Standardization and evaluation of DNA extraction protocol for Cryptosporidium oocysts from faecal samples in goats

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Abstract

DNA extraction from stools is the major hurdle in the detection of *Cryptosporidium* infection due to complex oocyst wall and presence of PCR inhibitors. There is no conventional full-proof DNA extraction method which efficiently recovers DNA from *Cryptosporidium*. Alternatively, commercial DNA kits costs dearer and unaffordable in smaller laboratories with limited funding. To address this, the current study explored for an efficient DNA extraction method from *Cryptosporidium* oocysts. Four different DNA-extraction methodologies were developed at different Cetyltrimethylammonium Bromideconcentrations and temperature-cycles and analyzed for quality-quantity parameters. Four different types of recipes were used, in brief which includes 1. Sonication+3 cycles of chill-thaw+ (24:1) Chloroform:Isoamyl alcohol, 2. Liquid Nitrogen+ thaw (@70ºC)+ (24:1) Chloroform: Isoamyl alcohol, 3. Sonication + 3 cycles of freeze that + (24:1) Chloroform: Isoamyl alcohol and 4. Three cycles of 'snap-chill (@-80ºC) and boil (@95ºC) +(24:1) Chloroform: Isoamyl alcohol'. Among these, a protocol involving three cycles of 'snap-chill and boil' (Method-4) could successfully recover *Cryptosporidium* DNA with better quality-quantity parameters with consistency and repeatability and lack of PCR inhibitors as evidenced by the workability of this method confirmed by conventional-PCR and real-time PCR for 18 small-subunit-ribosomal RNA (*18SSU rRNA*). The repeated deep freezer and boil cycles successfully disrupted the thick chitin-rich oocyst wall of *Cryptosporidium* leading to precipitation of nucleic acids by chloroform-isoamyl alcohol. The current study aims to introduce a cost-effective method that overcomes the bottlenecks faced with the conventional DNA extraction techniques for *Cryptosporidium* directly from faecal samples.

Standardization and evaluation of DNA extraction protocol for *Cryptosporidium* oocysts from faecal samples in goats

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ABSTRACT

DNA extraction from stools is the major hurdle in the detection of *Cryptosporidium* infection due to complex oocyst wall and presence of PCR inhibitors. There is no conventional full-proof DNA extraction method which efficiently recovers DNA from *Cryptosporidium* . Alternatively, commercial DNA kits costs dearer and unaffordable in smaller laboratories with limited funding. To address this, the current study explored for an efficient DNA extraction method from *Cryptosporidium* oocysts. Four different DNA-extraction methodologies were developed at different Cetyltrimethylammonium Bromideconcentrations and temperature-cycles and analyzed for quality-quantity parameters. Four different types of recipes were used, in brief which includes 1. Sonication+3 cycles of chill-thaw+ (24:1) Chloroform:Isoamyl alcohol, 2. Liquid Nitrogen+ thaw (@70^{o}C)+ (24:1) Chloroform: Isoamyl alcohol, 3. Sonication + 3 cycles of freeze that + (24:1) Chloroform: Isoamyl alcohol and 4. Three cycles of 'snap-chill (@-80^{o}C) and boil (@95^{o}C) +(24:1) Chloroform: Isoamyl alcohol'. Among these, a protocol involving three cycles of 'snap-chill and boil' (Method-4) could successfully recover *Cryptosporidium* DNA with better quality-quantity parameters with consistency and repeatability and lack of PCR inhibitors as evidenced by the workability of this method confirmed by conventional-PCR and real-time PCR for 18 small-subunit-ribosomal RNA (*18SSU rRNA*). The repeated deep freezer and boil cycles successfully disrupted the thick chitin-rich oocyst wall of *Cryptosporidium* leading to precipitation of nucleic acids by chloroform-isoamyl alcohol. The current study aims to introduce a cost-effective method that overcomes the bottlenecks faced with the conventional DNA extraction techniques for *Cryptosporidium* directly from faecal samples.

