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Molecular Identification of *Eimeria* Species in Desi Chickens Using ITS-1 rDNA PCR Analysis

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ABSTRACT

Eimeria (E.) species are protozoan parasites responsible for coccidiosis, a widespread and economically significant disease affecting all chicken breeds. Coccidiosis negatively impacts poultry health and performance, leading to substantial economic losses in the global poultry sector. The present study aimed to assess the current status of coccidiosis in desi chickens reared on farms and processed at retail poultry dressing centers in Bengaluru, Karnataka, India. A polymerase chain reaction (PCR) assay targeting the internal transcribed spacer one (ITS-1) region of ribosomal DNA (rDNA) was performed to identify *Eimeria* species in desi chickens (dual purpose of broilers and layers) aged one to eight weeks. Among different DNA extraction methods tested for *Eimeria* oocysts in desi chickens, including liquid nitrogen, hypochlorite, direct, sonication, and glass bead techniques, the glass bead method proved to be the most efficient for oocyst disruption and genomic DNA extraction. Two species, *E. tenella* and *E. acervulina*, were successfully identified based on distinct bands of 278 base pairs (bp) and 145 bp, respectively, observed on a 2% agarose gel. The minimum number of oocysts required for DNA extraction was 32 oocysts (0.10 ng) for *E. tenella* and 127 oocysts (0.41 ng) for *E. acervulina*. Amplicon sizes of 278 bp for *E. tenella* and 145 bp for *E. acervulina* were consistently obtained. Among the identified species in the present study, *E. tenella* was the most predominant cause of coccidiosis in desi chickens.

Keywords: Characterization, Coccidiosis, Desi chicken, *Eimeria*, Identification, Internal transcribed spacer one, PCR, Ribosomal DNA

INTRODUCTION

Eimeria (*E.*) species, classified under the kingdom *Protozoa*, phylum *Apicomplexa*, class *Coccidia*, order *Eucoccidiorida*, family *Eimeriidae*, and genus *Eimeria*, are intracellular protozoan parasites that can lead to coccidiosis in poultry (Taylor et al., 2007). Seven *Eimeria* species have been identified in chickens, including *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*. While *E. acervulina*, *E. maxima*, *E. mitis*, and *E. praecox* exhibit relatively low pathogenicity, *E. tenella*, *E. necatrix*, and *E. brunetti* are highly pathogenic (Ahmad et al., 2024). The oocysts of Eimeria species exhibit remarkable resilience, allowing them to survive in poultry litter for several months despite exposure to different environmental conditions and disinfectants (Saeed and Alkheraije, 2023). Coccidiosis has been studied most extensively in commercial poultry, resulting in significant economic losses due to overcrowding. Coccidiosis is emerging in desi chickens due to overcrowding caused by the transition from a backyard rearing system to an intensive system (Saravanajayam et al., 2016).

Coccidiosis is highly prevalent in young chickens, and outbreaks often occur when the chickens are between three to eight weeks old. Feed and water can become contaminated with *Eimeria* oocysts as a result of fecal shedding by infected chickens, facilitating transmission through ingestion. Coccidiosis can spread to other farms through fecal contamination on workers and equipment. Both clinical and subclinical types of poultry coccidiosis can infect chickens (Mathis et al., 2024). Dehydration, lower feed intake, weight loss, mortality, and conspicuous diarrhea with or without blood are the clinical manifestations (Gussem, 2006). Kumar et al. (2014) highlighted the importance of molecular identification of *Eimeria* species, which became feasible through gene sequencing in 2002, utilizing unique conserved genes for each species.

Over the past two decades, different polymerase chain reaction (PCR) assays have been developed to target specific genomic regions of *Eimeria* species, including the small subunit rRNA and the *E. tenella 5S* gene (Fatoba and Adeleke,

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2018). The first and second internal transcribed spacer regions (ITS-1 and ITS-2) have been widely used for the molecular characterization of all the *Eimeria* species (Gasser et al., 2001). Traditional non-quantitative PCR methods have been employed for the molecular identification of seven *Eimeria* species that infect chickens, with one method specifically targeting the ITS-1 sequence-characterized amplified regions (Haug et al., 2007).

A review of existing literature revealed that there has been no comprehensive study on the occurrence and molecular identification of *Eimeria* species in desi chickens. Consequently, the present study was conducted to assess the morphological and molecular characterization of *Eimeria* species by amplifying the ITS-1 regions of ribosomal DNA (rDNA) that affect desi chickens in Bengaluru, Karnataka, India.

MATERIALS AND METHODS

Ethical approval

The present study did not involve any invasive procedures or experimental treatment on live animals. Samples were collected non-invasively (chicken droppings, intestines from slaughtered chickens, and litter materials from chicken farms or dead chickens); therefore, ethical approval was not required according to the university guidelines of KVAFSU in India**.**

Study area and sample collection

The study was conducted from September 2019 to December 2020. From a total of 793 samples, including chicken droppings, intestines, and litter collected from desi chickens aged one to eight weeks across four farms in and around Bengaluru and from local chicken retail centers in Bengaluru, India, 79 samples were selected for DNA extraction and PCR analysis. Fecal droppings and litter samples were collected from poultry sheds. Intestinal samples (jejunum and cecum) were obtained using sterile scissors and forceps under aseptic conditions from naturally deceased desi chickens on the farm, which were suspected to have died from coccidiosis, as well as from desi chickens slaughtered at local dressing centers.

Oocyst sporulation

The *Eimeria* oocytes from positive samples of droppings, litter, and intestines were allowed to grow for one week under laboratory conditions. The sediment of contents was transferred to a 5 mm thick petri dish containing a 2.5% potassium dichromate ($K_2Cr_2O_7$) solution (Jigs Chemical Limited, India). The petri dishes were partially covered to allow oxygen to pass through and were kept for a week at room temperature (25 to 30°C) and 65 to 80% relative humidity. To ensure the oxygenation for oocyst culture, the contents of the petri dishes were stirred daily. A drop of the mixture was examined daily for one week to record sporulation time, and then observed under the microscope (Olympus CX23 USB digital microscope, India) at 100x and 400x magnification to analyze the morphological characteristics of the oocysts (Mona et al., 2015).

