptosporidium* was performed using PCR-restriction fragment length polymorphism (RFLP) and

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DNA sequencing of a fragment from the GP60 gene. Differential expressions of various COWP genes in the total oocyst RNA were evaluated.

Material and methods

Experimental Plan

The experiment was designed to study the standard microscopical and molecular detection methods applied in the occurrence of cryptosporidiosis in goats of central India (Madhya Pradesh) and western semi-arid plains of northern India (ICAR-CIRG, Makhdoom) followed by the characterization, sequencing and phylogenetic analysis of the pathogen (Fig. 1). From September 2019 to April 2020 a total of 80 faecal samples were collected from juvenile goats (up to 6 months of age).

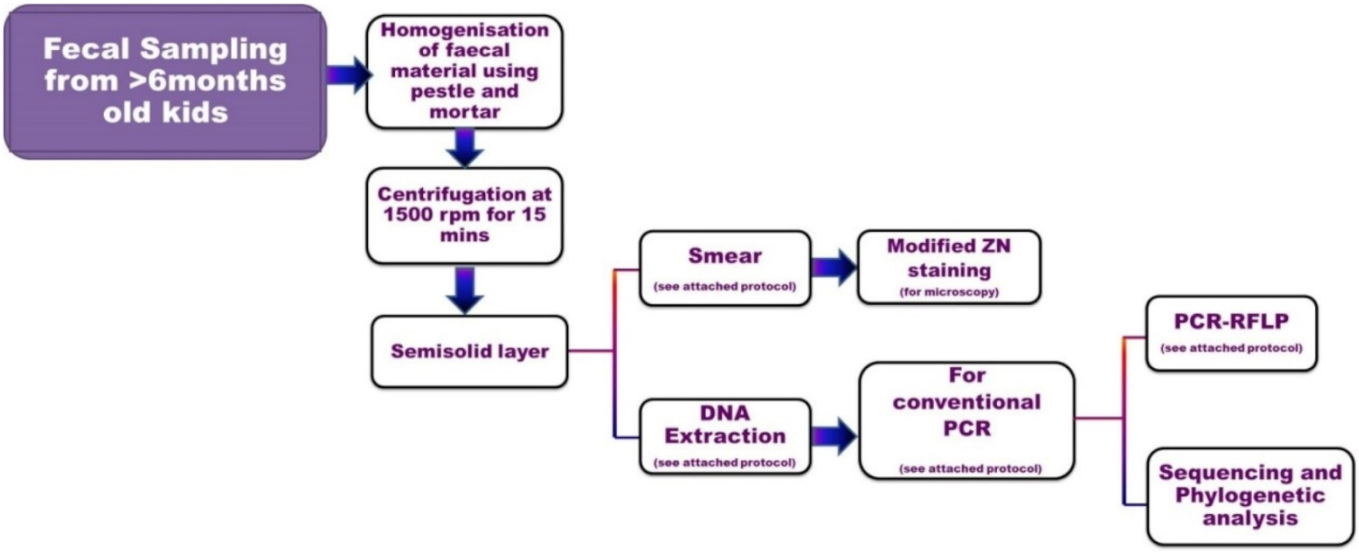

Figure 1. A technical illustration of the work-flow followed.

Collection of faecal samples and preliminary examination

Faecal samples were collected from each goat in sterile vials by rectal lavage or per-rectal collections and stored immediately by diluting in phosphate buffered saline (PBS) pH 7.2 Then, transfer to the laboratory under 4°C. The preliminary examination consisted of staining of the faecal smears by the modified Ziehl–Neelsen method (Henricksen and Pohlenz 1981, Venu *et al.* 2013) and examination under a microscope oil-immersion lens (100X).

Extraction of DNA from the Cryptosporidium oocysts

The faecal samples from goats were used for the extraction of genomic DNA following the technique outlined by Sambrook and Russel 2001 with minor modifications as described in the in-house protocol developed at Animal Health Division, CIRG, Makhdoom (U.P.) using CTAB as isolation buffer (100mM Tris-HCl, pH-8; 1.4M NaCl, 20mM EDTA - no salt; 2% CTAB) followed by 3 consecutive freeze- heat cycles and vortexing after every cycle. The eluted DNA was tested for its concentration by mixing 2µl of DNA sample with 200µl of QuantiFluor® One ds DNA dye and the concentration is subsequently measured using QuantusFluorometer® (Promega Technologies, Madison, USA).

Nested PCR of the 18SSU rRNA, HSP70 and GP60 gene

The primer-directed PCR amplification of 18SSUrRNA, HSP70, and GP60 genes was achieved from individual samples using the oligonucleotide primers (Table I). The PCR was carried out in a 25µl volume containing 1.0µl (10ng/ml) of genomic DNA, 10µl 2x PCR buffer (PrimeStar PCR Master Mix, TaKaRa®), 1.0µl (10pmol/ml) of each forward and reverse primer and nuclease-free water up to 25µl. The reaction mixture was initially incubated at 95°C for 5 min for initial denaturation and then a total of 35 cycles in a reaction were performed with denaturation at 95°C for 1 min,

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primer annealing of 18SSU rRNA, HSP70, and GP60 at 56°C, 61°C, and 52°C respectively for 1 min and strand extension at 72°C for 1 min. The final extension was done at 72°C for 10 min. For the nested PCR, 1µl of the primary product was used as the template, and primers were used as mentioned in Table I. The reaction and the cycling conditions for PCR amplification remained the same as for the primary PCR, except that the annealing temperatures were 57°C, 70°C, and 56°C for 18SSU rRNA, HSP70, and GP60 respectively. The amplification of a specific PCR product was evaluated by gel electrophoresis using 1.5% agarose in TAE buffer stained by Ethidium Bromide.

GENE	SEQUENCES (5′→3′)			REFERENCES
18SSU rRNA	EXTERNAL	F	TTCTAGAGCTAATACATGCG	Morgan *et al.* 2001
		R	CATTTCCTTCGAAACAGGA	
	INTERNAL	F	GGAAGGGTTGTATTTATTAGATAAAG	
		R	AAGGAGTAAGGAACAACCTCCA	
HSP- 70	EXTERNAL	F	GGTGGTGGTACTTTTGATGTATC	
		R	GCCTGAACCTTTGGAATACG	
	INTERNAL	F	GCTGSTGATACTCACTTGGGTGG	
		R	CTCTTGTCCATACCAGCATCC	
GP60	EXTERNAL	F	ATAGTCTCCGCTGTATTC	Quielz *et al.* 2008
		R	GGAAGGAACGATGTATCT	
	INTERNAL	F	TCCGCTGTATTCTCAGCC	
		R	GCAGAGGAACCAGCATC	

Table I. Primers used in the two-step nested PCR protocol.

18 SSUrRNA full gene

The in-house designed primers (Table II) were targeted for the full gene sequence of 18SSU rRNA of *Cryptosporidium* spp. using BioEdit version 7.0 (Hall 1919) and aligned by ClustalW algorithm and oligonucleotides were quality checked by OligoAnalyzer1.0.2. The PCR was carried out in 25µl volume containing 1.0µl (10ng/ ml) of genomic DNA, 10µl 2x PCR buffer (PrimeStar PCR Master Mix, TaKaRa®), 1.0µl (10pmol/ml) of each forward and reverse primer and nuclease free water up to 25µl. The reaction mixture was initially incubated at 95°C for 5 min for initial denaturation and then a total 30 cycles of reaction were performed with denaturation at 95°C for 30 sec, primer annealing at 60°C for 30 sec and strand extension at 72°C for 1 min 30 sec. The final extension was done at 72°C for 10 min. The amplification of a specific PCR product was checked by gel electrophoresis using 1.5% agarose.