Keywords: *Cryptosporidium* oocyst wall**,** DNA extraction, Faecal PCR inhibitors, Freeze-Boil, Sonication.

Significance of the study

- This method proved that it is simple and highly economical for laboratories with minimal funding, and on the other hand commercial faecal DNA kits are very costly and offer a limited sample size for the extraction of *Cryptosporidium* genomic DNA.
- Method 4 with minimal steps devoid of sonication and LN_2 steps could extract good quality DNA using the freeze-boil-CTAB method as assessed by validation with conventional PCR and real-time-PCR (RT-PCR).
- Most importantly this conventional protocol for DNA extraction developed in the current study could match the spin column-based kits in terms of successful removal of the PCR inhibitors as evidenced by the successful replication of results by molecular tests.

1. INTRODUCTION

Cryptosporidium is an intestinal protozoan parasite that infects a wide range of livestock and vertebrate hosts[1,2]. It is an opportunistic pathogen responsible for gastrointestinal disease, especially in the immune-compromised host[3]). *Cryptosporidium* infection is also responsible for mortality and morbidity in severely affected animals and less than five years of children lead to diarrhoea, weakness, weight loss and late maturity, reported 526,000 deaths annually worldwide in 2015 [4]. *Cryptosporidium* was detected worldwide in young sheep and goats in diarrheic and non-diarrheic conditions with a higher rate of prevalence. *Cryptosporidium*infection has been reported highest in Spain (62.7%) and Mexico (72.5%) in goat kids [5,6].

The oocysts of various *Cryptosporidium* species infecting humans and livestock are not readily distinguishable based on morphological characteristics by the conventional diagnostics or microscopic examination [7]. Moreover, microscopy-based methods require skilled personnel and are labour-intensive[8]. Nowadays, several molecular techniques have been developed to overcome this problem and increasing as first-line diagnosis procedures for the identification of *Cryptosporidium*infection in faecal samples [9,10]. A wide range of polymerase chain reaction (PCR) assays are available with higher sensitivity and specificity including simplex PCR[11-13], multiplex real-time PCR and duplex qRT-PCR for detecting *Cryptosporidium* spp. with the stage of infection and differentiating the live and dead oocysts[14]. Molecular assays are highly sensitive to the purity and quality of DNA material.

However, DNA extraction by conventional method is a multi-step procedure by using various reagents and incubation steps. The*Cryptosporidium* oocysts consist of three layers of filamentous glycoprotein (oocyst

wall protein (OWP)) and acid-fast lipids. COWP play a major structural and functional role in *Cryptosporidium*oocysts. The structural make-upof COWP comprises of two different amino acid motifs: Type I and Type II repeatspresent with six cysteine residues and the higher amount of glycine and proline residues. The structural arrangement of COWP consist two major domains: amino-terminal domain and carboxyl-terminal domain. The signal peptide (extracellular localization)of COWP consist 1622 amino acids, first 698 amino acids consist in ten Type I repeats of approximately 65 amino each and 772 amino acids consist in eight Type II repeats of approximately 53 amino acids. This complex structure of *Cryptosporidium* oocysts wall haywires the DNA extraction by conventional methods[15-18]. So there is dire necessity for an effective DNA extraction method that could effectively break the thick-walled oocyst of *Cryptosporidium* spp. and remove the PCR inhibitors from the faecal samples [19-26]. Also PCR based downstream analysis (PCR-RFLP, sequencing and phylogenetic analysis) offers better understanding in the disease dynamics compared to microscopy, while the latter also cannot assess the type of strains and sub-types of *Cryptosporidium* species in a particular geo-climatic zone. DNA extraction is the key bottleneck that needs to be addressed for a successful geo-epidemiological disease dynamics and effective management of cryptosporidiosis.

Hence in the current study, to overcome these constraints, a new conventional DNA extraction protocol was developed and evaluated for rapid screening of*Cryptosporidium* spp. in faecal samples by conventional and real-time PCR. The aim of this study is also to compare the current method with commercially available kits for DNA extraction in terms of quality, quantity and workability in PCR based tests.