Oocyst purification

The procedure for purifying sporulated oocysts followed that of Mona et al. (2015), with a slight modification to the centrifuge speed. The sporulated oocysts suspension was mixed thoroughly with an equal quantity of $K_2Cr_2O_7$ solution at a concentration level of 2.5%. The suspension was then filtered through a sieve, followed by a muslin cloth. The filtrate was centrifuged at 3000 rpm for five minutes and washed with water two to three times. Almost 90% of the supernatant was discarded, and the remaining portion in the centrifuge tubes was transferred to a fresh tube and mixed with saturated sodium chloride (NaCl) for flotation. The mixture was centrifuged at 2000 rpm for two minutes. The supernatant, with sufficient sporulated oocysts, was aspirated by pipetting and collected separately in a tube. The sediment was processed similarly until no sporulated oocysts remained in the supernatant. The supernatant thus collected was mixed with water (1:5) in a Falcon tube and kept undisturbed overnight at 4^{o}C. The sporulated oocysts that had settled on the bottom were collected by removing all water (one inch above the bottom of the tube) through suction using a pipette. The supernatant (3/4th portion) was removed, and the remaining mixture at the bottom, containing the sporulated oocysts, was centrifuged at 3000 rpm for five minutes. To the sediment, $K_2Cr_2O_7$ was added at a concentration of 2.5% to prevent fungal growth and ensure long-term viability. The mixture was then stored at 4°C until further use.

Oocyst counting

The Neubauer counting chamber (modified by Bright-Line, Germany) was used to quantify oocysts before DNA extraction. The early phases of this protocol are similar to the McMaster method (Conway and McKenzie, 2007). A coverslip was placed on top of the hemocytometer, and the silvered region was filled with the oocyst suspension. The total number of oocysts found in the four large corner squares and the central large square was tallied. The overall count of oocysts was then multiplied by 2000, a conversion factor that adjusted the total volume in the counting grid to one mL.

Extraction of genomic DNA by the glass bead method

In general, the DNA extraction procedure was performed according to the procedure and instructions of the DNA fast stool kit (QIAGEN, Germany), with minor adjustments made to the procedure (Figure 1).

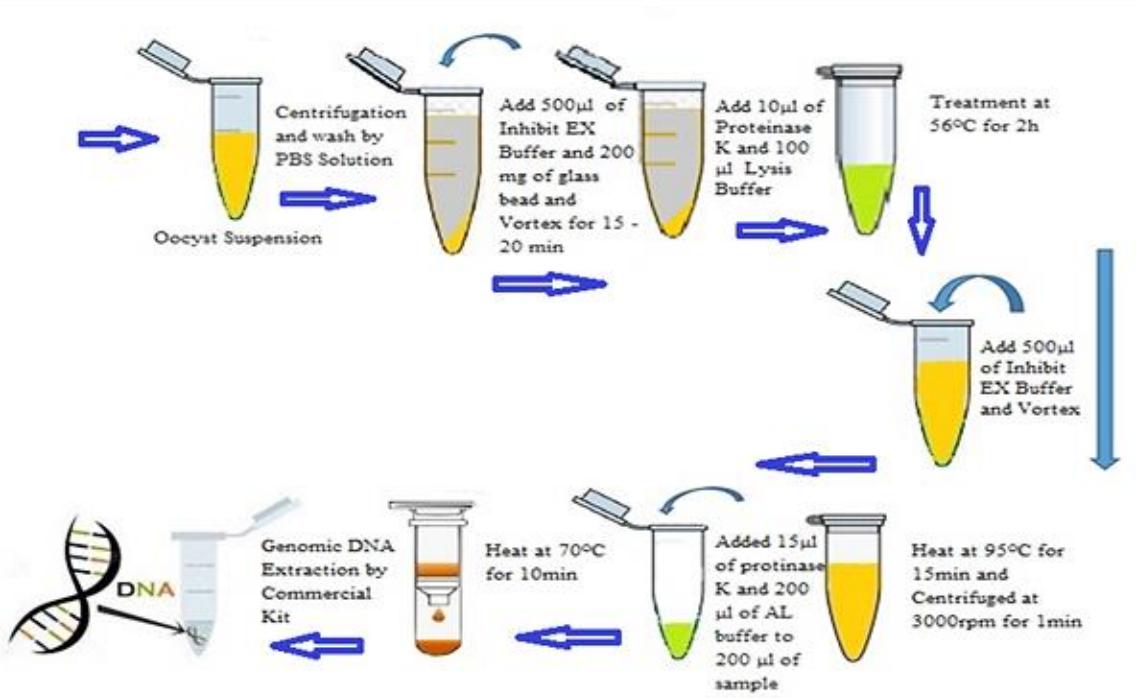

Figure 1. The flow chart of the modified DNA extraction by the glass bead method

Assessment of DNA quality and concentration

A Nanodrop spectrophotometer (Eppendorf AG, Germany) was used to determine the concentration and purity of DNA extracted from *Eimeria* oocysts of desi chickens. The absorbance at wavelengths of 260 and 280 nanometers (nm) was measured using the operating software of the Nanodrop Spectrophotometer (Eppendorf AG) to carry out the spectral measurements. To calculate the DNA concentration, the unit of ng/µl was employed. The ratio derived from the wavelength measurements of 260 and 280 nm indicated the purity (Siddiki et al., 2014).

The DNA confirmation by agarose gel electrophoresis

A total 0.375 grams of agarose (analytical grade) was weighed and then dissolved in a conical flask in 25 ml of 1x TBE buffer (diluted with 90 ml of distilled water and 10 ml of a 10x TBE buffer; Hi Media, India) and melted for 90 seconds in a microwave until a smooth, uniform suspension was obtained. Ethidium bromide (Sigma-Aldrich, Co., India) was added to the gel at a concentration of 0.5 µg/ml until the temperature of the molten gel cooled to 55°C. The melted agarose was poured into an acrylic comb-fitted casting tray and allowed to solidify. Once the gel had solidified, approximately 20 minutes later, it was transferred to an electrophoresis tank filled with 1x TBE buffer. The buffer level was maintained at least 0.5 cm above the gel, and eight µL of DNA and two µL of gel-filling dye (6x) were added to each well. Electrophoresis was conducted at 100 volts for 60 minutes, and the DNA was visualized using a UV transilluminator (Haug et al., 2008).

Primers for *Eimeria* species

Primers targeting the ITS-1 region of the rDNA gene, specific to each *Eimeria* species, were used for all seven species of *Eimeria* spp., including *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. praecox*, *E. mitis*, and *E. brunetti* (Haug et al., 2007). These primers were supplied by Bio-Serve Biotechnologies, India Pvt Ltd (Table 1).