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S.No	Primer name	Sequence (5′→3′)	Amplicon size (bp)	
			Hi-fidelity polymerase	Taq Polymerase
1.	Cryp_18s_full_F	CTGGTTGATCCTGCCA GTAGTC	1711-1774	1712-1775
2.	Cryp_18s_full_R	CAGGTTCACCTACGG AAACCTTG		

Table II. In-house designed primers for 18SSUrRNA full gene.

PCR-RFLP of the 18SSU rRNA gene of Cryptosporidium spp.

A volume of 5µl of purified PCR product (834 bp) were separately subjected to the restriction endonucleases digestion with enzymes *Ssp*I, *Vsp*I and *Mbo*II (10IU/ml) in 30 µl reaction mixture for 1 h in a humid chamber at 37°C followed by inactivation at 65°C for 5 min (Feng *et al.* 2007). The confirmation of the digested product was performed by electrophoresis in 1.8% agarose gel.

Sequencing and Phylogenetic analyses

The nested amplicon of GP60 gene was generated and sequencing was done on both strands using Sanger's dideoxy method by using BigDye® terminator v1.1 cycle sequencing kit (Applied biosystems, Tokyo, Japan). The chromatogram file with raw data containing un-aligned nucleotide sequences of GP60 gene was opened in BioEdit version 7.0 (Hall 1919) and were proof-read with matching nucleotide for each nucleotide peak and corroborated with NCBI database for possible errors in the coding region. Finally, the forward and reverse strands which were proof-read previously were subsequently aligned to obtain the final coding region containing all the necessary elements of the gene. The aligned GP60 gene was compared for point mutations in the coding region plotting sequence identity using the graphical view of the BioEdit version 7.0 (Hall 1919). The Clustal W aligned GP60 gene was further subjected to phylogenetic analysis using MEGA 6.0 tool (Tamura *et al.* 2013).

For comparative phylogenetic analysis, reference sequences retrieved from the GenBank™ were aligned with representative sequences of each species of *Cryptosporidium*. The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei 1992). The optimal tree with the sum of branch length =0.00868418is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura *et al.*2004). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree (Saitou and Nei 1987). The analysis involved 11 nucleotide sequences comprising of 18SSU rRNA gene of *Cryptosporidium* strains. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 231 positions in the final dataset.

Extraction of RNA from Oocysts

The RNA was isolated from purified oocysts samples (Oocyst concentration = 5,00, 000/ml) or unknown faecal samples by standard Trizol® method (Chomczynski 1993). The quantity and quality of the RNA were assessed using QuantusFluorometer by binding 2µl of RNA to Quantifluor RNA dye followed by vortexing for 20 sec and incubation at dark in RT for 5 minutes before reading the concentration. The quantified RNA samples were further stored at -20°C.

c-DNA synthesis

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Total RNA was converted to copy DNA (cDNA) using Primescript™ Ist strand cDNA synthesis kit (TaKaRa, Japan) as per manufacturers protocol. The synthesized cDNA product was taken out and kept at -20°C till further use for real-time PCR.

Quantitative reverse transcription real-time PCR (qRT-PCR)

Premix Ex-Taq II SYBR green master mix (TaKaRa, Japan) was used for quantitative analysis of COWP 3, 4, 6 and 9 genes in both cDNA and gDNA of oocysts. Gene expression was studied using reverse transcription real-time SYBR green PCR assay (CFX96 Real-time PCR system®, Bio-Rad) with the respective primers (Table III) used at 0.5pmol final concentration, 0.5µl of cDNA template equivalent to 50ng of total RNA, 1x master mix in a final reaction volume of 20µl in duplicates per sample per gene. For DNA the aforesaid protocol is replaced with gDNA with same components and volume. The reaction condition has been followed as described by the manufacturer with the annealing temperature and extension at 60°C. Reaction controls like No template control (NTC) were also incorporated to rule out the carry-over contaminations and background amplifications. The samples were subjected to amplification using SYBR green qRT-PCR. After cycle extension, the products were subjected to melt curve analysis to assess the melting temperature (Tm) of the generated product using the melting peak or Tm call generated by the software CFX96™ manager (Bio-Rad).

S. No.	Gene of interest	Oligo sequences 5′- 3′	Remarks
1.	COWP 3	F: GCCATAATGGTTCACAGTCT	Templeton *et al.* 2004
		R: TGGGATACATTTTGGAAGTC	
2.	COWP 4	F: CGATTTGGTTGATGAGAGTT	
		R: CTCTCGGTTATTTCTGTTGG	
3.	COWP 6	F: TGAAAAGGGTTTCCAACTTA	
		R: ATTGGTCTCTCATCCATACG	
4.	COWP 9	F: CTGGATTTCCAGGATGTGCT	
		R: TGCTTTGTATTCCCGATTCTG	

Table III. Primers of target genes used in qRT-PCR.

For determining the standard curve of respective COWP genes $\log_{10}$ dilution was done, where 1µl of gDNA/cDNA from the original sample was taken and serial dilutions were done with each dilution dispensed into corresponding reaction mixes in duplicates and subjected to thermal conditions as described above.

The transcript copies for each gene were computed using the interpolation of the standard curve generated regression co-efficient Y=mX+C, where 'Y' is the Cycle quantification (Cq), 'm' is the slope, 'X' is the transcript copies and 'C' is the Y-intercept.

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Statistical Analyses

Chi square test was applied to analyze the data as per Snedecor and Cochran 1994. For the calculation of kappa value QuickCalcs, GraphPad Prism 8 web tool was used (Landis and Koch 1977).

Results

In mZN staining, out of 80 faecal samples assayed, 7 (8.75 %) were found positive where *Cryptosporidium* oocysts stained red and on pale green/blue background of malachite green in smears appeared as spherules, however the degree and proportion of staining differed with individual oocysts (Fig. 2 A and B). Out of the total faecal samples collected, nPCR based method of detection revealed specific amplification of ~834bp, ~334bp and ~850bp for the 18SSU rRNA, HSP70 and GP60 gene respectively (Fig. 3) in 12 samples. 18SSU rRNA full gene PCR amplified products (using in- house designed primers) were of ~1700bp (Fig. 3) when subjected to 0.8% agarose gel electrophoresis. The kappa value calculated between conventional nPCR and mZN microscopy was 0.704 and the agreement was found to have substantial agreement (Table IV).