2. MATERIALS AND METHODS

2.1. Collection of faecal sample

Faecal samples were collected (October 2018 to October 2021) from ICAR- Central Institute for Research on Goats Mathura, Uttar Pradesh, India (27.100N, 78.020 E and 169.2 m above MSL) at Barbari (382), Jamunapari (217) and Jakhrana (45) breeds of goats. The samples were collected from both diarrhoeic and non-diarrhoeic animals, confirmed by microscopy using modified Ziehl-Neelsen's staining (mZN staining), the standard method for identification of *Cryptosporidium* oocysts. Also known purified positive cryptosporidial oocysts (5 x 10^5) were spiked to known negative faecal samples (per gram) as an internal control for assessing the workability of the various DNA extraction methods as described in the following sections.

2.2. Ethical Approval

Ethical approval for collection of bio samples used in the current study was obtained from the Institute animal ethics committee (IAEC) vides 05/1010680/dated 20.02.2021.

2.3. Solution used

Extraction buffer was prepared in 2.0% cetyltrimethylammonium bromide (CTAB), which consist 100mMTris-HCl (pH 8.0), 1.4 M NaCl, 20mM ethylenediaminetetraaceticacid (EDTA). Absolute Isopropanol, 70% ethanol (v/v); Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v), Chloroform: Isoamyl alcohol (24:1, v/v), TE (Tris/EDTA) buffer preparation: 10mM Tris-HCl (pH 8.0); and 1mM EDTA (pH 7.4).

2.4. Method 1

Initially, in method 1, the physical force in the form of sonication is applied to the sample (kept in falcon tubes placed in ice) to rupture the cell wall at medium amplitude with three cycles per sample. The sonicated samples are transferred to a new microcentrifuge tube and spiked with 800 µl of 2% CTAB isolation buffer, followed by three cycles of snap-chill-thaw, and added 800 µl of Chloroform: isoamyl alcohol (24:1) and finally with 600 µl Phenol: Chloroform: isoamyl alcohol (25:24:1) with supernatant discarded and pelleted by centrifugation for each of the above reagent steps. The pelleted DNA was subsequently washed with 1 ml of 70% (70%EtOH+30% Nuclease free water V/V) ice-cold ethanol and re-suspended in 30-40 µl of TE buffer (pH 8) and quality/quantity checked using Quantus fluorimeter method (Cat# E6150 Promega Technologies, Madison, USA).

2.5. Method 2

The major challenge for the extraction of *Cryptosporidium* DNA is to lyse the thick wall of oocysts. Since pretreatment steps like snap-freeze and thawing facilitate rupture of the oocyst wall, we have employed the same using Liquid nitrogen (-196C) and incubation at 70C respectively, instead of the sonication step used in the previous method. The remaining steps/procedure performed was similar to method 1.

2.6. Method 3

In this method, the samples were subjected to both sonication and snap-freeze-thaw method (as per Method 2) for efficient lysis of the cell wall of *Cryptosporidium* oocysts. The rest protocol was same as method 1

2.7. Method 4

In this method sonication step was not included, instead, the 'snap-freeze-thaw' method is replaced with snap-chill (at -80C) for 10 minutes and thaw (with higher temperature) at 95C (instead of 70C) for 5 minutes and this snap-chill-boil cycle repeated two more times with vortexing for 30 seconds between each cycle (Table 1). 10 µl of DNase-Free-RNase were spiked in the sample after cooling in ice for 2 minutes and incubated at 37^{o}C for 10 minutes to improve the quality of DNA, The remaining protocol was performed as per method 1.

(Table 1 here)

2.8. Commercial Kits I and II

To further assess and compare the developed DNA extraction process from faecal samples spiked with cryptosporidial oocysts, we have used commercial kits (spin column based faecal specific DNA extraction kits) manufactured from two standard companies (Promega and Nucleopore) as positive internal control. The protocols followed were strictly adhered according to the manufacturer's instructions.

2.9. Quantification of DNA

The obtained DNA was quantified by Quantus^{TM}Fluorometer® (Cat# E6150 Promega Technologies, Madison, USA) as per the manufacturer's protocol and kept at -20 °C for further use in molecular diagnosis.