Polymerase chain reaction

A gradient thermal cycler (Eppendorf AG, Germany) was used, and PCR was performed according to the procedure described by Haug et al. (2008). Optimized single PCR assays targeting ITS-1 were employed for all *Eimeria* species. The genomic rDNA ITS-1 sequences unique to each species were amplified with a gradient thermal cycler, utilizing a combination of specimens, primers, nuclease-free water, and PCR master mix (Bio-Serve Biotechnologies, India Pvt Ltd), in accordance with the sample processing flow chart (Figure 2).

Table 1. The primers used for *Eimeria* species target the internal transcribed spacer 1 regions of ribosomal DNA

***Eimeria* spp.**	**Amplicon Size (bp)**	**Primer sequence 5′ to 3′**	**Type**	**Reference**
E. maxima	205	*5′-GTGGGACTGTGGTGATGGGG-3′* *5′-ACCAGCATGCGCTCACAACCC-3′*	Forward Reverse	Haug et al. (2007)
E. acervulina	145	*5′-GGGCTTGGATGATGTTTGCTG-3′* *5′-GCAATGATGCTTGCACAGTCAGG-3′*	Forward Reverse	
E. brunetti	183	*5′-CTGGGGCTGCAGCGACAGGG-3′* *5′-ATCGATGGCCCCATCCCGCAT-3′*	Forward Reverse	
E. mitis	330	*5′-GTTTATTTCCTGTCGTCGTCTCGC-3′* *5′-GTATGCAAGAGAGAATCGGGATTCC-3′*	Forward Reverse	
E. praecox	215	*5′-CATCGGAATGGCTTTTTGAAAGCG-3′* *5′-GCATGCGCTAACAACTCCCCTT-3′*	Forward Reverse	
E. tenella	278	*5′-AATTTAGTCCATCGCAACCCTTG-3′* *5′-CGAGCGCTCTGCATACGACA-3′*	Forward Reverse	
E. necatrix	160	*5′-AGTATGGGCGTGAGCATGGAG-3′* *5′-GATCAGTCTCATCATAATTCTCGCG-3′*	Forward Reverse	

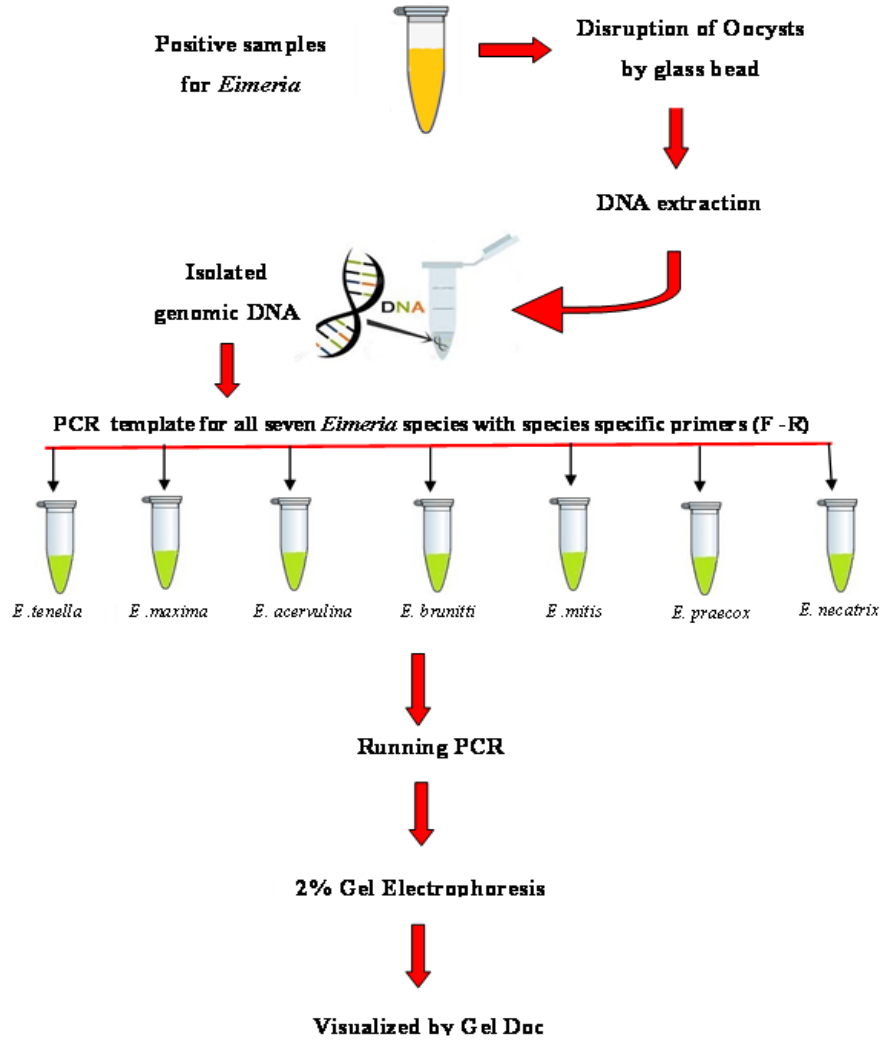

Figure 2. Complete flow chart of sample processing procedure for all sample types (fecal, litter, and intestinal) for different species of *Eimeria*

A first denaturation phase at 95°C for five minutes was followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58 or 65°C for 30 seconds, and extension at 72°C for one minute. The annealing temperature of 65°C was used for PCR amplification of *E. tenella*, *E. mitis*, *E. maxima*, *E. praecox*, and *E. acervulina*, while 58°C was used for the other two species, *E. brunetti* and *E. necatrix* (Sharma et al., 2018). The PCR procedure concluded with an extended extension phase at 72°C, after which electrophoresis was performed on agarose gel treated with ethidium bromide, and the products were identified using a 100 bp standard. The decomposed water served as a negative control. No template control (NTC; without DNA template) was maintained in all PCR amplifications (Tang et al., 2018).

Genus and species-specific polymerase chain reaction

A PCR was carried out, targeting the ITS-1 region of the rDNA gene for the identification of different *Eimeria* species in desi chickens. Seventy-nine samples were subjected to PCR analysis, namely 51 samples from farms and 28 from retail poultry dressing centers in Bengaluru, India. Among these samples, 25 samples (10 fecal samples, 10 litter samples, and five from caecum and colon) were from poultry farm KVAFSU, Bengaluru, India. 11 samples (Five fecal and six litter) from poultry breeding and training center, seven samples (Three fecal and four litter) from AKN farm, eight samples (Four fecal and four litter) from CPDO, and 28 samples (15 fecal and 13 intestines) were from retail poultry dressing centers in Bengaluru, India were subjected to PCR.