Based on the nPCR results the epidemiological study was considered under parameters like age, faecal consistency, sex, breed and rearing system of the animals. The age wise occurrence of *Cryptosporidium* was observed highest in kids of less than one-month age (20.8 %) and lowest (5 %) in kids above 3 months (Table V, Fig. 4a). The occurrence was non-significantly higher in diarrhoeic kids (18.18%) as compared to that of non-diarrhoeic (12.76%) kids (Table V, Fig. 4a). Among male goats, 4 (12.9 %) and in females, 8 (16.32 %) samples were detected positive for *Cryptosporidium* (Table VI, Fig. 4b). In breeds, particularly from Madhya Pradesh and ICAR-Central Institute for research on Goats (CIRG), Makhdoom, Mathura, (U.P.) no significant difference was found rather highest occurrence rate was reported in Barbari (18.80 %) and lowest in Sirohi (7.7 %) respectively (Table VII, Fig. 4c). The infection was slightly higher in kids kept in farm conditions (19.44%) than that of kids kept in field conditions (11.36%) but the difference was non-significant (Table VIII, Fig. 4d).

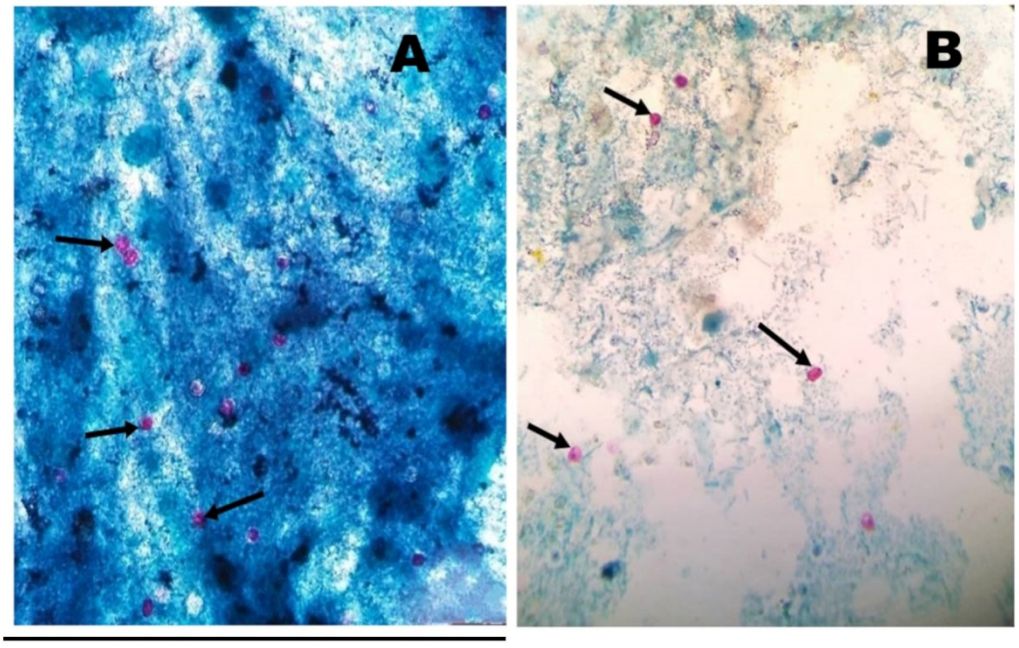

Figure 2. Cryptosporidium oocysts under 1000x magnification (mZN Staining).

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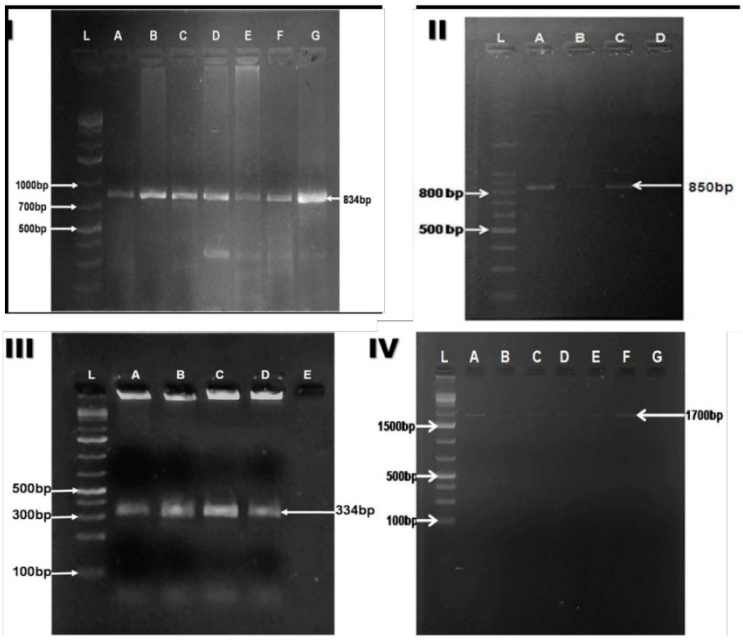

Figure 3. Nested and full gene amplification targeting various Cryptosporidia genes: I- 18 SSU rRNA gene[L- 1Kb DNA Ladder; A, B, C, D, E, F- Nested products (834bp); G- Positive control]; II -GP 60 gene [L- 100bp DNA Ladder; A, B, C - Nested products (850bp); D - Negative Control]; III - HSP 70 gene[L- 1Kb DNA Ladder; A, B, C, D - Nested products (334bp); E – Negative control];IV - Full gene amplification of 18 SSU rRNA gene using in-house designed primers [L – 1Kb DNA Ladder; A, B, C, D, E, F – Full gene PCR product size (~1700bp); G – Negative Control]

Comparison of nPCR with mZN				Fischer's Exact Test (*p*-value)	Kappa value	SE of Kappa	Strength of agreement	Sensitivity	95% CI	Specificity	95% CI
mZN			Total	0.0001*	0.704**	0.122	**Substantial	100%	59% to 100%	93.2%	85% to 98%
nPCR	+	-									
+	7	5	12	* Statistically significant							
-	0	68	68								
Total	7	73	80								

Table IV. Comparison and kappa value estimation between conventional nPCR and mZN microscopy.

Age (Months)	Number positive/ Number screened		
	Faecal consistency		Total
	Diarrhoeic	Non-Diarrhoeic	
< 1	3/10 (30.0%)	2/14 (14.3%)	5/24 (20.8%)
1 – 2	1/9 (11.1%)	2/8 (25.0%)	3/17 (17.6%)
2 – 3	2/6 (33.3%)	1/13 (7.7%)	3/19 (15.8%)
3 – 6	0/8 (0.0%)	1/12 (10.0%)	1/20 (5.0%)
Total	6/33 (18.18%)	6/47 (12.76%)	

Table V. Occurrence of Cryptosporidium infection based on nPCR with respect to age group and faecal consistency. *Statistically insignificant ($\chi 2 = 0.4$; $p>0.05$)

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Faecal Sample	Male	Female
Screened	31	49
Positive	4	8
Percent Positive	12.9	16.32

Table VI. Gender-wise occurrence of Cryptosporidium infection based on nPCR. $\chi 2$ value = 0.86** statistically insignificant ($p>0.05$)

Goat Breeds	Screened	Positive	Percent Positive
Barbari	16	3	18.8
Sirohi	13	1	7.7
Jamunapari	22	4	18.2
Beetal	10	1	10.0
Non- Descript	19	3	15.8

Table VII. Breed-wise occurrence of Cryptosporidium infection based on nPCR. $\chi 2$ value = 1.10** statistically insignificant ($p>0.05$)

Faecal Samples	Organized Farms	Unorganized Farms
Positive	36	44
Screened	7	5
Percent Positive	19.44	11.36

Table VIII Occurrence of Cryptosporidium infection by nPCR in relation to rearing system. $\chi 2$ value = 1.01** statistically insignificant ($p>0.05$).

