RESEARCH ARTICLE

Phenotypic and Molecular Characterization of Most Prevalent *Eimeria* Species of Dairy Cattle from Indian Subtropical Region

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Abstract

A total of 2469 faecal samples were examined from 12 different villages in Mathura district of Uttar Pradesh to determine the presence and to conduct morphological and molecular characterization of *Eimeria* species. Morphological characterizations were performed by the faecal flotation method. The analytic survey revealed that the overall prevalence of *Eimeria* species in dairy cattle was 11.05% (273/2469). A total of nine *Eimeria* species were identified. *E. zuernii* and *E. bovis* species were reported in a maximum number of dairy cattle with a prevalence rate of 6.95% and 5.49%, respectively. The internal transcribed spacer-1(ITS-1) region of the ribosomal RNA genes of *E. bovis* and *E. zuernii*, were amplified, sequenced and studied in the current work to determine their evolutionary relationships. Pair-wise comparison showed over 90% homology between sequences from the same species. Furthermore, species-specific PCR tests based on amplification of the ITS-1 region were developed to differentiate between *E. zuernii* and *E. bovis*. The ITS-1 region of each *Eimeria* species displayed enough interspecies sequence variation to create primer sets that specifically amplified the target species. This PCR method demonstrated greater sensitivity than traditional methods for identifying and distinguishing between *Eimeria* species. This marks the first genomic-level attempt to identify two *Eimeria* parasites in cattle, potentially offering valuable tools for the diagnosis and study of cattle coccidiosis.

Keywords: Cattle, DNA, Eimeria, ITS-1, PCR, Ribosomal RNA genes

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Introduction

Protozoan parasites are considered more detrimental than other parasitic infections due to their rapid multiplication within the host, leading to significant pathogenic effects on the body. This often results in substantial economic losses for farmers in India (Akoolo *et al*., 2022). Among protozoa, coccidiosis is one of the major parasites that infect dairy cattle globally (Pal and Firaol, 2024). Coccidiosis is a generalized term used for infection caused by a group of sporozoa of the family Eimeridae. Bovine coccidiosis caused by various species of *Eimeria* contributes significantly towards reduced milk production in lactating animals and is also considered a major cause of calf diarrhea under field conditions. Family Eimeridae is commonly found in the host intestinal tract and cause mild to severe grade of enteritis depending upon factors like species of coccidia, host species, immune status of the host and age (Lopez-Osorio *et al*., 2020). Members of the family Eimeridae are highly specific to their host and site of predilection. A major population of cattle suffers sub-clinically, resulting in considerable economic losses to the cattle industry (Li *et al*., 2021). Although global data on economic losses from cattle coccidiosis is lacking, in South Africa, the annual loss is estimated at approximately $400 million, primarily due to *Eimeria* infections in cattle and buffaloes (Matjila and Penhorn, 2002). Different types of cattle coccidia are distinguished from one another using their morphological traits. However, it can occasionally be challenging to distinguish an *Eimeria* species solely by looking at its shape. The current study was designed to provide molecular characterizations of the most common *Eimeria* species in cattle and to conduct a phylogenetic analysis of these species in Indian subtropical region.

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Materials and Methods

Sample Collection and Characterization of *Eimeria* spp.

A prevalence study of coccidiosis was conducted between January 2018 and December 2019 in DUVASU, Mathura (UP,

India). Mathura, located approximately 56 km north of Agra and 150 km south of Delhi, lies between the parallels of 27014' N to 27058' N latitudes and 77017' E to 78012'E longitudes, 187 meters above sea level. A total 12 villages of Mathura district, including ILFC Farm of DUVASU were selected to study the cattle *Eimeria* species. The study areas had major cattle populations consisting of non-descript, Haryana, crossbred, and Sahiwal breeds. A total of 2469 faecal samples were examined from all these cattle breeds during the study period, which were collected during 4 seasons, *viz,,* Summer (Apr-Jun), Monsoon (Jul-Sep), Winter (Oct-Jan) and Spring (Feb-Mar).

