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Changes in the morphology and structure of some *Eimeria* oocysts in two cattle calves under some experimental observations

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Abstract
The study was carried in two cattle calves of age around one year with the aim to study the changes in the morphology of *Eimeria* oocysts with time and under different pooling conditions. One calf was highly infected with *Eimeria alabamensis* and *Eimeria bovis* with an oocyst per gram (opg) count of about 30000 and other was highly infected with *Eimeria zuernii* with an opg count of about 24000. However a very lesser percentage (5-10 percent) of other *Eimeria* species (*E. auburnensis*, *E. ellipsoidalis*, *E. cylindrica*, *E. subspherica*) was also present in both the cases. Different pooling procedures were applied for DNA extraction. Some portion of pooled and intact faecal samples from both the calves were kept preserved in a refrigerator for about two months. On examination after pooling under different protocols, a decrease in the oocyst count of *E. alabamensis* and *E. bovis* in sample first and also some unique changes in the morphology of some oocysts along with a small black rounded and vibrating cylindrical mass was observed. However, no decrease in opg count or any distinct changes were noticed in the oocysts of *E. zuernii* in sample second under same conditions. A change was also observed in some oocysts in preserved samples. The identity of *E. alabamensis*, *E. bovis* and *E. zuernii* was ascertained at morphological level and confirmed by molecular sequencing while the rest of species were identified only on morphological basis.

Keywords: *Eimeria*, morphology, change, black round mass, different protocols

Introduction
Bovine coccidiosis caused by *Eimeria* species are strictly host specific and more than 20 species of *Eimeria* are defined in cattle worldwide (Daugschies and Najdrowski, 2005) [7]. Thirteen species have so far been described from Europe (Joyner *et al*., 1966) [13], nine species from Hamdan province of Iran (Heidari *et al*. 2014) [11]. In Indian state of Assam, seven (Das *et al*. 2015) [6] and union territory of Jammu and Kashmir eight species of *Eimeria* (Pandit, 2009) [15] have been reported with *E. zuernii* and *E. bovis* as most predominant in Kashmir valley. The main work done on the *Eimeria* has been more focused on the prevalence and identification of the species and little work has been done to note the changes in the morphology. However changes in the morphology particularly the size of oocysts have been reported by a number of investigators in a number of organisms including sparrows, poultry and rats (Becker *et al*. 1955, 1956, Cheissin 1947, Cordero 1959, Fish 1931, Jones 1932, Donald 1971) [2, 4, 5, 10, 12, 8] but no such a work has been carried out in cattle. This study was aimed to note morphological changes in *Eimeria* oocysts of cattle with time in the laboratory and using different methodological procedures.

Material and Methods
Collection of samples
Faecal samples of two male cattle calves of age around one year were brought from two local farmers from district Ganderbal of Kashmir Valley.

Identification and pooling of oocysts
Oocysts were identified based on shape, presence or absence of micropyle and its cap, presence or absence of residual bodies, sporulation time (Soulsby, 1982 and Eckert *et al*. 1995) [18, 9]. Micrometry was done as per the procedure laid down by Shahardar *et al*. (2020) [17]. McMaster's technique (1986) was used to determine the oocyst count per gram of faeces (opg). Pooling of oocysts was done by standard procedure of repeated centrifugation followed by sedimentation and washing. A total of 3 Centrifugations (2500-3000 rpm) were carried

each for 5-7 minutes for each protocol described below. However in addition to standard procedure of pooling certain modifications were performed under different protocols to ascertain the changes in opg and morphology if any:-

Protocol-1 (Collection of oocysts done in a single day followed by repeated washing and centrifugation.
Float of about twenty test tubes (cover slips and 2ml supernatant) containing predominantly *E. alabamensis* and *E. bovis* and a very less percentage of other species like *E. zuernii* and *E. subspherica* was collected and pooled in a single day by standard procedure of washing, centrifugation and sedimentation.

Protocol-2 (Collection of oocyts done for about two months in same 6-8ml water and washed after two months)
Same sample (as in protocol -1) containing mainly *E. alabamensis* and *E. bovis* and few other species was selected for oocyst pooling using standard salt floatation technique. Pooling was done in a 15 ml test tube for about two months by dipping and washing cover slips using about 6-8 ml of pure water and kept stored in refrigerator at 4^0c for further use. All the rest cover slips containing oocysts were washed in the same 6-8ml of water for about two months to obtain maximum number of oocysts if possible. The same procedure was done for sample containing mainly *E. zuernii* oocysts. The samples were washed after about two months by the same standard procedure.

Protocol-3 (Collection done for three days in 20 ml plain water)
Oocysts of *E. alabamensis* and *E. bovis* were harvested for two days by same salt floatation technique from the same sample and cover slips dipped in about 20ml of plain water in a container and stored at 4^0c in a refrigerator. The container was vigorously hand shacked on third day and solution was observed under microscope.