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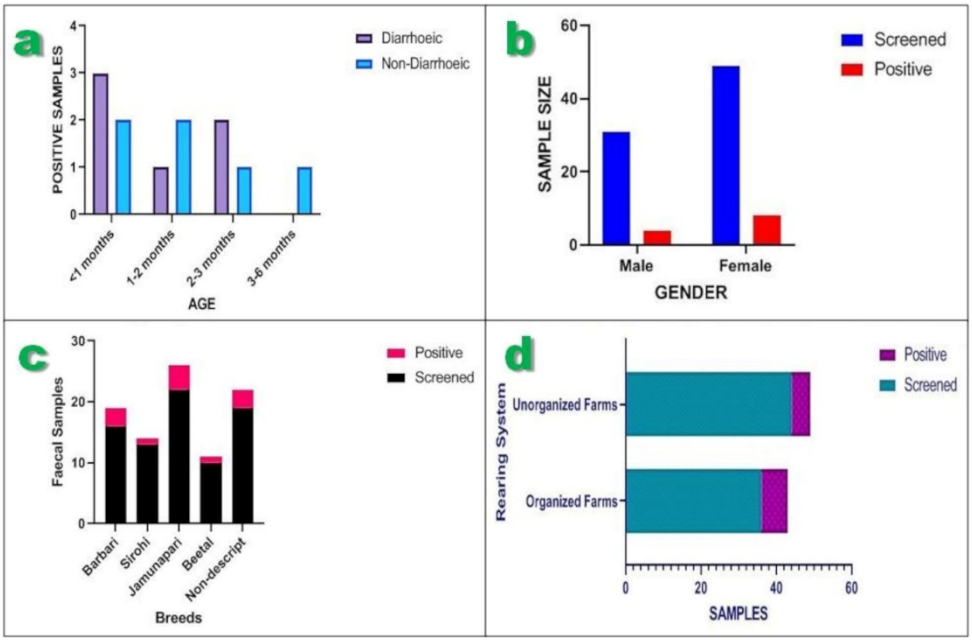

Figure 4. Graphical representation of effect of certain variables on the occurrence Cryptosporidium infection in goats based on nPCR; a-age and faecal consistency, b- Gender c- breed and d-rearing system.

The amplicons in the present study of the 18SSU rRNA nested PCR were subjected to PCR-RFLP with restriction enzymes viz., *Ssp*I, *Vsp*I and *Mbo*II (Fig. 5, Table IX) where the 12 isolates were identified as *C. parvum* among which 4 isolates were found together with *C. bovis* as mixed infection.

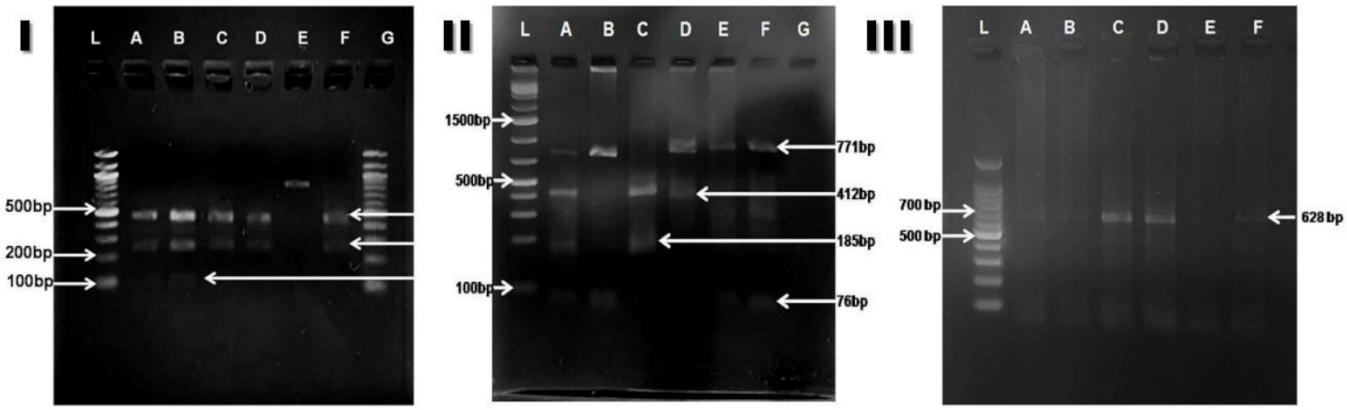

Figure 5. PCR-RFLP of nested amplicons of 18 SSU rRNA gene by restriction enzymes. I- SspI PCR-RFLP- L and G - 100bp DNA Ladder; E - Undigested PCR product (834bp); A, C, D, F- SspI Digested Products (449bp and 267bp); B - SspI Digested Products (449bp, 267bp and 108bp); II- MboII PCR-RFLP, L - 1kb DNA Ladder; A –MboII Digested Products (771bp, 412bp, 185bp and 76bp); B, E, F –MboII Digested Products (771bp and 76bp); C –MboII Digested Products (412bp and 76bp); D –MboII Digested Products (771bp and 412bp); III- VspI PCR-RFLP, L – 100bp DNA Ladder; A, B, C, D, E, F –VspI Digested Products (628bp).

Field isolate of *Cryptosporidia* was sequenced by Sanger's dideoxy method for GP60 gene. The 5′ end of the gene which falls in the coding region showed a single point mutation at 275thnucleotide position from A→C (Fig. 6).

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Sample ID	Enzyme	RFLP fragments	Suggested *Cryptosporidium* Species	Remarks
2	*Mbo* II	771 and 76 bp	*C. parvum*	Mixed infection
		412, 185 and 76 bp	*C. bovis*	
	Vsp I	628 bp	*C. parvum*	
	Ssp I	449 and 267 bp	*C. parvum*	
7	*Mbo* II	771 and 76 bp	*C. parvum*	Single infection
	Vsp I	628 bp		
	Ssp I	449, 267 and 108 bp		
19	*Mbo* II	771 and 76 bp	*C. parvum*	Mixed infection
		412, 185 and 76 bp	*C. bovis*	
	Vsp I	628 bp	*C. parvum*	
	Ssp I	449 and 267 bp	*C. parvum*	
20	*Mbo* II	771 and 76 bp	*C. parvum*	Mixed infection
		412 and 185 bp	*C. bovis*	
	Vsp I	628 and 115 bp	*C. parvum*	
	Ssp I	449 and 267 bp	*C. parvum*	
33	*Mbo* II	771 and 76 bp	*C. parvum*	Single infection
	Vsp I	628 and 115 bp		
	Ssp I	449, 267 and 108 bp		
37	*Mbo* II	771 and 76 bp	*C. parvum*	Single infection
	Vsp I	628 and 115 bp		
	Ssp I	449 and 267 bp		
50	*Mbo* II	771 and 76 bp	*C. parvum*	Single infection
	Vsp I	628 and 115 bp		
	Ssp I	449, 267 and 108 bp		
56	*Mbo* II	771 and 76 bp	*C. parvum*	Single infection
	Vsp I	628 bp		
	Ssp I	449, 267 and 108 bp		
61	*Mbo* II	771 and 76 bp	*C. parvum*	Single infection
	Vsp I	628 and 115 bp		
	Ssp I	449, 267 and 108 bp		
63	*Mbo* II	771 and 76 bp	*C. parvum*	Mixed infection
		412 and 185 bp	*C. bovis*	
	Vsp I	628 bp	*C. parvum*	
	Ssp I	449, 267 and 108 bp	*C. parvum*	
69	*Mbo* II	771 and 76 bp	*C. parvum*	Single infection
	Vsp I	628 and 115 bp		
	Ssp I	449, 267 and 108 bp		
77	*Mbo* II	771 and 76 bp	*C. parvum*	Single infection
	Vsp I	628 bp		
	Ssp I	449, 267 and 108 bp		

Table IX PCR-RFLP with restriction enzymes.