2.10. Qualitative analysis of DNA

Qualitative analysis of extracted DNA from goat faecal samples was done by 1.0% agarose gel electrophoresis. Agarose gel was stained with ethidium bromide (EtBr) and run in 1x TAE buffer along with a 100bp DNA ladder. The DNA presence was confirmed after gel electrophoresis under the UV trans-illuminator and photographed using a gel documentation system.

2.11. Conventional PCR amplification of *18SSU rRNA* gene

The PCR product for *18SSU rRNA* gene of about 1325bp was amplified by primary PCR from each sample using the oligonucleotide primers Cryp_18ssuP_F (5´-TTCTAGAGCTAATACATGCG-3´) and Cryp_18ssuP_R (5´-CCCTAATCCTTCGAAACAGGA-3´) in the primary PCR[27]. The PCR was carried out in 20µl volume containing 2.0µl (10ng/µl) of genomic DNA, 10µl 2X Master Mix (Takara, Prime Star Max 2X PCR Master Mix), 2µl Forward Primer, 2µl Reverse Primer, and 4µl Nuclease Free Water up to 20µl. The reaction mixture was initially incubated at 94°C for 5 min for initial denaturation and then a total 35 cycles of reaction were performed with denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min, strand extension at 72°C for 1 min. The final extension was done at 72°C for 10 min.

For the secondary/nested PCR product of 834bp was amplified, 2µl of the primary product was used as the template and primers viz., Cryp_18ssuS_F (5´ -GGAAGGGTTGTATTTATTAGATAAAG-3´) and Cryp_18ssuS_R (5´-AAGGAGTAAGGAACAACCTCCA-3´) were used [27]. The PCR reaction and the cycling conditions were the same as for the primary PCR was used in the nested PCR and the annealing temperature was 57°C. The amplification of specific PCR products was checked by gel electrophoresis in 1.8% agarose.

2.12. TaqMan® probe based Real Time PCR for *18SSU rRNA*

For *18SSU rRNA* TaqMan® probe Real-time PCR, we have used the oligonucleotide primers and probes designed in-house[14] in a previous study. The real-time PCR master mix was prepared in duplicates for each sample in a final reaction volume of 25µl in 8 strip PCR tubes with optically compatible caps assayed in the real-time thermal cycler (CFX96 Real-time PCR system®, Bio-Rad) with the reagent preparation and thermal conditions as mentioned in the table (Table 2). The 109bp amplicon post-conventional PCR was confirmed by gel electrophoresis.

(Table 2 here)

In the present study, CTAB method was used for the DNA extraction from *Cryptosporidium* oocysts. The major problems of *Cryptosporidium* DNA extraction were associated with the presence of thick wall of *Cryptosporidium* oocysts and other organic and bile salts available in faecal material that inhibits PCR enzymes.In the current study, extractions weredone with CTAB buffer that effectively removes the faecal organic compounds and act as a detergent which solubilizes the cell wall, lipid membrane and denature the protein of *Cryptosporidium* oocysts along with thermal manipulation steps.

In the present study, DNA extraction from *Cryptosporidium* oocysts was standardized by the CTAB method. The main bottlenecks of *Cryptosporidium* DNA extraction are due to the thick *Cryptosporidium* oocyst wall and other organic and bile salts available in faecal material that inhibits PCR enzymes. In the current study, CTAB based extractions has effectively managed to remove the faecal organic compounds and with its detergent action, it solubilized the cell wall, lipid membrane and denature the protein of *Cryptosporidium* oocystsalong with thermal manipulation steps.