Agarose gel electrophoresis

Upon completion of the PCR reaction, the amplified DNA products were isolated using a 2% agarose gel with 0.5 µg/ml ethidium bromide in a horizontal electrophoresis setup. Three µl of standard DNA gel loading buffer and one µl of 100 bp ladder (Marker) were used as markers and loaded into the well. The positive control, negative control (distilled water), and eight µl of each PCR product mixed with two µl of (6x) gel loading dye were loaded. Electrophoresis was carried out at 100 volts until the tracking dye just reached the 3/4 portion of the gel (approximately one hour and 30 minutes). A UV transilluminator visualized the amplified DNA fragments, and the images were captured in a gel documentation unit (M/S Major Science, USA; Kumar et al., 2018).

Standardization of single-species polymerase chain reaction

The sensitivity of the optimized PCR assays was assessed by conducting PCR on serial dilutions (1/10-fold) of template genomic DNA sourced from each *Eimeria* species to determine the minimum number of oocysts necessary for PCR amplification. Three microliters of standard DNA gel loading buffer and one microliter of a 100 bp ladder (marker) were utilized as a marker and placed into the well. The positive control, negative control (distilled water), and eight microliters from each PCR product, mixed with two microliters of (6x) gel loading dye, were applied (Tang et al., 2018).

RESULTS

Oocyst disruption

Different methods, including direct, glass bead, sodium hypochlorite, sonication, and liquid nitrogen, were evaluated for disrupting purified sporulated oocysts of *Eimeria* (5×10^5) to determine the most effective approach. The genomic DNA was extracted from the disrupted oocysts, and DNA extraction was performed using a DNA fast stool kit (QIAGEN, Germany) according to the manufacturer's instructions with slight modifications. Among the five methods, the glass bead method and the liquid nitrogen method were effective in disrupting the oocyst's wall, yielding a proper amount of DNA. Other methods, such as sodium hypochlorite, direct, and sonication, failed to produce the required quantity of DNA from *Eimeria* oocysts.

The glass bead method was much easier, faster, and more efficient. The yield of DNA using the liquid nitrogen method was similar to that obtained with the glass bead method. However, the liquid nitrogen method, as mentioned, was time-consuming and required critical analysis during the DNA extraction procedure. The estimated DNA yields using the glass bead method and liquid nitrogen method were 118.24 and 113.82 ng/µL, respectively.

In the glass bead method, glass beads sized 425 to 600 µm were pre-acid-washed and added to disrupt the oocysts. Tests with varying vertexing durations indicated that at least 20 to 25 minutes was necessary to achieve rupturing of 95 to 100% of the oocysts. The ruptured oocysts after vertexing are illustrated in Figure 3.

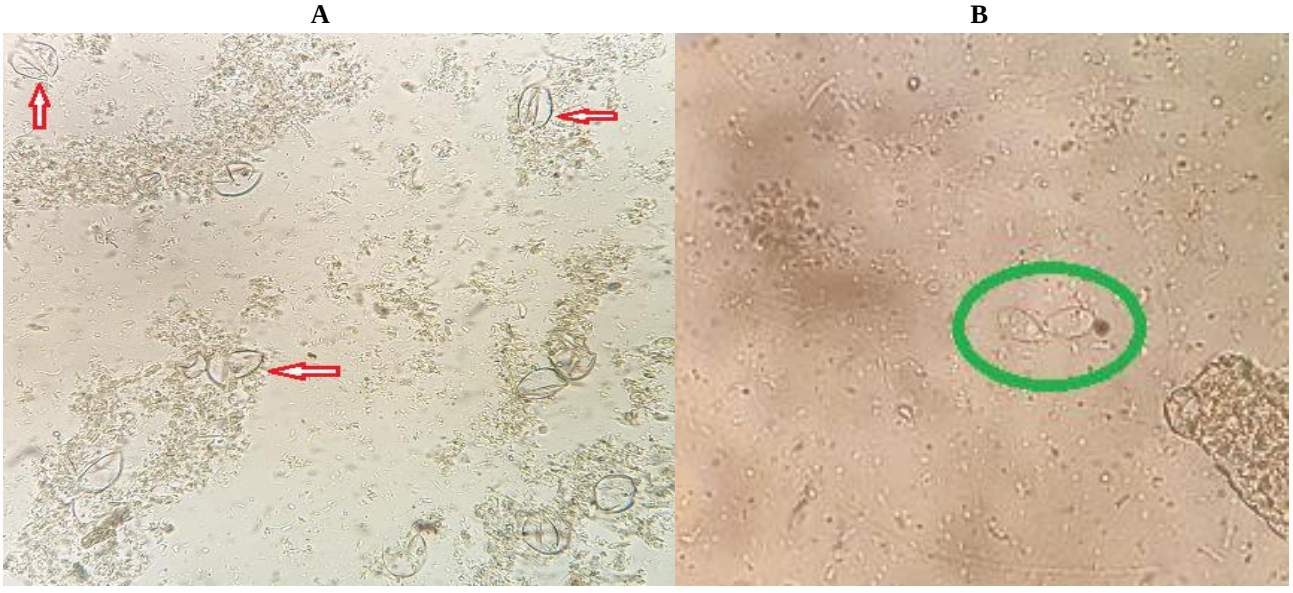

Figure 3. Mechanical disruption of *Eimeria* oocysts and sporocyst release. **A**: Disruption of *Eimeria* oocyst walls using the glass bead method, **B**: Release of sporocysts following oocyst wall rupture.

Polymerase chain reaction sensitivity

Sensitivity tests were conducted using individual primer sets on DNA samples from *Eimeria* species, with detection limits ranging from 0.41 ng for *E. acervulina* to 0.10 ng for *E. tenella* (Figures 4 and 5).

