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ORIGINAL ARTICLE

Phylogenetic analysis of *Eimeria tenella* isolates from chicken of sub-tropical mountains of Meghalaya, India

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Abstract

Background Coccidiosis is the most common and pathogenic intestinal disease caused by different species of *Eimeria* is chicken. In this study, we describe the prevalence, molecular diagnosis and evolutionary insight of *Eimeria tenella* in chicken of Meghalaya's sub-tropical mountainous area.

Methods and results Faecal samples (337 no.) and dead chicks (298 no.) were collected every month from January to July' 2023 from poultry farms (4nos.) in and around Umiam, Ri-Bhoi, Meghalaya. The chicks were categorized into different age groups viz. < 3, 3–6 and > 6 weeks. Samples were examined by flotation techniques and post-mortem. The oocysts were sporulated in 2.5% potassium dichromate solution. *Eimeria tenella's* 18 S rRNA gene genomic DNA was extracted, amplified, and sequenced. Fecal sample and postmortem examinations revealed 24.04% and 33.22% infections of *Eimeria* sp., respectively. Oocyst per gram (OPG) was recorded highest and lowest in July (26,500) and February (9800), respectively. Amplification of the 18 S rRNA small subunit gene (SSU) by Polymerase Chain Reaction (PCR) revealed a 1790 bp band size. The amplicon was sequenced and deposited in the NCBI database. BLAST analyses of the SSU rRNA gene of *E. tenella*, Umiam, Meghalaya isolate (OR458392.1) revealed sequence similarities of more than 99% with SSU rRNA gene sequences available in the NCBI database. Pair wise alignment exhibited nucleotide homology ranging from 71.59 to 100.0% with the maximum sequence homology (100.0%) shared with the *E. tenella* isolate from Turkey (HQ680474.1) and the lowest homology of 95.6% with UK (HG994972.1). Umiam isolate were found to have 97.08% and 100.0% nucleotide similarities with *E. tenella* from both the UK (AF026388.1) and the USA (U40264.1), respectively. However, nucleotide similarities of 98.24%, 85.33%, 84.75% and 81.35% were observed with *E. tenella* strain Bangalore (JX312808.1), *E. tenella* isolate Kerala-1 (JX093898.1), *E. tenella* isolate Kerala-3 (JX093900.1) and *E. tenella* isolate Kerala-2 (JX093899.1), respectively. Phylogenetic analysis of SSU rRNA sequences of *E. tenella* Umiam, Meghalaya isolate with cognate sequences throughout the world revealed these sequences are distinct but at the same time share a close phylogenetic relationship with Indian isolates from Bangalore and Andhra Pradesh. In addition, the distant phylogenetic relationship was observed with cognate gene sequences of United States of America, Canada, China.

Conclusion Phylogenetic analysis of SSU rRNA sequences of *E. tenella* Umiam, Meghalaya isolate with cognate sequences throughout the world revealed these sequences are distinct but at the same time share a close phylogenetic relationship with Indian isolates from Bangalore and Andhra Pradesh. Distant phylogenetic relationship was observed with cognate gene sequences of United States of America, Canada, China.

Keywords Phylogeny · *Eimeria tenella* · Chicken · Meghalaya

Introduction

Coccidiosis is a common intracellular intestinal parasitic disease in chicken caused by different species of *Eimeria*. In poultry birds, nine *Eimeria* species have been identified, with *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella* being the most pathogenic; *E. acervulina*, *E. mitis* and *E. mivati* being less pathogenic, and *E. praecox* and *E. hagani*

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being the least pathogenic [1, 2]. *Eimeria* sp. spreads via the fecal–oral pathway. Infection begins with the ingestion of sporulated (infectious) oocysts, and after asexual and sexual replications, un-sporulated oocysts are expelled with feces [3]. Commercial poultry producers tend to be concerned about coccidiosis due to the costs associated with effective chemoprophylaxis and immunoprophylaxis as well as the losses endured as a result of acute infection, digestion difficulty, decrease in egg production, morbidity and mortality as high as 80% [4, 5]. Furthermore, Mesa-Pineda et al. [6] state that the chicken enterprise is one of the primary source of protein, but it faces various obstacles, including coccidiosis, one of the diseases with the greatest impact on production performance. According to Blake et al. [7], the global financial cost of coccidiosis in chickens is estimated to be £10.4 billion.