3. RESULTS AND DISCUSSION

3.1. Modified DNA extraction procedure (Method 4)

The final DNA extraction protocol was developed which gives a higher yield that also facilitates better purity of the DNA. Briefly, described the procedure for efficient DNA extraction, a total of 500 µl of faecal lavage/washing (Suspended in 1X PBS) was taken in a 2 ml microcentrifuge tube. The microcentrifuge tube was further added with 800 µl of CTAB isolation buffer (100mMTris-HCl, pH-8; 1.5M NaCl, 25mM EDTA (no salt); 2.5% CTAB) and vortexed for 30 seconds. The mixture was frozen in a -80^{o}C deep freezer for 15minutes. The frozen mixture was transferred to a -20C cooler to carry and immediately given heat shock in a water bath at 95^{o}C for 5 minutes. This cycle was repeated two more times (a total of 3 cycles of freeze and boil) with brief vortexing for 30 seconds between each cycle. The mixture was centrifuged at 12000rpm for 5 minutes at room temperature (RT; 28°C). Following this, ˜800 µl supernatant was carefully collected and transferred to a new sterile 1.5 ml microcentrifuge tube and spiked with 10 µl of DNase-Free-RNase mix (Cat# 12091021, Invitrogen). The mixture was then incubated in the water bath at 37°C for 20 minutes followed by the addition of an equal volume (˜800 µl) of Phenol: chloroform: isoamyl alcohol (25:24:1) mixture and mixed vigorously through vortexed for 30 seconds till the content turned white/milky emulsion and centrifuged (conditions as above). Approximately ˜600µl of the upper aqueous phase was collected in a new microcentrifuge tube and added an equal volume of chloroform: isoamyl alcohol (24:1) reagent mixed vigorously through vortexed to form a white emulsion and centrifuged again. 500µl of the upper aqueous phase was transferred in a new microcentrifuge tube and added 0.8 volume of (˜400 µl) of isopropanol. The content of the tube was mixed by gently inverting the tubes several times and incubating the mixture at -20°C for 30 minutes, centrifuging at 12000rpm for 10 minutes at room temperature. The supernatant was carefully discarded without disturbing the DNA pellet at the bottom. The pellet was further washed with 1 ml of 70% ice-cold ethanol (70% ethanol + 30% Nuclease free water V/V) and centrifuged; the step was repeated. After washing, ethanol was removed by carefully decanting the content and the pellet was dried in the fume hood for 5 minutes. The DNA pellet was re-suspended in 30-40µl of TE buffer (pH 8) and stored at -20^{o}C for further molecular analysis.

3.2. Quantification of DNA

The DNA thus extracted was measured for its concentration by Quantus™ Fluorometer® as per the ma-

nufacturer's protocol. For the method 1, the final concentration of genomic DNA was 4.11ng/µl, similarly, in method 2, method 3 and method 4 are 11.49ng/µl, 17.81 ng/µl and 53.98ng/µl respectively (Figure 1). The concentration of method 4 is higher than the other three methods and approximately equal to the commercial faecal DNA extraction Kits 1 and 2. These commercial kits were used as an internal quality control while extracting the DNA by current methods. The DNA yield obtained for Kit 1 and 2 is 49.76ng/µl and 56.93ng/µl respectively.

(Figure 1 here)

3.3. Qualitative analysis of DNA

Present protocol (Method 4) overcomes the issue of difficulty in lysing the thick oocyst wall and further removing the contaminating agents that act as PCR inhibitors. The four critical changes incorporated with this protocol; 2.5% CTAB, freeze at -80^{o}C and boil at 95^{o}C as 'snap-chill-boil' instead of 'freeze-thaw' and proteinase-K steps, followed by treatment with DNase-Free-RNase mix to enhance the DNA quality and final precipitation of DNA with isopropanol and also considerably reducing the time taken for the total process. The results showed that the modified DNA extraction protocol in the current study generated DNA with high purity and workability, as evidenced by the absence of RNA contamination during gel electrophoresis (Figure 2A) and Nested PCR amplification of *18SSU rRNA* gene showed the *Cryptosporidium* positive faecal on 1.8% agarose gel stained with ethidium bromide respectively (Figure 2B).