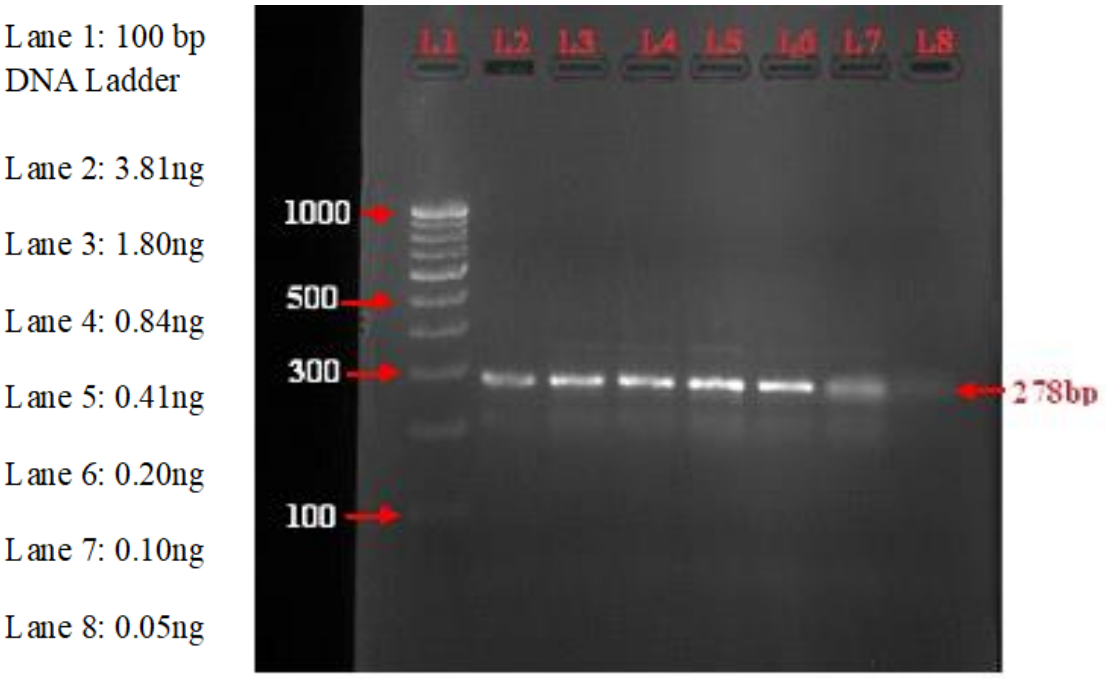

Figure 4. Minimum quantity of *Eimeria tenella* DNA (278 bp) for polymerase chain reaction

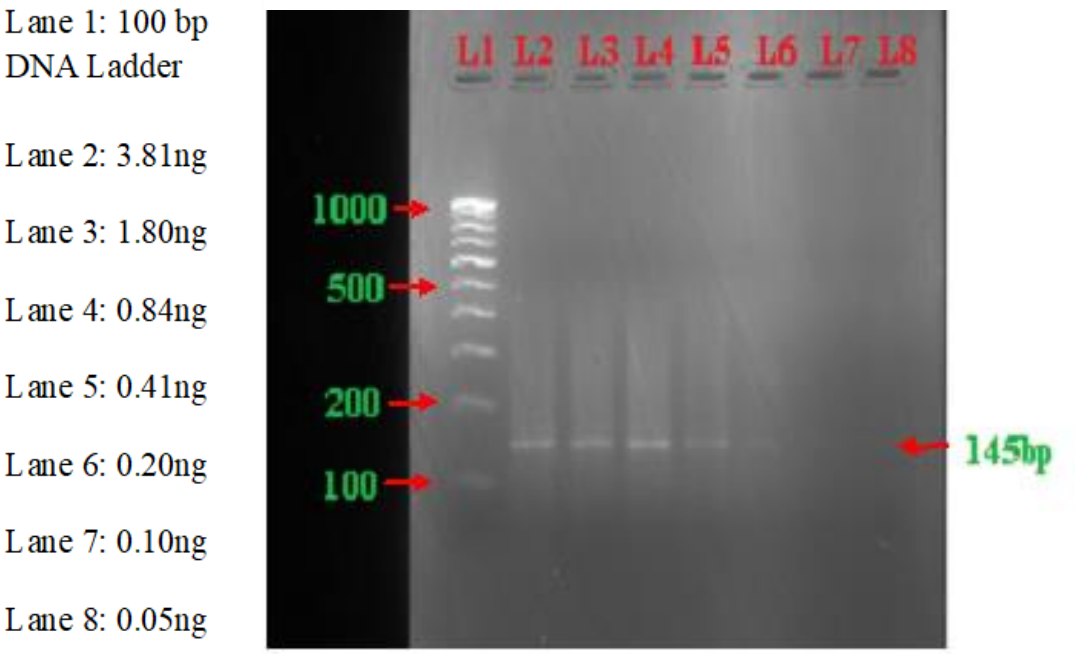

Figure 5. Minimum quantity of *Eimeria acervulina* DNA (145 bp) for polymerase chain reaction

The detection threshold for *E. tenella* and *E. acervulina* required approximately 32 and 127 oocysts, respectively, to optimize PCR. The sensitivity of PCR amplification, assessed with varying DNA concentrations, is illustrated in Figures 6A and 6B. In contrast, the minimum oocyst quantity required for PCR detection of *E. tenella* and *E. acervulina* is provided in Tables 2 and 3, respectively.

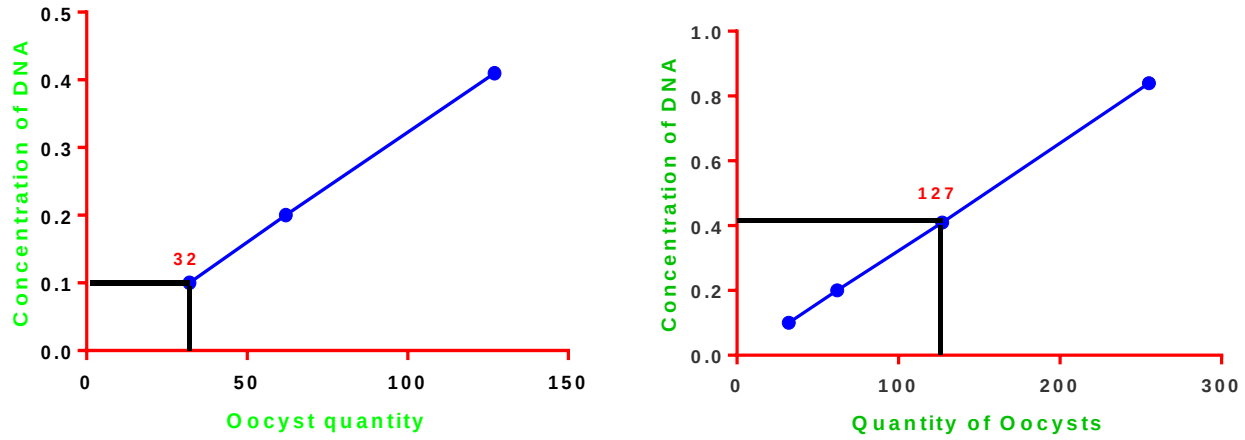

Figure 6. The minimum number of *Eimeria* oocysts required for running the polymerase chain reaction from desi chickens. **A**: The minimum number of *E. tenella* oocysts from desi chickens running polymerase chain reaction, **B**: The minimum number of *E. acerculina* oocysts from desi chickens running polymerase chain reaction