Caecal coccidiosis is caused by *E. tenella* and is characterized by diarrhoea and severe caecal haemorrhages. It is a substantial threat to birds that are between 15 and 50 days old and is associated with morbidity of 50–70% [8, 9]. The afflicted birds' growth and feed consumption are severely hampered, which reduces output and inflicting enormous financial losses to the farm enterprise [10]. It damages the intestinal epithelium due to deep penetration of the cecum tissues, causing cecal subepithelial hemorrhagic lesions [11] and anaemia [12, 13]. Secondary *Clostridium perfringens* infection may ensue, predisposing infected birds to additional gut infections such as necrotic enteritis. Its prevalence in poultry is increasing as a result of higher stocking densities and intense husbandry practices [14].

ITS-1 and ITS-2 genomic area sequences, as well as 18 S rDNA, the small subunit rRNA, are commonly utilized for parasite identification, ecological genetic research, and phylogenetic and evolutionary analysis at the taxonomic level, including *Eimeria* [10, 15, 16]. Though there have been reports on the incidence and phylogenetic analysis of *E. tenella* in chickens from various parts of the world viz. Bangladesh [17], Vietnam [18], Pakistan [19, 20], China [10], North India [21], Haryana [22], South India [23] and Tamil Nadu [24]. Phylogenetic analysis helps us to estimate the relationship of the isolate from the region with other isolates of the world. It will also provide useful information for prevention and control of caecal coccidiosis in the region. However, no report on the phylogenetic analysis of *E. tenella* in chickens of Meghalaya and North East region of India is available. Thus, the present investigation was attempted for molecular diagnosis and phylogenetic evaluation of *E. tenella* in chickens of the Meghalaya's subtropical mountainous terrain.

Materials and methods

Study area and period

The current investigation was carried out in and around Umiam, Meghalaya's Ri-Bhoi district, which lies between 25°6768′ North latitude and 91°9270′ East longitude. The study was conducted from April'2022 to October'2022.

Sample collection

Fresh fecal droppings and dead chicks were gathered every month from chicken farms (4nos.) within and around Umiam, Ri-Bhoi, Meghalaya in the clearly labelled plastic pouches/vials during January-July'2023. All of the chicks were divided into three groups based on their age: 3 weeks, 3–6 weeks, and > 6 weeks. In total, 337nos. fecal samples from chicken chicks of different ages have been collected viz. < 3 weeks (106nos.), 3–6 weeks (119nos.) and > 6 weeks (112nos.). 298nos. of dead chick samples from various age groups have been collected viz. < 3 weeks (83nos.), 3–6 weeks (123nos.) and > 6 weeks (92nos.).

Sample examination

To identify *Eimeria* oocysts in the specimens of chick feces, the samples have been examined using the direct flotation technique with saturated salt and sucrose solution [25]. Samples that were not tested on the same day were refrigerated (4 °C) for analysis the following day. Sporulation of the oocyst was accomplished by combining positive samples containing *Eimeria* oocysts in a 1:5 volume ratio with 2.5% potassium dichromate solution [26, 27]. The oocysts were morphologically characterized using an Olympus BX51 microscope at 100x and 400x magnifications [25]. Post-mortem examination of dead chicks was done. Wet smears were made for microscopic evaluation of *Coccidia oocysts* with deep scrapings from the duodenum, jejunum, ileum, and caecum as per the method described by Flek and Moody [28].

Genomic DNA extraction

Before DNA extraction, repeated centrifugation and resuspension of sporulated oocysts in distilled water was performed to remove potassium dichromate. Oocysts are cleaned 3–4 times by centrifugation at 13,000 rpm for 5 min with distilled water. The pellet having 5000 oocysts was subsequently processed by glass bead DNA extraction protocol [29] with slight modification i.e. repeated thawing (57 °C) and freezing (− 80 °C) till oocyst wall gets ruptured. In

the oocyst pellet, 200 µL of STES buffer (0.1% SDS (w/v), 0.2 M Tris-HCl, EDTA 0.01 M, pH 7.6) was added followed by freezing (− 20 °C) and thawing (57 °C) 3–4 times. Then 100 µL phenol-chloroform-isoamyl alcohol and a few glass beads (0.5 mm) were added to the suspension, and vortexed for 5 min. The solution was then centrifuged for 5 min at 13,000 rpm, the supernatant was extracted and passed to a fresh sterile tube, where it was precipitated with cold 2 V (volume) absolute ethanol and 0.1 V 5 M NaCl. After a period of 30 min, the solution was spun in a centrifuge for 10 min at 12,000 rpm. The pellet had been washed two times with 70% ethanol before being air dried. The genomic DNA eluted in 100 µL of 1X TE buffer. Thermo-Fisher Scientific's NanoDrop 1000 UV–Vis Spectrophotometer was used to quantify genomic DNA concentration. Further confirmation was done by using gel electrophoresis on an ethidium bromide-stained agarose gel (0.8%).