Fresh fecal samples from each animal were collected directly via the rectum using sterile disposable plastic gloves and transported to the Department of Parasitology, DUVASU for fecal examination. Samples found positive in qualitative examination for *Eimeria* spp. were pooled and further treated with faecal flotation method. The sporulation of oocyst was conducted as per the method described by Floriao *et al*. (2016). A thin layer of 2.5% solution of potassium dichromate (Hi-AR^{TM}/ACS) was poured in disposable 10 mm size of glass petridish and purified oocysts were mixed thoroughly in the solution with the help of clean glass dropper. Each petridish was kept in BOD incubator (Scientech, India) at 28°C to allow sporulation. Identification of *Eimeria* oocyst after sporulation was done with the help of morphometric characteristics (Floriao *et al*., 2016). Sporulated oocysts with potassium dichromate solution were kept at -20°C in a deep freezer till further use for molecular characterization.

Animals were divided into three groups on the basis of their age (<6 month, 6 month to 1 year and > 1 years). The male and female cattle studied were 78 and 2391, respectively.

DNA Isolation of *Eimeria* spp.

The purification and isolation of sporulated oocysts were performed by washing three times with distilled water to completely remove the potassium dichromate solution from oocysts. Oocysts were then isolated using the floatation method as well as the Percoll density gradient method as per technique of Dulski and Turner (1987) with slight modification. The pooled sporulated oocysts with approximately 20,000 oocysts/mL of distilled water were used for whole genomic DNA extraction of *Eimeria* species. Genomic DNA was extracted using a commercial kit QIAamp® DNA Stool Mini Kit (Germany) as per the manufacturer's instructions with some modifications, mainly in breakdown of oocyst wall protocol. DNA was also isolated from individual oocyst as per the method described by Kokuzawa *et al*. (2013).

PCR Assays with *Eimeria*-Common Primers

The PCR primer based on the ITS-1 gene of *Eimeria* spp. was used as per Kawahara *et al*. (2010). The primer was synthesized by outsourcing from Imperior Life Science Pvt. Ltd. Gurgaon, Haryana. The up and downstream primer sequences of *Eimeria* common sequence of the internal transcribed spacer 1 (ITS-1) region were: F: 5'- GCA AAA GTC GTA ACA CGG TTT CCG -3', R: 5'- CTG CAA TTC ACA ATG CGT ATC GC-3' with expected product sizes of 348-546 bp. A volume of 20 µL of reaction mixture comprised of 10 µL master mix (HotStarTaq MM Qiagen), 0.8 µL of the 200 nM primer (0.4 µL each), 0.5 µL of extracted DNA and 8.7 µL nuclease free water. Reaction conditions incorporated were an initial denaturing phase at 94°C for 5 min followed by 35 cycles each at 94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min with a final extension at 72°C for 5 min in a Thermocycler (C1000 Touch, BIO-RED) as per Kawahara *et al.* (2010). Electrophoresis (BIO-RAD PowerPacTM Basic) was performed at 80V/1h. The gel was visualized in an E-gel Imager camera, Thermo-Fischer Scientific (Model number NA1800281), after staining with ethidium bromide on a UV transilluminator and images were stored.

Cloning and Isolation of Recombinant Plasmid DNA

The amplified samples were subjected to cloning. The PCR products that tested positive with the genus-specific primer set were purified using the GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific K0831). The purified PCR products were then cloned using the pGEM®-T and pGEM®-T Easy Vector System (Cat A1360, Promega). Recombinant plasmid DNA was extracted following the manufacturer's protocol, with slight modifications, using the GeneJET Plasmid Midiprep Kit (Thermo Fisher Scientific).

Sequencing, Phylogenetic Analysis and Designing of Species-Specific Primer

The transformants containing the recombinant vector plasmid underwent DNA isolation, followed by outsourced sequencing (Eurofins Genomic India Pvt. Ltd., Bengaluru, India). The nucleotide sequences of each sample were analyzed using NCBI BLAST to assess their homology with other published sequences. Species-specific primers for the diagnosis of *Eimeria* species were designed using Primer3 software and synthesized by Imperial Life Sciences Pvt. Ltd. PCR reactions for species-specific *Eimeria* diagnosis were standardized based on Tm values. Additionally, gradient PCR was performed at varying annealing temperatures to optimize the specificity of the PCR reaction.