Table 2. Sensitivity of polymerase chain reaction amplification for *Eimeria. tenella* in desi chickens using DNA extracted from feces, litter, and intestinal samples, estimated by different concentrations of DNA and oocyst quantities

Sample numbers examined	The quantity of DNA template for each PCR reaction	Quantity of oocysts/ PCR reaction in 5 (µl)	Quantity of oocysts/ (µl)	Concentration of DNA /(µl)	Visualization of DNA in a 2% Agarose gel
1	5 µl	8330.00	1666.00	27.60 µg	+
2		4165.00	833.00	13.50 µg	+
3		2080.00	416.00	7.52 µg	+
4		1025.00	205.00	3.81 µg	+
5		515.00	103.00	1.80 µg	+
6		255.00	51.00	0.84 µg	+
7		127.50	25.50	0.41 µg	+
8		62.50	12.70	0.20 µg	+
9		32.00	6.40	0.10 µg	+
10		16.00	3.20	0.05 µg	-

+ means DNA band revealed, - means no DNA band revealed

Table 3. Sensitivity of polymerase chain reaction amplification for *Eimeria. acervulina* in desi chickens using DNA extracted from feces, litter, and intestinal samples, estimated by different concentrations of DNA and oocyst quantities

Sample numbers	Amount of template used per PCR reaction	Number of oocysts per PCR reaction in 5 (µl)	Concentration of oocysts per (µl)	Concentration of DNA per (µl)	Visibility of DNA in a 2% Agarose gel
1	5 **µl**	8330.00	1666.00	27.60 µg	+
2		4165.00	833.00	13.50 µg	+
3		2080.00	416.00	7.52 µg	+
4		1025.00	205.00	3.81 µg	+
5		515.00	103.00	1.80 µg	+
6		255.00	51.00	0.84 µg	+
7		127.50	25.50	0.41 µg	+
8		62.50	12.70	0.20 µg	-
9		32.00	6.40	0.10 µg	-
10		16.00	3.20	0.05 µg	-

+ means DNA band was detected in the gel documentation unit, - means the DNA band was not detected in the gel documentation unit

Out of the 79 samples analyzed, which included fecal matter, litter, caecum, and colon, 18 samples (22.7%) tested positive for a single infection of *E. tenella*, while 61 samples (77.21%) were found to have mixed infections of *E. tenella* and *E. acervulina*, along with other species of *Eimeria*. *Eimeria*. *brunitti*, *E. maxima*, *E. praecox*, *E. mitis*, and *E. necatrix* were not detected (Figure 7 and 8).

Lane 1: *E. brunitti* (183)
Lane 2: *E. acervulina* (145bp)
Lane 3: *E. maxima* (205bp)
Lane 4: 100 bp DNA Ladder
Lane 5: *E. necatrix* (160)
Lane 6: *E. tenella* (278bp)
7: No template control (NTC)
8: Negative control

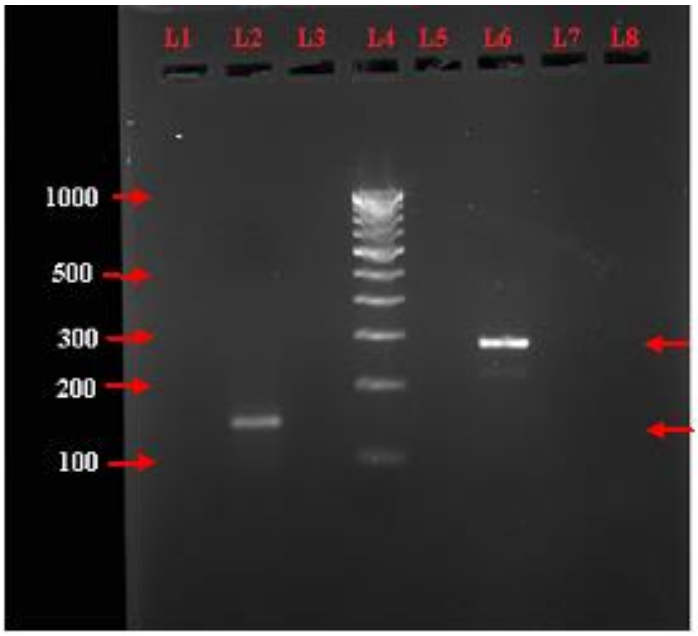

Figure 7. The mixed infection of *Eimeria*. *tenella* and *Eimeria acervuline* amplification of the 278 bp and 145 bp DNA fragments

Lane 1: 100 bp DNA Ladder
Lane 2: *E. tenella* (278bp)
Lane 3: *E. maxima* (205bp)
Lane 4: *E. praecox* (215bp)
Lane 5: *E. acervulina* (145bp)
Lane 6: *E. mitis* (330bp)
7: No template control (NTC)
8: Negative control