Amplification and sequencing of 18 S rRNA of *Eimeria tenella*

The extracted DNA's small subunit (SSU) of the 18 S rRNA was amplified utilising species-specific primers (EtF: 5′-ACCTGGTTGATCCTGCCAG-3′ and reverse EtR: 5′-CTTCCGCAGGTTCACCTACGG-3′) as already stated by Schwarz et al. [30]. The amplification has been carried out in a 25 µL reaction mixture comprising 12.5 µL of master mix (DreamTaq Green PCR Master Mix (2X), 2 µL of genomic DNA template, 1 µL of each primer, along with 8.5 µL of nuclease free water. A thermal cycler was used for amplification with cyclic settings: 5 min of initial denaturation at 95 °C, 60 s of denaturation at 95 °C, annealing at 62° for 60 s, 120 s extension at 72 °C and final extension at 72 °C for 5 min. The PCR result was separated by electrophoresis on a 1% agarose gel, stained by ethidium bromide, and visualized using a UV transilluminator. The size of the PCR product was identified by using a 1Kb DNA ladder. The sequencing of PCR amplified fragments was performed by the Sanger sequencing method.

Sequence alignment and phylogenetic analysis

The nucleotide sequence of *E. tenella* obtained after sequencing was established by using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/) and then multi-aligned utilizing the CLUSTAL W algorithm [31] from the suite of Molecular Evolutionary Genetic Analysis (MEGA X) program package. The evolutionary history was deduced using the Maximum Parsimony and Neighbor-Joining Method [32], respectively with a bootstrap value of 1000 replicates [33] to depict the evolutionary history of the taxa analyzed.

Results and discussion

Fecal sample and postmortem examinations revealed 24.04% and 33.22% *Eimeria tenella* infections in chicken, respectively of the sub-tropical hilly region of Meghalaya. Fecal sample examination revealed 10.38% (< 3), 43.70% (3–6) and 16.07% (> 6) infections in chicks, statistically significant ($P < 0.05$). However, post-mortem examination revealed 6.02%, 61.79% and19.57% infections in < 3, 3–6 and > 6 weeks old chicks, respectively which was significant statistically ($P < 0.05$) (Table 1). Oocyst per gram (OPG) was recorded highest and lowest in July (26,500) and February (9800), respectively. Earlier Kalita et al. [34], Thenmozhi et al. [23], Mares et al. [35] and Hassan et al. [36] reported 62.5%, 46.6%, 10.84% and 5.88% *E. tenella* infections in chicken, respectively. The discrepancy in percent prevalence observed in the current study could be attributed to differences in climate, environment and geographical region. *E. tenella* infects and destroys the epithelial cells of Lieberkhun's caecal crypts, causing various degrees of hemorrhage depending on the amount of the parasite's infective dosage and host characteristics such as age, genotype, and pre-exposure [37]. This parasite may trigger moderate to extreme morbidity, reduction in weight or gain, dehydration, diarrhoea, blood loss, and, in severe cases, death [38, 39]. According to Choi et al. [40], *E. tenella* infection reduces cecal volatile fatty acid (VFA) production, which harms feed efficiency and small intestine health. VFA is linked to feed efficiency by supplying more energy to the host or affecting chicken metabolism.

The use of species-specific primers to amplify the small subunit (SSU) of the 18 S rRNA gene from isolated genomic DNA confirmed *Eimeria tenella* infection in chicken. An expected size of 1790 bp PCR product was visualized on agarose gel electrophoresis (Fig. 1).