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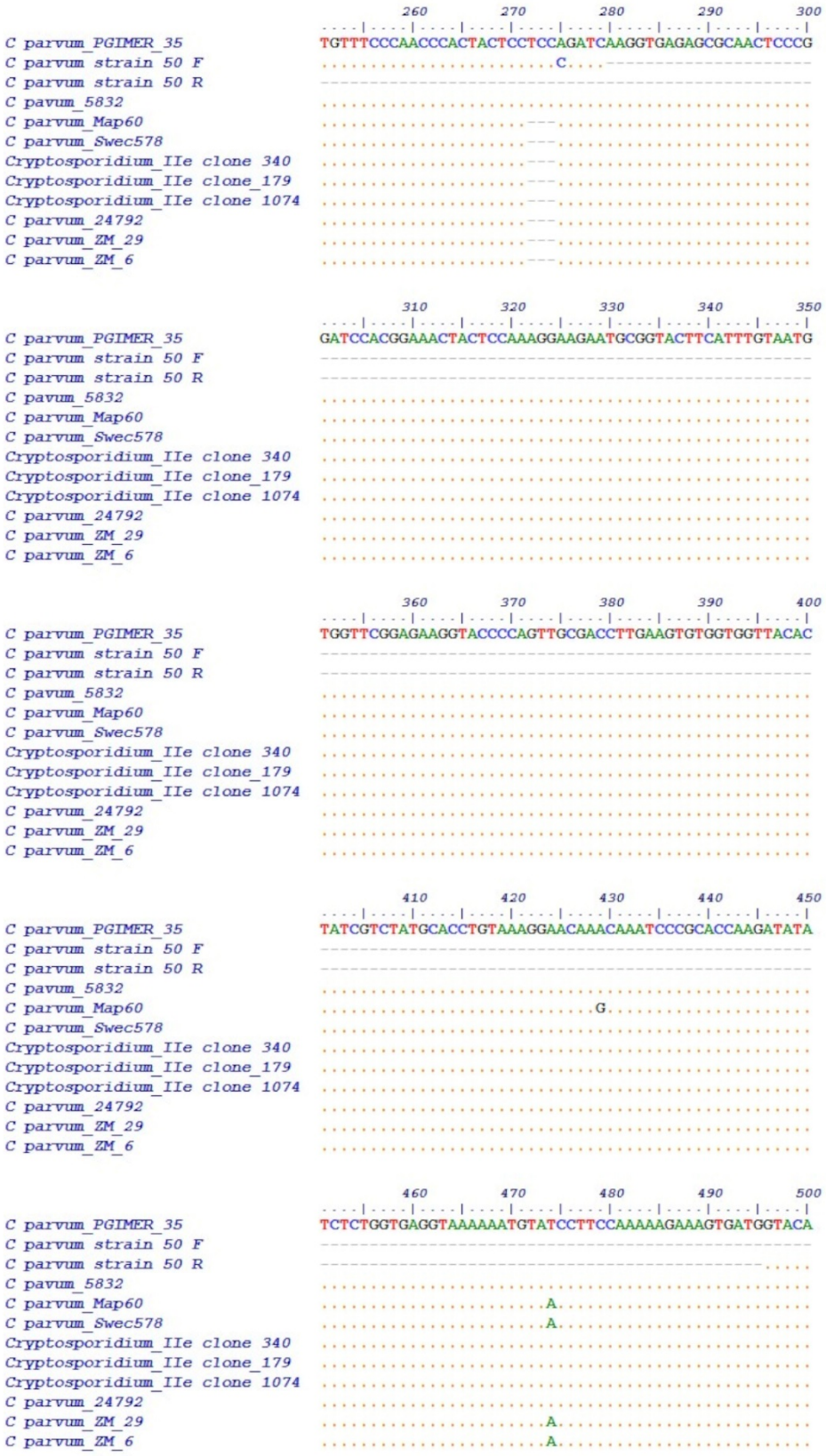

Figure 6. Nucleotide Sequence plot of C. parvum strain 50 NDVSU showing 275th Nucleotide position a single mutation from A→C.

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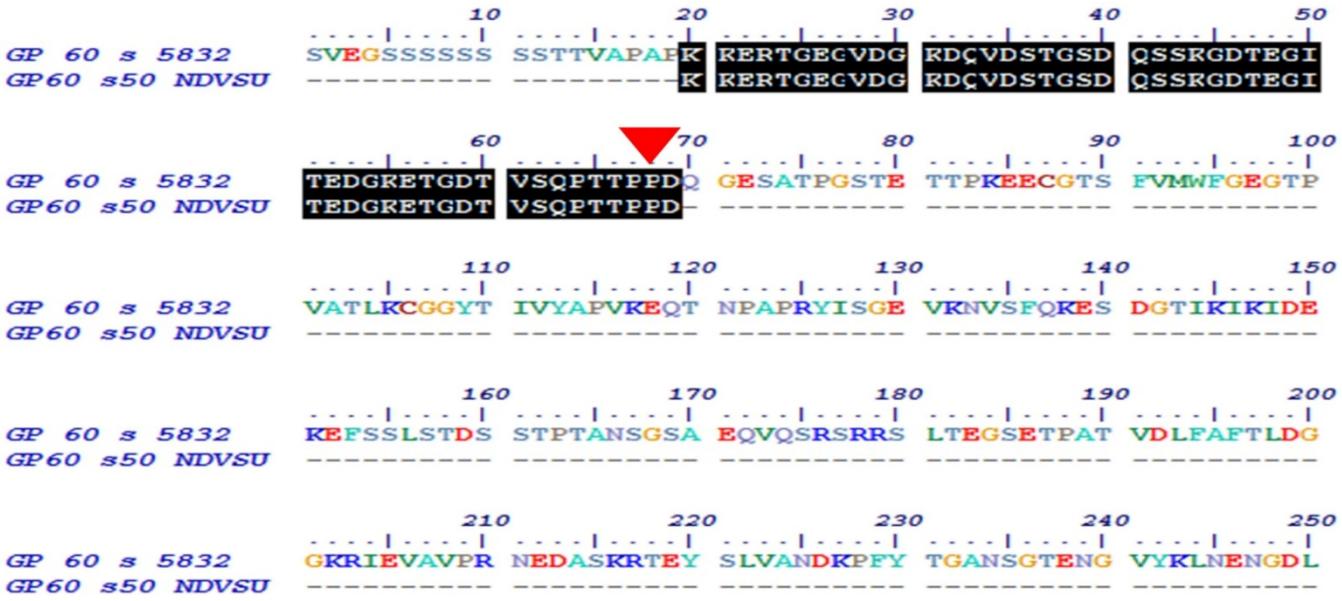

Figure 7. Amino acid Sequence plot of C. parvum strain 50 NDVSU showing silent mutation (not depicted) without any change in the amino acid proline at 68th position (in figure) and residue 50 (as per original sequence) for the codon CCC instead of CCA.