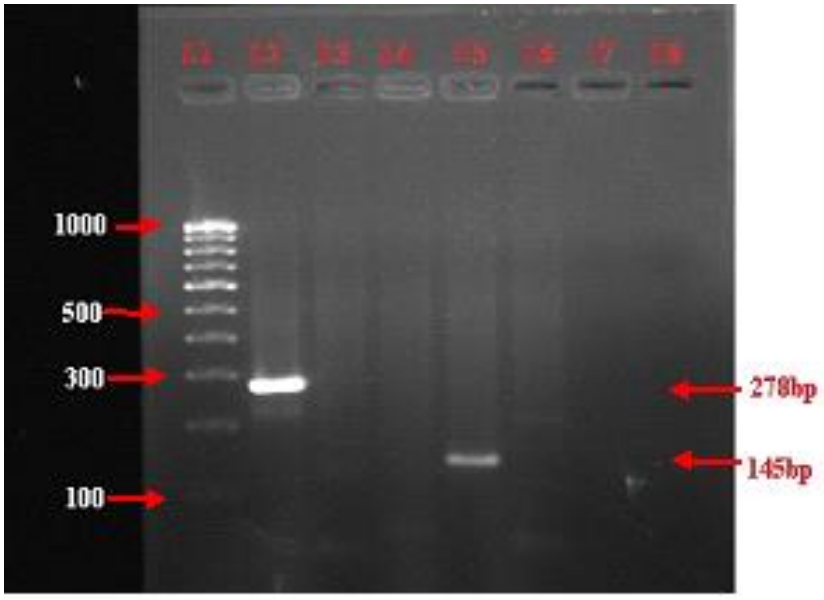

Figure 8. The mixed infection of *Eimeria tenella* and *Eimeria acervulina* amplification of the 278 bp and 145 bp DNA fragments

Statistical data analysis revealed that the samples collected from farms indicated no significant difference in the prevalence of *Eimeria* infection among different types of samples in each farm. Additionally, there was no significant difference ($P > 0.05$) in the prevalence of *Eimeria* infection among four farms (poultry farm KVAFSU Bengaluru, AKN, poultry breeding and training center, and CPDO, Bengaluru, India) for each type of sample, whether in single or mixed infection.

DISCUSSION

Approximately five hundred thousand purified sporulated oocysts of *Eimeria* were exposed to disruption by several methods, including the direct method (Dawood Bawer et al., 2021), glass bead (Thabet et al., 2019), sodium hypochlorite, sonication (Haug et al., 2007), and liquid nitrogen methods (Sharma et al., 2018), and assessed for reliability and practicability. The DNA quick stool kit (QIAGEN, Germany) was used for the DNA extraction, with minor adjustments made according to the manufacturer's instructions. Jenkins et al. (2006), Haug et al. (2008), and Hamidinejat et al. (2010) have extensively utilized specific primers targeting the ITS-1 and ITS-2 sequences, which are

derived from the rDNA precursor through post-transcriptional processing, for the identification of individual *Eimeria* species in chickens. The molecular characterization of *Eimeria* species in desi chickens identified *E. tenella* and *E. acervulina.*

The purification of *Eimeria* oocysts using saturated saline flotation (Chere et al., 2022) improved PCR sensitivity with the stool DNA kit (QIAGEN, Germany). The glass bead method was effective in disrupting *Eimeria* oocyst walls and yielded High-integrity DNA. The findings of the current study aligned with those of Carvalho et al. (2011a), who reported that glass-bead grinding effectively disrupts *Eimeria* oocysts, noted prolonged processing time in the presence of fecal debris. In contrast, the present study, using purified oocysts, demonstrated that 20 to 25 minutes of vortexing with glass beads achieved 95 to 100% disruption, yielding high-quality DNA (118.24 ng/µl). Dulski et al. (1988), Tahir (1998), Thabet et al. (2019), and Dawood Bawer et al. (2021) observed that the glass bead method has been the most commonly used procedure for the disruption of *Eimeria* oocyst walls for the successful extraction of DNA.

The high-integrity DNA obtained through the glass bead method relies on the use of appropriately sized (425-600 µm) spherical glass beads, the quantity of oocysts for DNA extraction, the presence of contaminants in the sample, and the duration of grinding during the disruption process (Tsuji et al., 1999). However, in the current study, the sonication, direct, and sodium hypochlorite methods used for disrupting oocysts were unsuccessful in yielding DNA. These methods were unsuccessful in disrupting oocysts due to the thick membranes of the oocysts, which are highly resistant to both mechanical and chemical forces (Conway and McKenzie, 2007). Conversely, the sonication method was effective in breaking down the *Eimeria* oocyst wall and sporocysts, allowing for the release of sporozoites (Kaya et al., 2007). *Eimeria* DNA was obtained using a stool mini kit (QIAGEN, Germany). Different techniques for lysing the cell wall of oocysts have been documented, including sonication, repeated freezing and thawing according to Jinneman et al. (1998), and hot phenol incubation as reported by Stucki et al. (1993).

In the current study, different quantities of oocysts were used to extract DNA to determine the minimum number of oocysts required. The yield of 27.60 µg of DNA (60 µl of elution buffer) was obtained when $10x10^4$ oocysts were used for extraction. The minimum number of oocysts required for DNA extraction using a fast DNA stool kit (QIAGEN, Germany) was 383 for *E. tenella* and 1531 for *E. acervulina* species. The current results aligned with the findings of Lalonde and Alvin (2008) from Canada, who used the QIAamp DNA Micro Kit (QIAGEN, Germany) to extract DNA from a coccidian parasite (*Cyclospora cayetanensis*) with 100 coccidia oocysts, achieving successful PCR amplification. On the contrary, Tang et al. (2018) used approximately 50 oocysts for DNA extraction using QIAamp fast DNA stool mini kit (QIAGEN, Germany).

The identification and distinction of *Eimeria* infections in impacted chickens are typically carried out based on clinical signs and biological characteristics, including the pre-patent period, the location of development within the intestine, and the morphological features of oocysts and endogenous stages present in the intestinal mucosa (Karim and Begum, 1995). Although the identification techniques discussed have limitations due to the similarity of traits across species, and the need for highly trained personnel (Lee et al., 2011). Furthermore, the presence of mixed infections complicates the accurate identification of species through morphological methods. In India, studies on the prevalence of *Eimeria* have been recorded by Panda et al. (1997), Rana and Tikaram (1999), and Chere et al. (2022) utilizing conventional methods.Different target genes, such as the small subunit ribosomal 18s rRNA gene ITS-1 identified by Tsuji et al. (1997), Schnitzler et al. (1998), and ITS-2 identified by Gasser et al. (2001), along with the sequence-characterized amplified region (SCAR) developed from random amplified polymorphic DNA (RAPD) profiles (Fernandez et al., 2003; Su et al., 2003; Fernandez et al., 2004), have been utilized in PCR assays for distinguishing *Eimeria* species. The current findings regarding the prevalence of *Eimeria* species in mixed infections aligned with the observations of Meireles et al. (2004) and Shirley (2007), who noted that mixed infections involving multiple *Eimeria* species are quite prevalent, particularly those involving *E. tenella* and *E. acervulina*, worldwide.