(Figure 2A and 2B here)

3.4. TaqMan® probe based Real Time PCR targeting *18SSU rRNA* gene

Further to the confirmation by "*18SSU rRNA* nested PCR", the goat faecal DNA was also validated with the TaqMan®probe-based Real-Time PCR amplification (Table 3). The results were encouraging as evidenced by a higher 'RFU' value and better 'Cq' value in the amplification plots. TaqMan® probe-based Real-Time PCR confirmed the purity and yield of the extracted DNA from the modified CTAB extraction protocol (Figure 3).

(Figure 3 and Table 3 here)

4. DISCUSSION

Cryptosporidium oocyst wall is highly resistant to physical disruption due to its constituent integral membrane protein and short hydrophobic regions at signal peptide. All COWP subunits are present inside the inner oocyst wall and in wall-forming bodies of mature macrogametes. COWP1 has a striking cysteine periodicity with two cysteine-rich domains, with well conserved motifs. Further, an extensive disulfide-bonded globular structure or intermolecular disulfide bonds adds rigidity to the Cryptosporidium oocyst wall[15,28,29]. The current study explored four different DNA extraction protocols to assist oocyst wall disruption with varying the thermal conditions and reagents concentrations. All the four methods were CTAB-based and attempted on positive *Cryptosporidium*spp. oocyst spiked faecal samples till reproduction of consistent results. The conditions used in the Method 4 were effective in reproducing the results comparable to that of commercial faecal DNA extraction kits. The protocol thus developed was simple, less time consuming and economical with no requirement of additional equipment like the Mini Bead-beater or sonicator for oocyst wall disruption.

Molecular techniques demands DNA of high purity and quality for PCR and sequencing analysis that aids in the detection and characterization of *Cryptosporidium* spp. from faecal samples[30]. The difficulty that arises while extracting DNA from faecal samples is the presence of bile salts and other PCR inhibitors directly influencing the efficiency of PCR despite good recovery rate after disintegrating the thick-walled *Cryptosporidium* oocysts. This protocol could also prove useful in cases of autoinfection with thin walled oocysts or in subclinical conditions with minimal faecal oocyst shedding that goes unnoticed by microscopy and conventional DNA extraction based PCR.

Traditionally, the microscopy based detection method is employed for confirmation of *Cryptosporidium* infection, while it cannot differentiate the species and subtypes of *Cryptosporidium* species that will be useful in

the epidemiological analysis and disease dynamics. On the contrary, molecular techniques could aid species differentiation, possibly further characterization by sub-type grouping and phylogenetic analysis using sequencing methods. Similarly, Real-time PCR can also be an alternate for conventional PCR with its ability for quick detection and non-sequencing based characterization like 'endpoint genotyping' or multiplexing [14,31]. Hence, in the current study, we have used *18SSU rRNA* gene-based TaqMan® probe real-time PCR to assess the effectiveness of our extracted DNA for such protocols. Based on the machine call, we obtained encouraging results for Method 4, while Method 3, despite failing in conventional PCR could detect the cryptosporidium in *18SSU rRNA* gene-based TaqMan® probe real-time PCR.

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Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

Authors declare that they have no competing interests.

Authors' contributions

AKS and KG conceptualized the research work and designed the work plan. AKS also conducted the experimented and wrote the manuscript. RS analysed data. AG analysed data and edited the Manuscript. SP and DKS designed the experiment protocols and analysed the data. All authors reviewed and approved the manuscript.

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Legends to Figures

Figure 1: Total DNA extracted from *Cryptosporidium*faecal sample (Lane 1- Method 1, lane 2- Method 2, lane 3- Method 3, Lane4- Method 4, Lane 5- Kit 1, Lane 6- Kit 2) using present modified method visible on 1.8% agarose gel stained with ethidium bromide. Lane M is 100bp ladder.

Figure 2 (A): Cyclic conditions for DNA duplex TaqMan® probe real time PCR (**B)** Nested PCR amplicon of *18SSU rRNA* gene on 1.8% agarose gel with arrow indicates the *18SSU rRNA* gene product (Lane 1- Method 1, lane 2- Method 2, lane 3- Method 3, Lane4- Method 4, Lane 5- Kit 1, Lane 6- Kit 2) and DNA ladder (M is 100bp ladder).