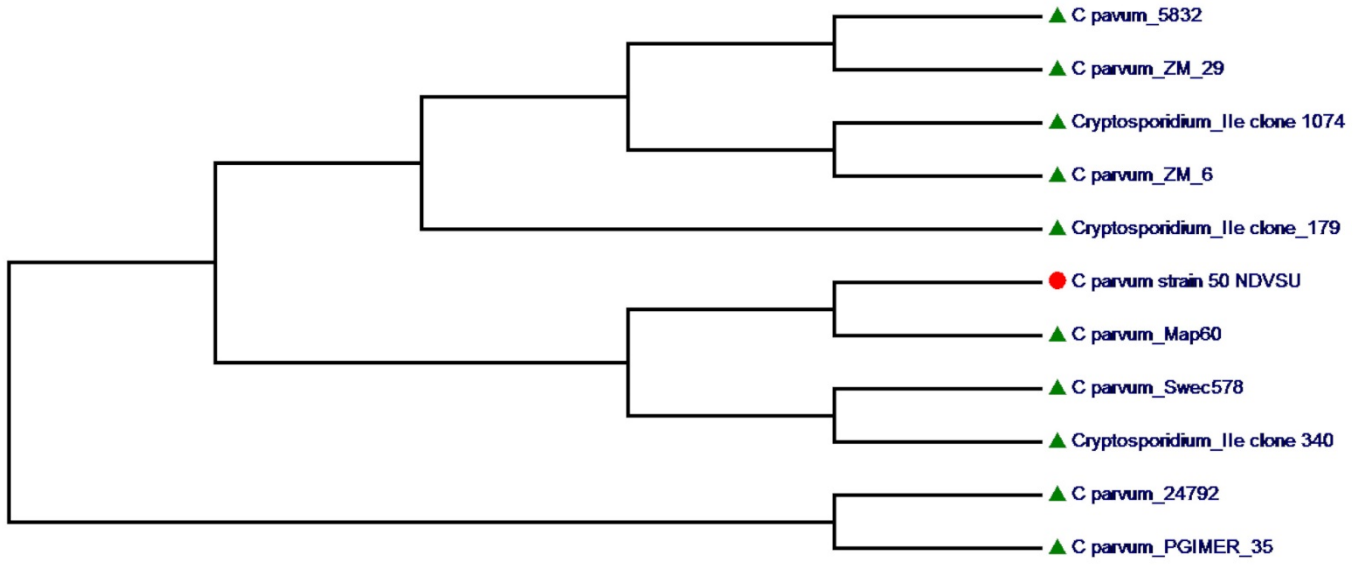

Figure 8. Phylogenetic tree of current Cryptosporidium parvum strain 50 NDVSU isolate in comparison with the global isolates for GP60 gene.

ΔCT method was used in determining the relative expression (fold change) of target genes in total RNA of cryptosporidial oocysts collected from diarrhoeic goat kids (Fig.9). Absolute quantification was done for the COWPs while relative quantification could not be done with other constitutively expressing genes of *Cryptosporidia* spp. However, the relative Cqs for the cDNA (Fig. 10) were compared with the gDNA copies for the same genes. The ΔCT was done only for the RNA transripts for each COWP component genes studied. There was up regulation of COWP6 followed by COWP4 gene with subsequent down regulation of COWP9 and least in COWP3 gene (Table X).

Besides, the highly positive samples were quantified for the transcript copies of the COWP component genes viz. COWP3, COWP4 and COWP6 using quantitative reverse transcription real-time PCR. Sample 40 despite high load of parasite was not having any transcriptional activity (Table XI) for COWP4 and COWP6, while the rest of highly positive samples showed higher transcripts for all the three COWP component genes.

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The genomic DNA was used as template for primary PCR followed by nested PCR to amplify 18SSU rRNA [Veil*et al.* 2007, Shanmathi *et al.* 2020.], HSP- 70 (Mendonca *et al.*2007, Lassen *et al.* 2009) and GP60 (Wielinga *et al.* 2008) gene for confirmation of the *Cryptosporidium* DNA.

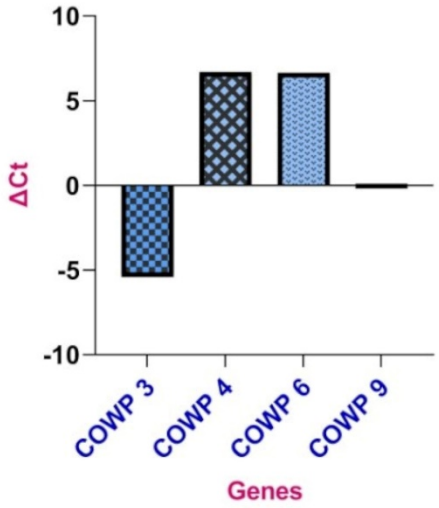

Figure 9 ΔCT of various cell wall oocyst protein (COWP) genes of Cryptosporidium spp. collected from diarrhoeic goat kids.