In the present study, a PCR assay was carried out to amplify the ITS-1 regions of the rDNA gene for the coccidian species *E. tenella* and *E. acervulina*. *E. tenella* and *E. acervulina* were successfully identified by the presence of distinct 278 and 145 bp bands on a 2% agarose gel. Lee et al. (2011) carried out a PCR assay based on the amplification of ITS-1 regions of rDNA for the diagnosis of chicken coccidian species such as *E. tenella* and *E. maxima*, which resulted in amplicon sizes of 278 bp and 205 bp, respectively. Whereas, Olufemi et al. (2020) in Nigeria reported the presence of five *Eimeria* species by quantitative PCR (qPCR), including *E. acervulina*, *E. tenella*, *E. mitis*, *E. necatrix*, and *E. maxima*. However, *E. brunetti* and *E. praecox* were not detected in all samples. *Eimeria mitis* had the highest abundance with a prevalence of 50.9%, followed by *E. acervulina* at 37.8%.

While Patra et al. (2002), Sun et al. (2009), and Carvalho et al. (2011b) reported seven *Eimeria* species and Schwarz et al. (2009) identified six, in the present study, only *E. tenella* and *E. acervulina* were detected, likely reflecting geographical or host-specific variations in species distribution. The genetic distribution of seven *Eimeria* species that infected broiler chickens in Tamil Nadu, India, was studied by Aarthi et al. (2010). The ITS-1 regions of the *Eimeria*

genome were amplified using genus-specific primers in a PCR process, while species-specific primers were utilized for species identification in a nested PCR. Among the 43 tissue samples from the caecum, duodenum, and jejunum that were analyzed, 25 tested positive in the ITS-1 region via PCR, and all seven species, including *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. brunetti*, *E. mitis*, and *E. praecox*, were recognized. The differences in the prevalence of these species might be attributed to varying management practices among desi chickens and broilers.

The present study employed molecular characterization using *Eimeria* species-specific primers to amplify the ITS-1 region of the rDNA. Patra et al. (2010) collected fecal samples from broiler chickens aged three to four weeks during a coccidiosis outbreak in Mizoram, India. The samples were subjected to molecular characterization of *Eimeria* species to amplify the ITS-1 region of *E. tenella* yielded PCR products of approximately 520 bp and 270 bp, respectively. The ITS-1 region of rDNA from *Eimeria* species was effectively used for PCR amplification as demonstrated by Guven et al. (2013), Khaier et al. (2015), and Jenkins et al. (2019), leading to the identification of different *Eimeria* species. In a study by Khaier et al. (2020), multiplex PCR targeting the ITS-1 primers revealed three distinct *Eimeria* species from different farming systems in Khartoum State, Sudan, namely *E. praecox*, *E. mitis*, and *E. necatrix*, which corresponded to band sizes of 368 bp, 306 bp, and 285 bp, respectively.

In the present study, 0.41 ng (410 pg) of DNA was the detection limit for *E. acervulina*, while *E. tenella* species was detected with as little as 0.10 ng (100 pg). The detection thresholds for *E. acervulina* and *E. tenella* required approximately 127 and 32 oocysts for PCR amplification, respectively. Tang et al. (2018) performed PCR amplification of ITS-1 for all seven chicken coccidia species using genomic DNA extracted from 50 oocysts. The current findings aligned with those of Procunier et al. (1993), who reported the sensitivity of optimized PCR assays using serial dilutions of pure template DNA from individual *Eimeria* species and demonstrated that as little as 0.32 pg of DNA or less was sufficient for successful amplification. The levels of DNA detection varied among 2 and 50 oocysts, as documented by Zhao et al. (2001), Fernandez et al. (2003), and Su et al. (2003). In contrast, Taylor et al. (1995) and Haug et al. (2006) utilized 100 oocysts per gram of feces. Carvalho et al. (2011a) found that a minimum of 50 oocysts was necessary to produce reliable results in PCR. Meireles et al. (2004) indicated the sensitivity of PCR in determining the limit number of oocysts for two species, specifically *E. mitis* or *E. praecox*, using the commercial kit (DNA-Zol). A species-specific DNA band was amplified in samples with at least 50 oocysts per gram of excrement. Tang et al. (2018) used genomic DNA extracted from 50 oocysts to perform PCR amplification of the ITS-1 *Eimeria*, targeting ITS-1 sequences ranging in length from 116 to 383 bp.

The observation of the present study did not agree with You (2014), who found a lower quantity of DNA concentration for amplification in multiplex PCR. Five pg of DNA was the detection limit for *E. acervulina*, while *E. maxima* and *E. tenella* were detected with as little as one pg of DNA. The higher number of oocysts used for DNA extraction in the present study, compared to earlier studies, may be because *Eimeria* oocysts processed from fecal samples were resistant to chemical agents and other mechanical forces, such as proteinase K and glass beads. Therefore, disrupting and rupturing the oocysts became imperative for obtaining quality DNA.

CONCLUSION

The present study highlighted the prevalence of *E. tenella* and *E. acervulina* as significant causative agents of coccidiosis in desi chickens maintained in farms and retail poultry dressing centers in Bengaluru, Karnataka, India. The PCR assay targeting the ITS-1 region of rDNA proved to be a highly sensitive and specific tool for identifying *Eimeria* species, with *E. tenella* emerging as the predominant species. The glass bead method demonstrated efficiency in disrupting oocysts and extracting genomic DNA, enabling reliable detection even with minimal oocyst quantities. Based on the present findings, it is suggested to implement targeted control strategies in Bengaluru's poultry farms, incorporating regular surveillance and PCR-based early detection to manage *Eimeria* species. Additionally, integrating efficient oocyst disruption methods into diagnostic protocols can help mitigate the economic impact of coccidiosis.

DECLARATIONS

Availability of data and materials

All relevant data and materials supporting the findings of the present study were fully described and included within this article. Further details are available from the corresponding author upon reasonable request.

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Authors' contributions

Mohammad Dawood Bawer conducted the study and performed the data analysis. Faisal Danish and Mohammad Dawood Bawer prepared the first draft of the manuscript. Mahboobullah Ahmadi, Mohammad Naeem Azizi, G. S. Mamatha, G. C. PuttalaKshmamma, and G. S. Naveen Kumar contributed to the manuscript's writing, proofreading, and preparation. All authors reviewed and approved the final version of the manuscript for submission.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been checked by all the authors.

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