After downloading the 18S rDNA sequences from GenBank, they were aligned, and their parental similarity with existing sequence resources was determined using the Basic Local Alignment Search Tool (BLAST®; http://blast.ncbi.nlm.nih.gov/Blast.cgi). With *Sarcocystis* spp. as an out-group, the evolutionary analyses and percent identity/mean genetic distance across sequences were carried out using the MEGA6 software (Tamura *et al*., 2013). The maximum likelihood technique, which is based on the Tamura-Nei model, was used to infer the evolutionary history (Tamura and Nei, 1993). For *E. bovis* and *E. zuernii*, the maximum log likelihood tree displayed was -337.7076 and -444.8294, respectively.

By employing the Neighbor-Join and BioNJ algorithms on a matrix of pair-wise distances calculated using the Maximum Composite Likelihood (MCL) technique, the initial tree or trees for the heuristic search were automatically generated. The topology with the highest log-likelihood value was selected. The branch lengths of the scaled-up tree represent the number of replacements at each location. The analysis included 14 nucleotide sequences, covering the first, second, and third codon positions, as well as noncoding regions. Positions with missing data and gaps were excluded, resulting in a final dataset with 55 positions.

Validation of Designed Primers on Field Samples

An overall 100 faecal samples were taken from the different dairy animals and subjected for qualitative and quantitative examination for *Eimeria* oocyst. Samples that tested positive were subjected to DNA isolation, and the DNA concentration was measured. For further validation, PCR-based amplification of the extracted DNA was performed using the specific primer.

Results and Discussion

Morphological examination of parasites among 2469 faecal samples revealed an overall prevalence of *Eimeria* spp. as 11.05% (273/2469). Studies elsewhere worldwide have reported overall prevalence rates ranging between 8% and 100% (Bangoura *et al*., 2012; Das *et al*., 2018; Gupta and Pilania 2020). In India, different studies have reported changeable prevalence rates*, viz.,* 26.7% from southern part (Krishnamurthy and D'souza, 2016), 57.84% from northern part (Nain *et al*., 2017), 11.97% from eastern part (Das *et al*., 2018), 27.73% (Gupta and Pilania 2020) and 28% (Sodha *et al*., 2021) from northwestern part of the Indian subcontinent. The same varying scenarios has been documented from different parts across world like 37.14% in Egypt (El-Ashram *et al*., 2019), 75.5% in Colombia (Lopez *et al*., 2020), and 38.91% in North-Central Nigeria (Olafadunsin *et al*., 2020).

In the current study, nine different species of *Eimeria* were identified based on morphological characteristics such as oocyst shape, sporulation time, oocyst outer wall, micropyle, micropylar cap, oocyst residuum, polar granule, and micrometry analysis of sporulated oocysts. These *Eimeria* species included *E. zuernii* (54.57%), *E. bovis* (23.07%), *E. brasiliensis* (12.82%), *E. subspherica* (24.54%), *E. bukidonensis* (6.59%), *E. auburnensis* (7.29%), *E. alabamensis* (43.58%), *E. elllipsoidalis* (34.06%) and *E. cylindrica* (41.39%). Further among 273 samples found positive, 18.32% (50/273) had a single species of *Eimeria* infection (*Eimeria zuernii*, *E. bovis*, *E.cylindrica*, or *E. ellipsoidalis*), 3.29% (9/273) had two species, 38.82% (106/273) had three species, and 39.56% (108/273) had more than three species (Table 1). The most common species found in both single and mixed infections across all positive samples were *E. zuernii* and *E. bovis*.

Table 1: Prevalence of different *Eimeria* species (n=273)

Infection of *Eimeria* species	No. of samples	Per cent prevalence
With single species	50	18.32
With two species	09	3.29
With three species	106	38.82
With >3 species	108	39.56

The number of oocysts beside the species of *Eimeria* was very low in mixed cases of coccidiosis. Nine species of *Eimeria* identified, and their presence as single or multiple species concurred with report of Deb *et al*. (2022) from Bangladesh, who found the mix infection (24.73%) of *Eimeria* species in cattle calves, though they observed combinations of five *Eimeria* species. Ekawasti *et al*. (2022) found 100% (36/36) mix infection in cattle with maximum number of coccidia five, while minimum number was two from Indonesia. Sufi *et al*. (2017) from Indonesia found 79 samples having mono-infection, 59 samples having bi-infection, 24 samples having three species, 15 samples having four species and 2 samples having five species of infection out of 179 positive faecal samples of dairy cattle. This indicates that *Eimeria* species can be found in various combinations in dairy cattle and *E. zuernii* and *E. bovis* are the most prevalent species in mono and mixed infection of cattle coccidiosis. The findings of the present study are in agreement with Manya *et al*. (2008) and Yadeessa *et al*. (2014).