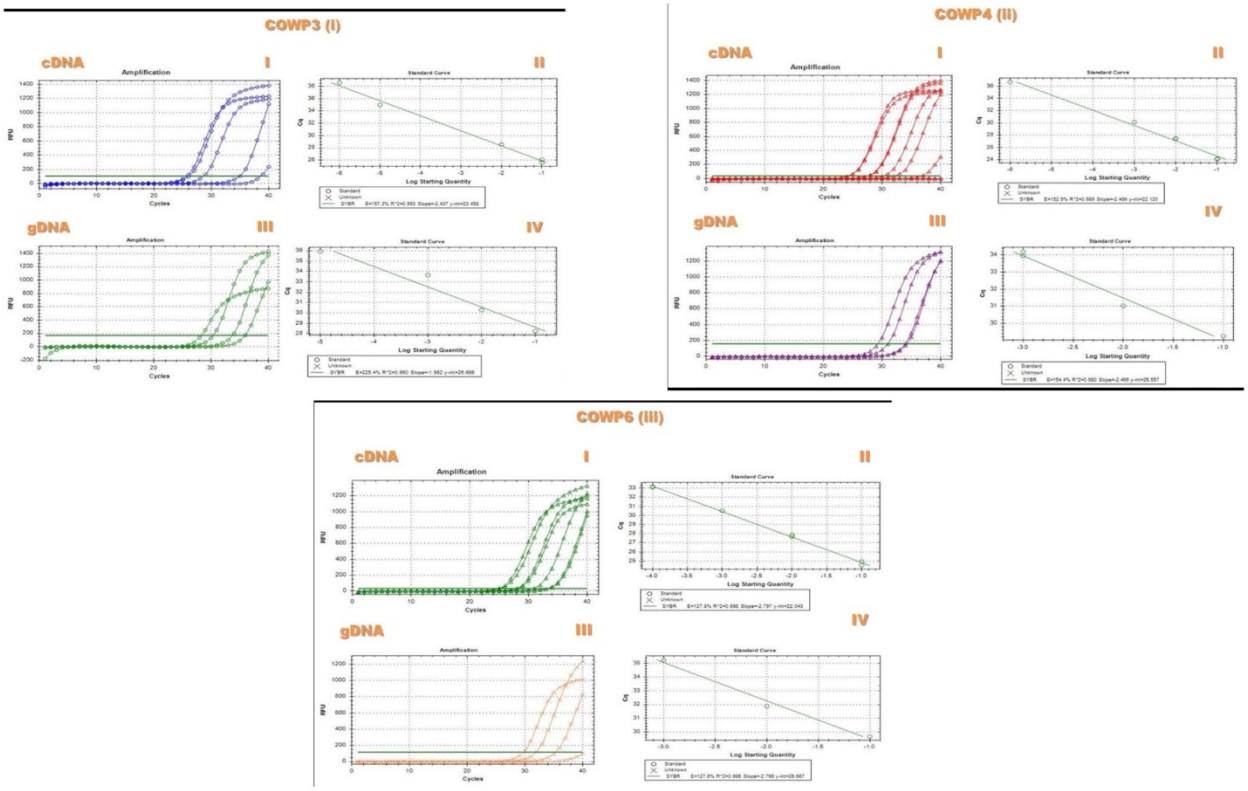

Figure 10. Amplification plot and standard curve for (I) - COWP 3 gene: I and II – cDNA, III and IV – gDNA; (II) - COWP 4 gene: I and II – cDNA, III and IV – gDNA; (III) COWP 6 gene: I and II – cDNA, III and IV – gDNA.

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GENE	E cDNA	E (cDNA) Actual	E gDNA	E (gDNA) Actual	Ct (gDNA)	Ct (cDNA)	ΔCt	Relative Expression
COWP3	99	1.99	95	1.95	27.85	33.26	-5.41	0.024166005
COWP4	99	1.99	98	1.98	28.11	21.47	6.64	96.46825603
COWP6	99	1.99	99	1.99	27.72	21.03	6.69	99.84517469
COWP9	99	1.99	99	1.99	35.04	35.23	-0.19	0.877440983

Table X ΔCT for determining the relative expression of various COWPs in total RNA.

Sample No.	COWP3		COWP4		COWP6	
	Avg. Cq	Transcript Copies	Avg. Cq	Transcript Copies	Avg. Cq	Transcript Copies
40	5.331	10,318	-	-	-	-
45	3.767	9,014	3.28	7,872	3.656	8,680
50	2.967	5,934	3.479	6,958	3.617	7,372

Table XI Transcript copies based on absolute quantification.

Discussion

mZN staining or Kinyoun staining is stated as 'gold standard' method among the conventional microscopic methods for routine diagnosis of *Cryptosporidium* infection in faecal samples (Fayer *et al.* 2000). Many Authors have now adopted mZN staining method with slight modifications (Venu *et al.* 2013) which is specifically and sufficiently sensitive to detect a moderate or high number of *Cryptosporidium* oocysts from faecal samples (Quílez *et al.* 1996). The detection limit of mZN staining has been reported up to 20 to50, 000 oocyst per gram of faeces (Paul *et al.* 2014, Khursheed *et al.* 2018). The observations in this study are in conformity to the epidemiological data reported up to 3.48%, 3.54%and 2.5% in goats from Spain (Wang *et al.* 2014), Switzerland (Jafari *et al.* 2012) and Iran (Marreros *et al.* 2012) respectively but lesser than the data provided from Siberia (31%) and Serbia (31.8%) in kids (Misic*et al.* 2006, Zorana *et al.* 2006). In India, caprine cryptosporidiosis prevalence has been reported as 14.63%, 40.41% and 48% (Quílez *et al.* 2008, Yadav *et al.* 2016, Ahamed 2012) respectively. The internal structures take up stain to varying degrees some appear amorphous, whereas others have the characteristic forms of sporozoites. When only a few oocysts are present difficulties arise in discriminating between *Cryptosporidium* oocysts and other spherical objects of similar size, particularly with mZN. Some bacterial spores might be acid-fast, but they are too small to cause confusion while some times oocysts of other coccidia like *Eimeria* and *Isospora* species also stain red, but they are much larger (Xiao and Fayer 2008).

There is a similarity in the pattern of age related occurrence data from various parts of the world suggesting that, *Cryptosporidium* infection is more common in young animals (Zorana *et al.* 2006) because the oocysts of *C. parvum* have a detrimental effect on the butyrate producing bacteria present as normal gut microbiota in kids which may increase mucosal inflammation as butyrate serve as an anti-inflammatory molecule (Mammeri *et al.* 2020). Due to this gut epithelial disruption; resulting in diarrhoea (Chen *et al.* 2002) and malnutrition may lead to decreased triglyceride level and hypercholesterolemia causing liver damage. A significant reduction in serum levels of IgA has a suppressive effect of the protozoa on the immune system (Mahmoud *et al.* 2018). Further, it is now accepted that cryptosporidial infections are more frequent during the first few weeks of life with marked decrease in the severity of symptoms as well as oocyst output (Causape *et al.* 2002). On the contrary some researchers found an increase of *Cryptosporidium* prevalence in kids with age increase (Misic *et al.* 2006, Rieux *et al.*2013). The parasite's lifecycle is

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approximately four days and peri-parturient females, infected kidding area and low immunocompetence may be the reasons for more prevalence of this infection in young animals (Khursheed *et al.* 2018).

There was no significant difference between occurrence of *Cryptosporidium* spp. infection in diarrhoeic and non-diarrhoeic kids. However, infection percentage was prevalent in diarrhoeic kids similar to a report stating higher prevalence of the infection in diarrhoeic kids (82.5%) than that of non-diarrhoeic kids (18.5%) (Bejan *et al.* 2009). In one study it was found that out of 291 goats suffering from *Cryptosporidium* infection only 9 were diarrhoeic (Noordeen *et al.* 2000). Some workers have also reported that this protozoan is not always associated with diarrhoea (Quílez*et al.* 1996, Oslon *et al.* 1997).

Sex wise prevalence was only slightly higher in male kids as compared to that of female kids based on previous reports (Quílez *et al.* 2008, Dixit *et al.* 2018). On the contrary, Bejan *et al.* 2009 reported that the distribution of *Cryptosporidium* infection was almost uniform in male and female kids up to six weeks age whereas in the current study the percentage infection was slightly higher in female. The highest occurrence rate was reported in Barbari and lowest in Sirohi respectively however, no breed differences in occurrence of *Cryptosporidium* infection are reported by other Authors previously (Dixit *et al.* 2018).

The infection was slightly higher in kids kept in farm conditions than that of kids kept in field conditions but the difference was non-significant like and reported higher prevalence up to 48.14% and 100% respectively in kids kept in farm conditions (Dixit *et al.* 2018). The variation in the rate of occurrence could be attributed to a varied factors including over-crowding, seasonal stress, selection of study population, contamination of premises, animal husbandry practices, age of the animal, consistency of dung, concurrent infections and nutritional factors of goats sampled (Castro-Hermida *et al.* 2002a, Castro-Hermida *et al.* 2002b).