Figure 3: TaqMan® probe based real time PCR amplification of *18SSUr RNA* gene for method 1 to 4 with No template Control (NTC).

Table 1 . Comparison of different DNA Extraction methods

S.N.	Components/Steps	Method 1	Method 2	Method 3	Method 4
1	CTAB	2%	2%	2.5%	2.5%
2	Sonication	3 cycles at high amplitute	No	3 cycles at high amplitute	No
3	FREEZE-THAW	3 cycles at 70°C and -20°C	3 cycles at 70°C and Liquid Nitrogen	3 cycles at 70°C and Liquid Nitrogen	No
4	FREEZE-BOIL	No	No	No	3 cycles at 95°C for 5 minutes, -80°C for 10 minutes
5	DNase-Free-RNase	No	No	10µl/ml	10µl/ml
6	Chloroform: isoamyl alcohol (24:1)	˜800 µl	˜800 µl	˜800 µl	˜800 µl

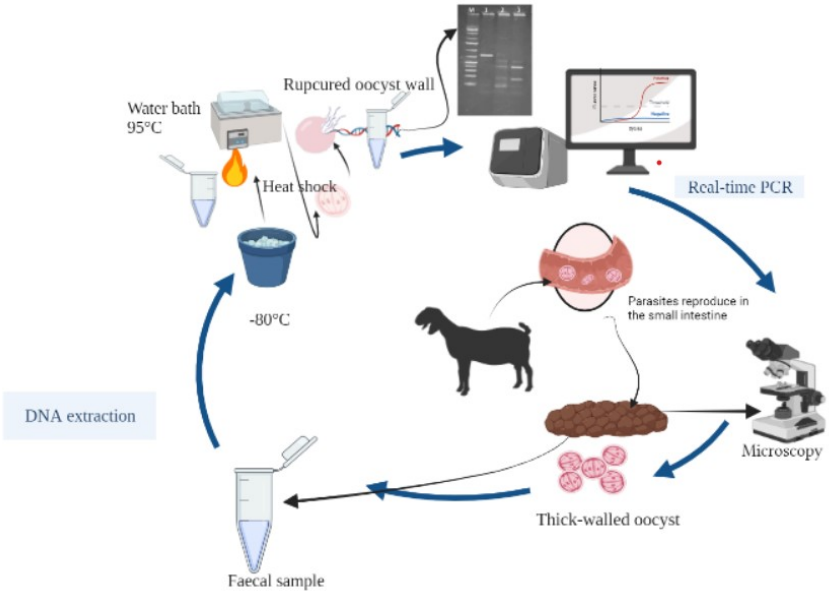
S.N.	Components/Steps	Method 1	Method 2	Method 3	Method 4
7	Phenol: Chloroform: isoamyl alcohol (25:24:1)	˜600 µl	˜600 µl	˜600 µl	˜600 µl
8	Isopropanol	˜400 µl	˜400 µl	˜400 µl	˜400 µl
9	Ice-cold Ethanol 70%	1ml, twice	1ml, twice	1ml, twice	1ml, twice
10	Nucleus Free Water	30 µl	30 µl	30 µl	30 µl
11	18ssu rRNA PCR	No	No	Yes	Yes