The present study observed the highest number of oocysts of *E. bovis* and *E. zuernii* in dairy cattle in the collected faecal samples irrespective of the age of the animals. The infection rate, varied greatly with different age groups in animals. Of 848 youngest animals of below 6 month of age 157 (18.51%) were positive for *Eimeria* spp., while 83 (10.61%) of 782 animals of above 6 month to one year, and 33 (3.93%) of 839 animals of more than one year age group were found positive for the presence of *Eimeria* oocysts in their faecal samples. The difference in the rate of infection between all three age categories was statistically highly significant ($p<0.01$).

Traditional methods for identifying *Eimeria* species at the clinical stage are challenging, time-consuming, and require highly trained personnel (Carvalho *et al*., 2011). These techniques involve identifying clinical symptoms in suspected animals, examining macroscopic lesions during necropsy, and studying the parasite's biology and the morphology of the oocysts. The PCR based methods using amplification of internal transcribed spacer-1 (ITS-1) region for the molecular identification of apicomplexan parasites have been developed by various workers and considered a reliable tool (Tsuji *et al*., 1997; Lew *et al*., 2003). Based on this high inter-species-specific diversity in the ITS-1 gene between various chicken *Eimeria* parasites species-specific molecular diagnostic tools were developed by various workers worldwide (Lew *et al*., 2003; Jenkins *et al*., 2006; Kawahara *et al*., 2008). Similarly, ITS-1 region based species-

specific PCR assays for bovine coccidiosis were developed in Japan (Kawahara *et al*., 2010).

In the present study, after DNA isolation from the positive samples, a total of four DNA samples were extracted from pooled sporulated oocysts. The purity of the DNA was assessed by measuring the OD 260/280 ratio using a Nanodrop spectrophotometer. Four DNA samples, with OD 260/280 ratios ranging from 1.81 to 1.86, were included in the study. However, the DNA extracted from single oocysts was found to be of insufficient concentration for PCR analysis. The extracted DNA samples from *Eimeria* oocysts were used for amplification of ITS-1 region using genus common primer (Kawahara *et al*., 2010) and 500 bp products were obtained (Fig. 1). Plasmid DNA was isolated from cloned PCR product, and sequencing of the transformants containing recombinant vector plasmid was outsourced from Eurofins Gnomic India Pvt. Ltd. Bengaluru, India.

The received result of nucleotide sequence of each sample was blasted through NCBI BLAST to know the homology of the received sequences with the other on-line published sequences available. The BLAST results revealed a 90-96% similarity with *E. bovis* and *E. zuernii*. The phylogenetic tree of *E. bovis* showed that the Mathura isolate (accession number OK486542) grouped with other global isolates, specifically clustering with the *E. bovis* from Iran. The phylogenetic tree of *E. zuernii* from the Mathura isolate (OL415193) indicated a close relationship with the isolate from Japan (Fig. 2 & 3).

Further, the primers were designed for *E. bovis* (F5'–GGGTTACATTTCTACCCA TATTC–3' & R5'–TCCCCCATAACGGCTATTATC–3') and *E. zuernii* (F5'–GTAACATG TTTCTACCCACTA–3' & R5'–ACCGCGATAAGGAGGAGGA–3') by using sequences of the present study with the help of Primer3 software for the development of species-specific diagnosis of *Eimeria*. The primers which were developed by Primer3 software were standardized with product size of 251 bp and 349 bp, respectively. The sequences of designed primers with product size and T_m are given in Table 2.