Another explanation is that the sampling was done only once per animal in the present study. If that specimen was identified as negative during a period when the animal was experiencing intermittent shedding of oocysts, the animal would be considered as negative (Castro-Hermida *et al.* 2007). But in some other studies the sampling might be a longitudinal or multiple per animal, so that the occurrence rate may vary to the present findings (Yang *et al.* 2014).

The PCR-RFLP findings were in agreement with the study conducted by pan-researchers (Dixit*et al.* 2018, Wang *et al.* 2014, Koinari *et al.* 2014, Singla *et al.* 2013, Lorenz *et al.* 2011, Geurden *et al.* 2008). In the present study for genotyping of *Cryptosporidium* species, *Mbo*II was useful in differentiating the *C. bovis*and *C. parvum* in cases where isolates could not be sequenced. Furthermore, PCR amplification of the 18SSU rRNA gene followed by *Ssp*I and *Vsp*I digestion failed to differentiate these species due to analogous digestion pattern. The use of *Mbo*II may be an economical alternative to sequencing for species identification (Feng *et al.* 2007). Thus, this method may be beneficial in identifying species in mixed infections, where direct sequencing of PCR products is likely to be unsuccessful (Feltus *et al.* 2008). In this study, RFLP is considered as discriminatory, less time-consuming and more cost effective alternative to sequencing for typing of *Cryptosporidium* isolates from domestic animals.

Sequence analysis of GP60 gene is often used in *Cryptosporidium* subtyping due its sequence heterogeneity and relevance to parasite biology. It is said to be most single polymorphic marker identified so far in the *Cryptosporidium* genome (Wielinga *et al.* 2008). Unlike other subtyping targets, such as double stranded RNA, which are generally considered non-functional, GP60 is located on the surface of apical region of invasive stages of the parasite, and is one of the dominant targets for neutralizing antibody responses (Xiao 2010). The codon no. 50 is actually changed from CCA→CCC which is proline for both the cases which means that there is a silent mutation in the 275thnt that encompasses in codon-50 and it is due to synonymous single nucleotide mutation. In a study conducted at PGIMER, Chandigarh, India in Human patients, phylogenetic analysis of *C. hominis* and *C. parvum* were identified belonging to eight subgenotype families. Most *C. hominis* were a part of subtype alleles Ie, Ia and Id, while *C. parvum* belonged to IIc, IId and IIe (Sharma *et al.* 2013). In a study conducted in Poland(Kaupke *et al.* 2017) subsequent GP60 subtyping revealed the presence of *C. parvum* IIdA23G1 subtype (detected for the first time) in goats whereas from the present study it can be corroborated based on the phylogenetic and sequence analysis that the subtype identified in the field isolate of *C. parvum* strain 50 NDVSU might be from the family IIe which is very similar to the information manifested in studies where 3% of the positive isolates belonged to this family (IIe) in humans (Khalil *et al.* 2017). Further, similar findings have been reported in the earlier studies from India (Sharma *et al.* 2013) where it was identified as subtype allele IIe (IIeA7G1) from humans. Based on the reports, it was understood that subtype allele IIa and IId are predominantly encountered in bovines (Khalil *et al.* 2017) and small ruminants respectively (Kaupke *et al.* 2017). But in the current study, here we report the presence of allele sub-family *C. parvum*IIe, which iterates the involvement of human origin subtypes in the caprine cryptosporidiosis. Further sequencing of multiple isolates could reveal the transmission dynamics and the involvement of other environmental factors leading to zoonotic infection in humans.

In the current study the COWP gene components were quantified for specific transcripts in the total RNA extracted from cryptosporidial oocysts. Of the three samples with high shedding of cryptosporidial oocysts in faeces, only two

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had shown transcriptional activity for all the three COWP genes viz., COWP3, 4 and 6. This shows that the shedding can be passive viz., dead oocysts which are not infective in nature and is cleared from the system in maintaining the immunological balance or active oocysts that are highly infective as evidenced by the high transcriptional activity. By this finding, it is also evident that merely microscopy cannot be an indicator of the immunopathology of the disease and it can only be a tool for assessing the presence in a herd. In a similar study from our lab, Sharma *et al* (2022) has developed a duplex reverse transcription TaqMan probe based Real-time PCR assay which targeted two genes viz., 18ssrRNA and COWP, which differentiated transcriptionally active cryptosporidial oocysts from that of dead ones. But the current study differentiated transcriptionally active COWP subunits viz., 3,4,6 and 9 and also quantified their absolute numbers and relative quantification.

The genetic expression of COWPs differs due to the variation in the ultra-structure of the cell wall protein of the oocyst. In this multigene family there are Type I and Type II domains along with histidine repeats and cysteine rich motifs which have participation in intermolecular cross-linking that provides stability to the oocyst wall (Del Coco *et al.* 2014). COWP 1, 2 and 3 genes have both type I and type II domains whereas COWP 4, 5, 6, 7 and 8 have only type I domain however, in COWP 9 there is only type I domain and less striking cysteine rich repeats which makes it less closely related to this family. In the present study COWP 6, 4, 9 and 3 was in correlation to RNA-Seq analysis where COWP 1, 6 and 8 being the most abundant, followed by significantly lower levels of COWPs 2, 3 and 4. However, COWPs 5, 7, and 9 are present in very low amounts (Templeton *et al.* 2004, Lippuner *et al.* 2018). COWP is found in the wall-forming bodies of early and late macrogametes, respectively which infer that expression of COWP genes is specific to macrogametocytes; they are good indicators for sexual stage development (Chatterjee *et al.* 2010).

Conclusion

In microscopy or nested PCR, it is difficult to differentiate the infectious and not-infectious oocysts. It is assumed that only live oocysts will be transcriptionally active compared to the dead one. In this study, we targeted the cryptosporidial oocysts wall protein (COWP) which is actively expressed during gametogony stage. The thick walled oocysts which abundantly express the COWP would mean active contagious infection, while thin walled also expresses the same leading to internal sporulation. In this aspect, our current study on qRT-PCR of various COWP components viz., COWP3, COWP4, COWP6 and COWP9 will be useful in assessing the stage of the infection based on the oocysts wall components expressed. The transcript copies for each COWP component were computed based on interpolation with the standard curve, generating data on the infection status, whether the infection is in peak or self-limiting. The functional significance of this multigene family can now be addressed through recent advances in molecular biology, and may lead to the identification of viable drug and vaccine targets for control of Cryptosporidiosis.

Author's Contributions

Supriya Sachan: Methodology, Investigation and original draft writing. Gururaj Kumaresan: Conceptualization, experimental design, Supervision, Project administration, writing- review & editing. Dinesh Kumar Sharma: Data analysis, writing- review & editing. Giridhari Das: Methodology, supervision. Suman Kumar: Conceptualization, Supervision, writing- review & editing. Ravi Khare: Visualization and Investigation, writing- review & editing. Anjali Pachoori: Methodology, experimentation and formal analysis. Souvik Paul: Investigation, Formal Analysis and Funding.

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