Table 1 *Eimeria tenella* infection in chickens of the hilly region of Meghalaya

Age group (Weeks)	Feces		Dead chicks	
	Sample examined (No.)	Sample positive (%)	Sample examined (No.)	Sample positive (%)
< 3	106	11 (10.38)	83	5 (6.02)
3–6	119	52 (43.70)	123	76 (61.79)
> 6	112	18 (16.07)	92	18 (19.57)
Total	337	81 (24.04)	298	99 (33.22)
χ^2, *df* - 2	–	23.19*	–	40.42*

Figures in parentheses indicate percent positivity

*P (< 0.05)

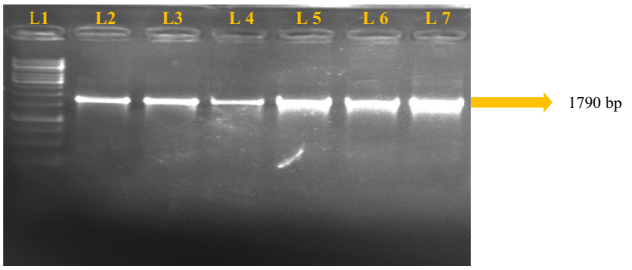

Fig. 1 PCR products electrophoresis on agarose gel. L1: 1Kb DNA ladder, L2, L3, L4, L5, L6, L7: 1790 bp (expected size)

The amplicon of SSU rRNA gene of *E. tenella* isolate from Umiam, Meghalaya was sequenced and deposited in the GenBank database of the National Centre for Biotechnology Information (NCBI). The sequence has been verified to be from the SSU rRNA gene of *E. tenella*, Umiam, Meghalaya isolate (Accession No. OR458392.1) using the Basic Local Alignment Search Tool (BLAST). Analyses revealed that these sequences shared similarities of more than 99% with SSU rRNA gene sequences of *E. tenella* available in the NCBI database with pair wise similarities ranging from 71.59 to 100.0%. The highest sequence homology of 100.0% was discovered between the Umiam isolate of *E. tenella* from Meghalaya and the *E. tenella* isolate from Turkey (HQ680474.1), and the lowest homology of 95.6% was found in the *E. tenella* genome assembly, chromosome: 12, UK (HG994972.1). The SSU rRNA gene sequences of the Umiam isolate were found to have 97.08% and 100.0% nucleotide similarities with *E. tenella* from both the UK (AF026388.1) and the USA (U40264.1), respectively (Fig. 2).

However, nucleotide similarities of 98.24%, 85.33%, 84.75% and 81.35% were observed with *E. tenella* strain Bangalore (JX312808.1), *E. tenella* isolate Kerala-1 (JX093898.1), *E. tenella* isolate Kerala-3 (JX093900.1) and *E. tenella* isolate Kerala-2 (JX093899.1), respectively. A similar range of nucleotide homology was also reported by Thenmozhi et al. [24], from ITS-1 sequences of *E. tenella*, isolated from feces from farms in Tamil Nadu, India with the American and European strains with nucleotide homology ranging from 95 to 100% respectively. A comparable sequence homology of SSU rRNA as observed in the present study was also reported amongst different *E. tenella* isolates from Kerala [23].