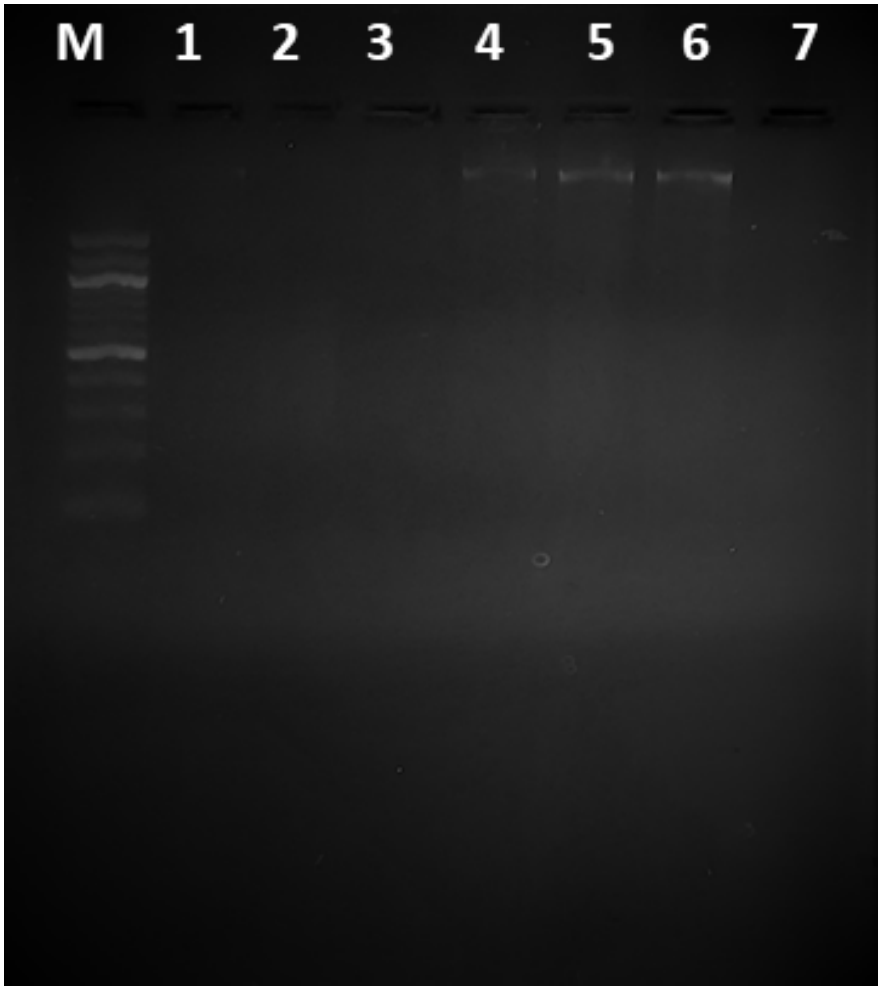
Table 2. Concentration and volume of reagents used in TaqMan® Probe based real-time PCR targeting *18SSUrRNA*

	Reagent	**Reagent**	**Volume**	**Conc. Used**
	Premix Ex Taq Probe qPCR Master Mix (cat# RR390A,TaKaRa, Japan)	Premix Ex Taq Probe qPCR Master Mix (cat# RR390A,TaKaRa, Japan)	12.5 µl	2x
3.	*188ssu rRNA*(HEX)	Forward primer	1.0 µl	(10 pmol/µl)
		Reverse primer	1.0 µl	(10 pmol/µl)
		Probe	1.0 µl	(10 pmol/µl)
5.	Template DNA	Template DNA	µl	(˜10ng)
6.	Nucleus Free Water	Nucleus Free Water	8.5µl	-
Total	**Total**	**Total**	**25 µλ**	**25 µλ**

Table 3. Percent positivity of cryptosporidia observed in samples collected from various breeds using conventional PCR (*18SSU rRNA*) and TaqMan® probe based Real Time PCR for *18SSU rRNA* assays.

Breeds	**Clinical Status**	**Total Sampled**	**Percent positivity by diagnostic Tests**	**Percent positivity by**
			Microscopy	*18SSUrRNA* **nested PC**
Barbari	Diarrheic	309	113(36.57%)	165(53.40%)
	Non-diarrheic	73	9(12.33%)	14(19.18%)
Jamunapari	Diarrheic	162	55(33.95%)	81(50.00%)
	Non-diarrheic	55	7(12.73)	8(14.55%)
Jakhrana	Diarrheic	27	11(40.74%)	14(51.85%)
	Non-diarrheic	18	2(11.11%)	2(11.11%)
Total		**644**	**197(30.59%)**	**284(44.10%)**





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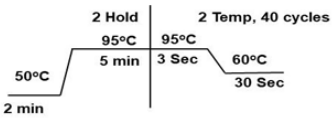

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