Protocol-4 (Faecal samples subjected to magnetic stirring)
Same faecal samples were directly subjected to magnetic stirring in salt and fresh water for varying times (2-15minutes) to note the opg count and observe the changes.

Protocol-5 (Storage of fresh faecal samples and pooled samples)
A portion among the two collected faecal samples, one containing mainly the oocysts of *E. alabamensis* and *E. bovis* and second containing mainly the oocysts of *E. zuernii* were stored for about two months in a refrigerator at 4^0c and processed after the said period by salt floatation technique. A sample of diluted and washed float in a test tube containing mainly oocysts of *E. alabamensis*, *E. bovis* and traces of other species like *E. subspherica*, *E. auburnensis* and *E. zuernii* was also stored in refrigerator for two months.

DNA Extraction and Molecular identification
DNA was extracted by standard procedure (Sambrook, 2001) [16] as obtained from four samples using protocol-1, protocol-2, protocol- 3 and protocol- 4 and named as sample S1, S2, S3, and S4 respectively.

DNA amplification and Primers
PCR was done by using genus and specific primers (**Table 1)** as described by Kawahara *et al*. (2010) [14] and amplified products were compared with 50bp gene ladder in case of *E. alabamensis* and *E. zuernii* and 100bp gene ladder in case of *E. bovis*. Sequencing was done at Agrigenome laboratory, Kerala, India. The raw sequences obtained were edited using GENE TOOL soft ware and the sequences were analyzed using BLAST (Altschul, 1990) [1] search of NCBI (National Centre for Biotechnology Information) for determining similarities with the sequences present in the nucleotide data base.

Table 1: Genus and species specific primers

Specimen	Forward primer	Reverse primer
Genus	5GCAAAAGTCGTAACACGGTTTCCG3	3CTGCAATTCACAATGCGTATCGC5
E. alabamensis	5CATTCACACATTGTTCTTTCAG3	3GCTTCCAAACTAATGTTCTG5
E. bovis	5TCATAAAACATCACCTCCAA3	3ATAATTGCGATAAGGGAGACA5
E. zuernii	5AACATGTTTCTACCCACTAC3	3CGATAAGGAGGAGGACAAC5
E. subspherica	5CAACGTTTTTCCTTTTCCTATCA3	3ACTGCGATGAGAGAGAGCG5
E. cylindrica	5GACATTTAAAAAACCGATTGGT3	3GGCTGCAATAAGATAGACATA5
E. auburnensis	5TAAATTGGTGCGATGAGGGA3	3GCAATGAGAGAAAGATTTAATA5

Results

Table 2: Summary of changes in oocysts (samples) under different protocols

Protocol/Sample	Changes in suspected species
First (Moderate changes)	Some demorphed oocysts of *E. alabamensis/E. bovis* suspect observed. A decrease in oocyst count and size in some oocysts was observed. However a good number of intact oocysts of *E. alabamensis* and *E. bovis* along with *E. auburnensis*, *E. zuernii* and other species were also reported.
Second (Major changes)	Some miniature empty oocyst ghosts were observed. Vibrant changes in more than 70% of oocysts of *E. bovis* and *E. alabamensis* suspect noticed (fig 1a, 1b, 1c). Steep decrease in oocyst count. Black rounded and vibrating cylindrical mass observed. No change in *E. zuernii* oocysts in morphology or opg observed. However a dense demorphed and enlarged oocyst of *E. zuernii* like was observed.
Third	Changes observed in *E. bovis* and *E. alabamensis* suspect (fig2a). Black rounded and vibrating cylindrical mass observed (fig 2b).
Fourth (Changed and enlarged oocysts)	Enlarged amoeboid and paramecium shaped oocysts of *E. bovis*, *E. alabamensis* suspect observed (fig 3a, 3b). Empty oocysts were seen in different broken stages with black mass both outside and inside the oocysts. An increase in size and miniature demorphing in shape was also observed in few oocysts of *E. zuernii*.
Fifth	A limp and dent in sides observed in some oocysts with micropyle like structure in faecal sample not seen two months before (fig 4a, 4b). Enlarged and demorphed *E. zuernii* like oocyst, Clear micropyle seen in *E. subspherica* like oocyst (fig 5a) and wider micropyle seen in china bowl type oocyst in stored float (fig 5b).