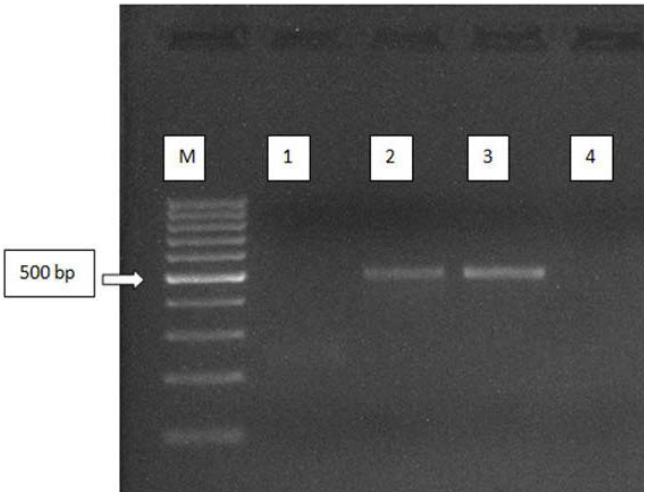

Fig. 1: Agarose get showing 500 bp *Eimeria* species product: M marker 100 bp; Lane 1 & 4 Negative; Lane 2 & 3 Positive

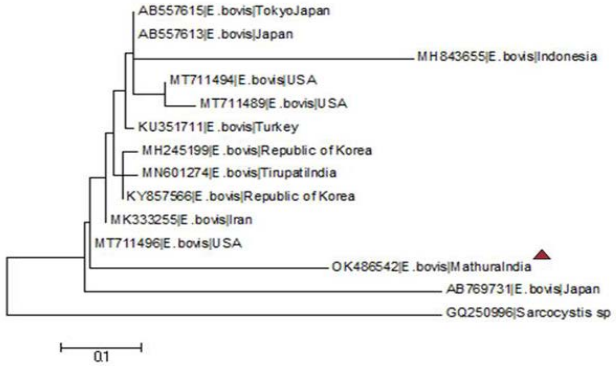

Fig. 2: Phylogentic analysis of *Eimeria bovis*

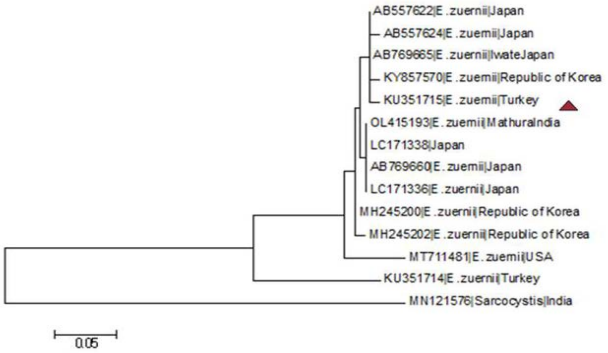

Fig. 3: Phylogentic analysis of *Eimeria zuernii*

For validation of above designed primer, 100 randomly selected faecal samples were taken from the field and microscopically total of eight samples were found positive for *Eimeria* oocyst. The standardized primer sets were validated on morphologically positive pooled faecal samples by PCR for *E. bovis* and *E. zuernii* (Fig. 4). The result indicates that the primer developed for this study was found to be suitable for identifying the *Eimeria* species present in the Mathura region

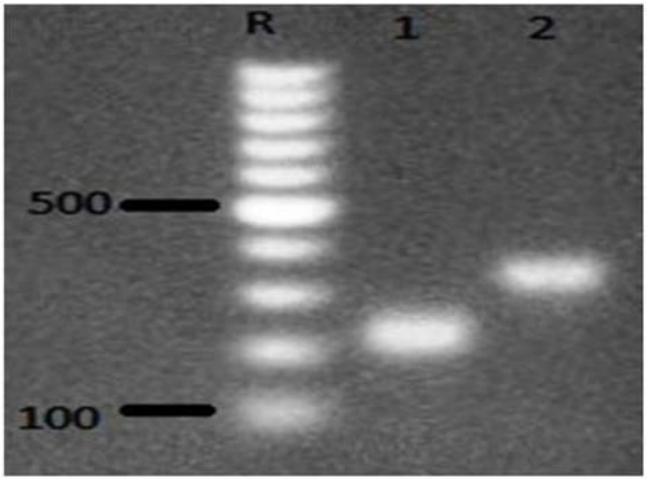

Fig. 4: Gel electrophoresis showing amplification of *E. bovis* and *E. zuernil* specific PCR products. R Marker of 100 bp; 1. *E. bovis* specific amplification at 251 bp; 2. *E. zuernil* specific amplification at 349 bp