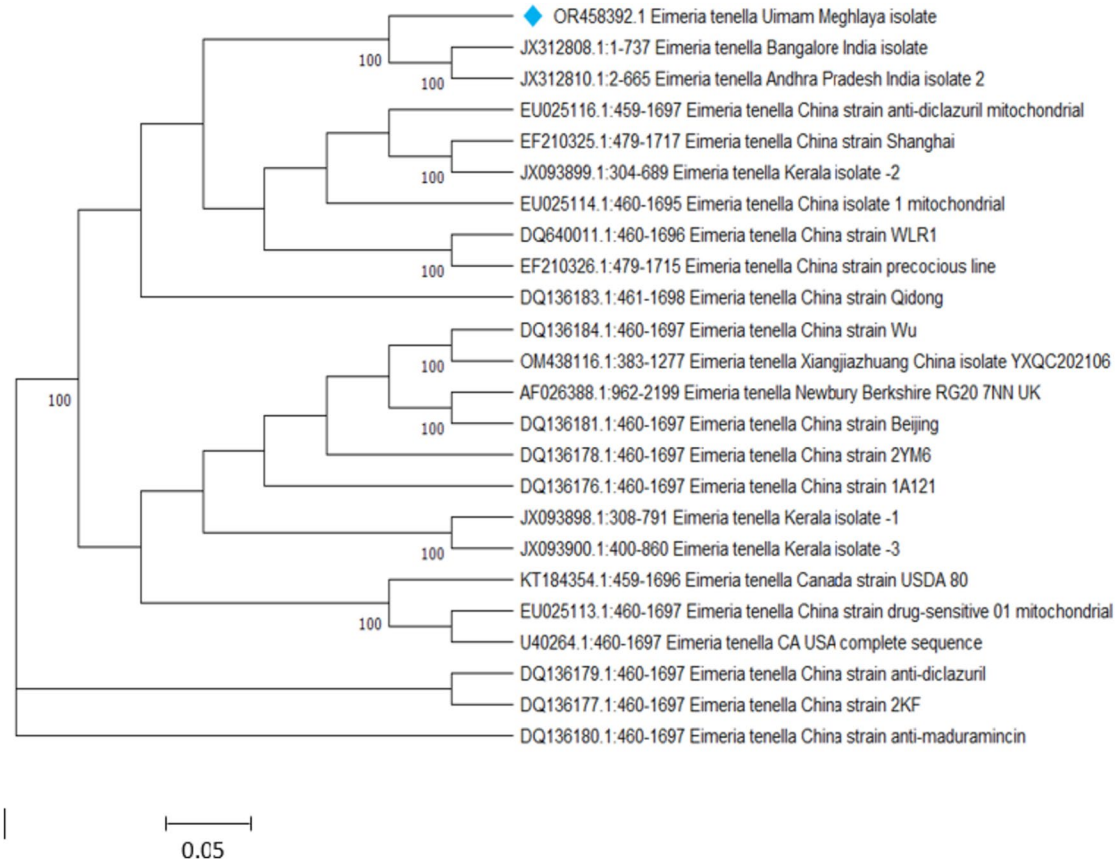

Fig. 2 Phylogenetic tree based on 18 S rRNA gene of *E. tenella* isolates

Phylogenetic analysis of SSU rRNA sequences of *E. tenella* Umiam, Meghalaya isolate with cognate sequences throughout the world revealed these sequences are distinct but at the same time share a close phylogenetic relationship with Indian isolates from Bangalore and Andhra Pradesh (Fig. 3).

In addition, the distant phylogenetic relationship was observed with cognate gene sequences of United States of America, Canada, China. Similar observations were also made by other researchers for ITS-l sequences of *E. tenella* Indian isolates, whereby Chinese *E. tenella* strains were clustered differently from the Indian isolates [24]. Interestingly, a rather distant relationship was observed between the *E. tenella* Umiam, Meghalaya isolates (OR458392.1) and three Kerala isolates. Furthermore, phylogenetic analysis of SSU rRNA sequences of *E. tenella* Umiam, Meghalaya isolate, with other Indian isolates showed three different clades (Fig. 3). Except for the Umiam, Meghalaya isolate (OR458392.1), being an out-group, three Kerela isolates (JX093898.1), (JX093900.1) and (JX093899.1) were clustered together in one group. Similarly, a monophyletic clade was observed between *E. tenella* strain Bangalore (JX312808.1) and *E. tenella* Andhra Pradesh isolate (JX312810.1) indicating a close relationship between these two isolates. Furthermore, the cladogram showed that the *E. tenella* Umiam, Meghalaya isolate (OR458392.1), is genetically distinct but shows some genetic relatedness with the *E. tenella* strain Bangalore (JX312808.1) and *E. tenella* Andhra Pradesh isolate (JX312810.1), but was observed to be genetically distant from the three Kerala isolates (JX093898.1, JX093900.1 and JX093899.1). A distant relationship between the *E. tenella* Umiam, Meghalaya isolate (OR458392), and the *E. tenella* Kerala isolates (JX093898.1, JX093900.1, JX093899.1) was observed with more than 2% of divergence based on the distance matrix.

Conclusions

The current investigation concluded that *Eimeria tenella* infection is common in different age groups of chickens of Meghalaya. *E. tenella* 18 S rRNA gene amplification and

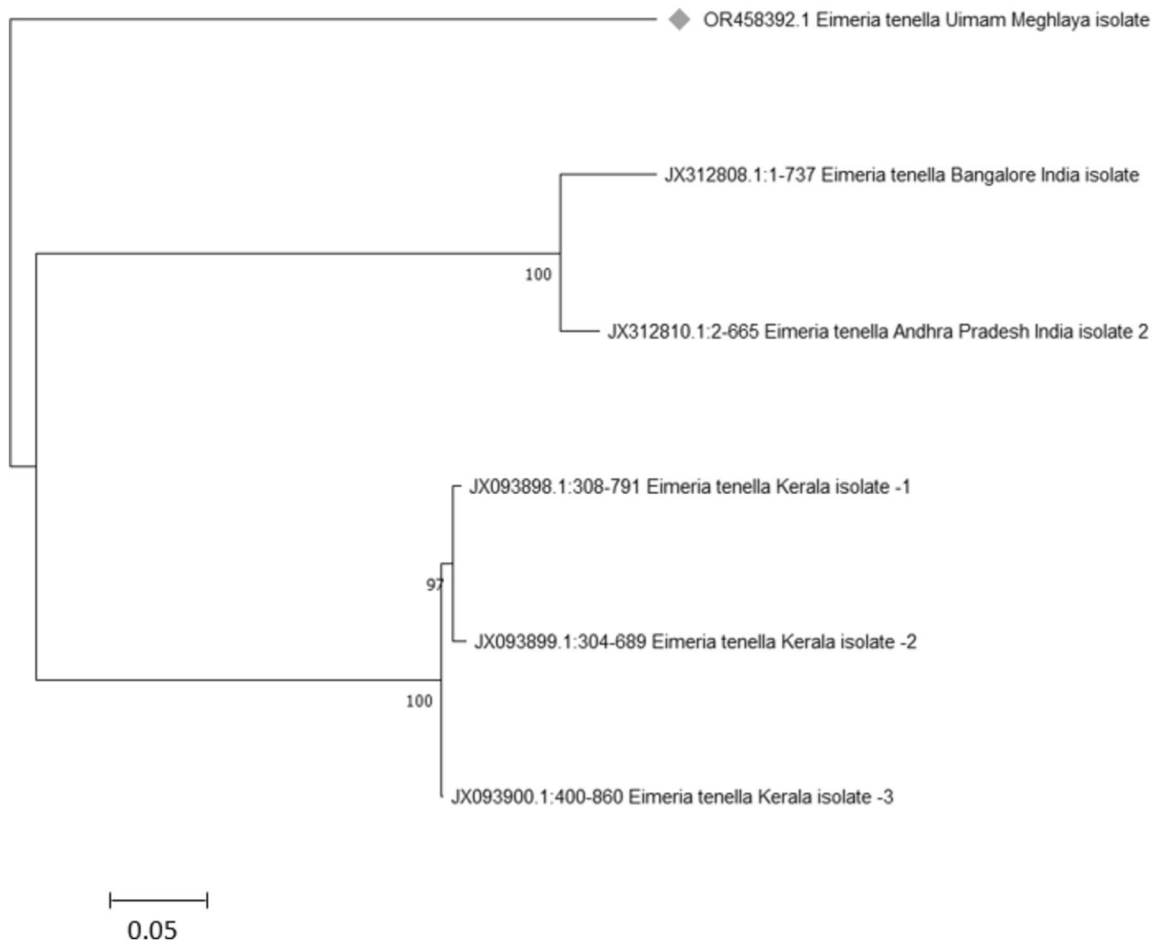

Fig. 3 Phylogenetic relationship of *E. tenella* Umiam, Meghalaya isolates with other E. tenella Indian isolates.

sequencing revealed that SSU rRNA gene of *E. tenella*, Umiam, Meghalaya isolate (OR458392.1) have sequence similarities of more than 99% with sequences available in the NCBI database. Pair wise alignment exhibited nucleotide homology ranging from 71.59 to 100.0% with the maximum sequence homology (100.0%) shared with the *E. tenella* isolate from Turkey (HQ680474.1). Phylogenetic analysis with cognate sequences throughout the world revealed that SSU rRNA sequences of *E. tenella* Umiam, Meghalaya isolate are distinct but also share a close phylogenetic relationship with Indian isolates from Bangalore and Andhra Pradesh. This report on phylogenetic analysis of *E. tenella* may be considered as the first report from Meghalaya as well as North East region of India.

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Author contributions MD: conceptualization, funding acquisition, drafted manuscript. NM: methodology, data analysis. MMM: sample collection, data curation.

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Data availability All data obtained during this study are included in the manuscript.

Declarations

Competing interest The authors declare no competing interests.

Ethical approval This study was done as per the guidelines of the Institute Ethics (OXX5395) and consent to publish manuscript approved by Institute. Owners of the poultry farm gave consent to us for collections of samples from chicks used in this study.

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