There is a need for extremely sensitive tests that can identify the lowest levels of parasitic infection, even if standard parasitological techniques will always be crucial for comprehending the biology, ecology, and molecular epidemiology of various parasite strains (Singh *et al*., 2015). In the current study, a total of seven samples were amplified using universal primer pairs of ITS-1 region of *Eimeria* spp. (Kawahara *et al*., 2008) and cloned in the commercial vector system followed by outsource sequencing. According to the NCBI BLAST results , variable similarities were found between the received gene sequences targeting the ITS-1 region of *Eimeria* and the other known sequences of the ITS-1 region of cattle infecting *Eimeria* species. .

Among all seven sequences, one of the sequences matched 96% with the same region of *E. zuernii* isolates of Korea region. While the other sequence of *Eimeria* spp. matched 89% with the *E. bovis* isolate of Iwate, Japan. It was also recorded that the two species in the majority of positive samples supported their presence in the same group. Lee *et al*. (2018) also reported *E. zuernii* and *E. bovis* in a separate group from other cattle *Eimeria* species based on the ITS-1 region. The rest five received sequences of *Eimeria* spp. under the current study showed a very low similarity with the previously published sequences of cattle *Eimeria* species. These sequences formed a monophyletic lineage similar to those of chicken *Eimeria* species. Kokuzawa *et al*. (2013) reported 10 *Eimeria* species of cattle divided in to 3 monophyletic groups.

The current study suggested that the PCR assays which were developed for different *Eimeria* species provide a simple, quick and precise tool to specifically detect these parasites from the field samples which are always found to be difficult to identify by faecal examination morphologically. The phylogenetic tree constructed based on ITS-1 region sequence revealed that the *E. bovis* of Mathura isolate (accession number OK486542) comes with the same clad of *E. bovis* Iran, while in a different clad with *E. bovis* Japan (accession number AB769731). However, the *E. zuernii* of Mathura isolate (accession number OL415193) is very closely related to Japan isolate (accession number LC171338) but formed a separate clad with USA and Republic of Korea isolate suggesting strain variation with geographical isolation. Similarly, Kawahara *et al*. (2010) analyzed the sequences of their study and constructed the phylogenetic tree to find the homology between *Eimeria* isolates of Japan. However, two species, *viz., E. bovis* and *E. zuernii* have been confirmed molecularly to be present in Mathura by amplifying 18S rRNA, Internal Transcribed Spacer-1 5.8S rRNA gene with designed primers in this study. However, seven other species, which have been reported based on morphological characteristics, are yet to be confirmed molecularly. *E. bovis* and *E. zuernii* are the most common cause of clinical coccidiosis and are highly pathogenic (Bangoura *et al*., 2012). The DNA sample was used with mixed species, *E. zuernii* and *E. bovis* primer as species specific primers amplified and produced single bands with the expected size. These results agree with Kawahara *et al*. (2010), who stated that the ITS-1 regions are flexible corresponding with species variation, showing a pattern of low intra-specific and high inter-specific variations in the DNA sequence, thus reducing the risk of cross-reactions with different species.

Table 2: Sequences of designed primers with product size and T_m

Eimeria species	**Primer Forward**	**Primer Reverse**	**Product size (bp)**	**T_m**
E. bovis	GGGTTACATTTCTACCCATATTC	TCCCCCATAACGGCTATTATC	251	53
E. zuernii	GTAACATGTTTCTACCCACTA	ACCGCGATAAGGAGGAGGA	349	53

Conclusion

From the study it is concluded that PCR technique for amplification of the ITS-1 gene is more rapid, convenient and accurate in the detection of *Eimeria* species of cattle than microscopic examination, which is very labour intensive and requires skillful technique. Primers which were designed in this study for *E. zuernii* and *E. bovis* were useful in detecting for these two *Eimeria* species of cattle.

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