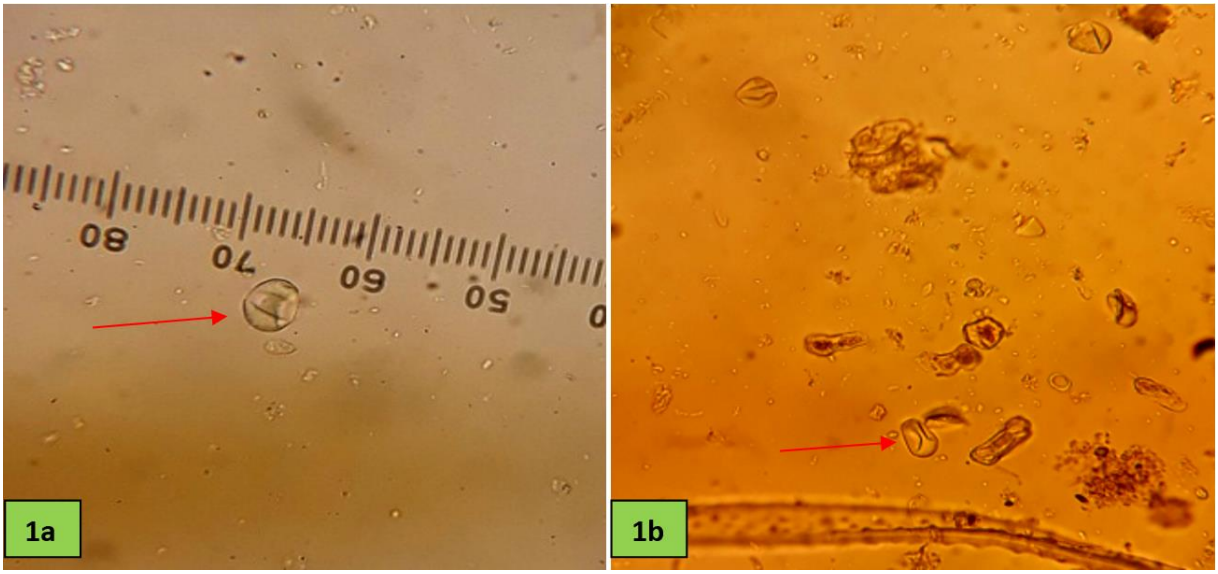

Fig 1a: (Transparent empty and changed oocyst by centrifugation, *E. alabamensis* suspect).
Fig 1b: (Changed oocyst with groove).

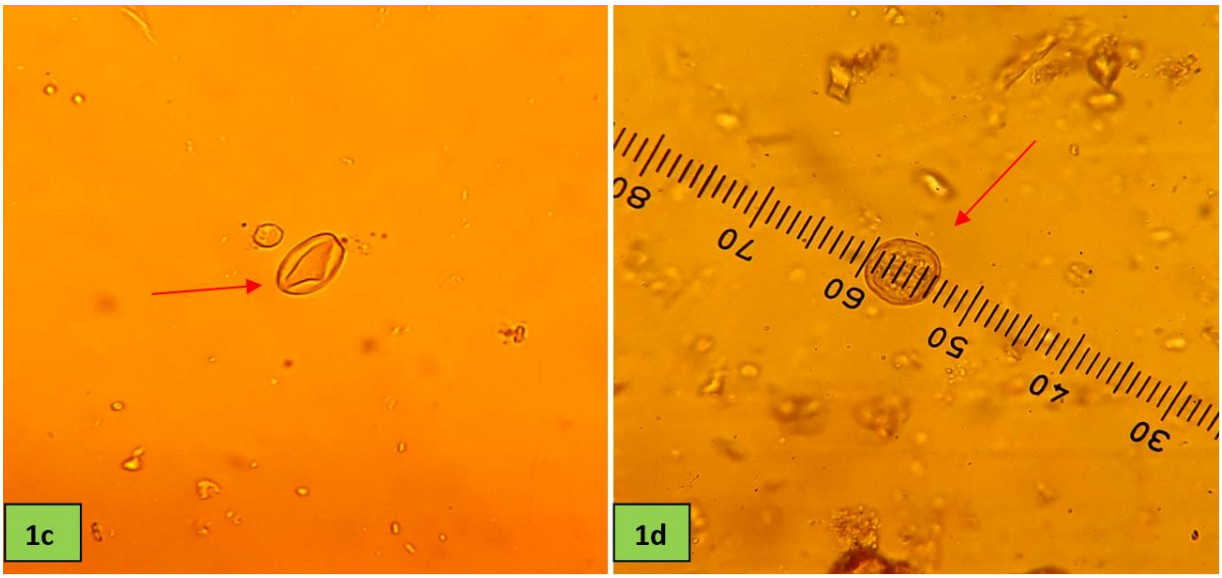

Fig 1c: (Emptied diamond shaped oocyst with round mass outside**).**
Fig 1d: (Enlarged and slightly distorted oocyst of *E. zuernii* suspect)

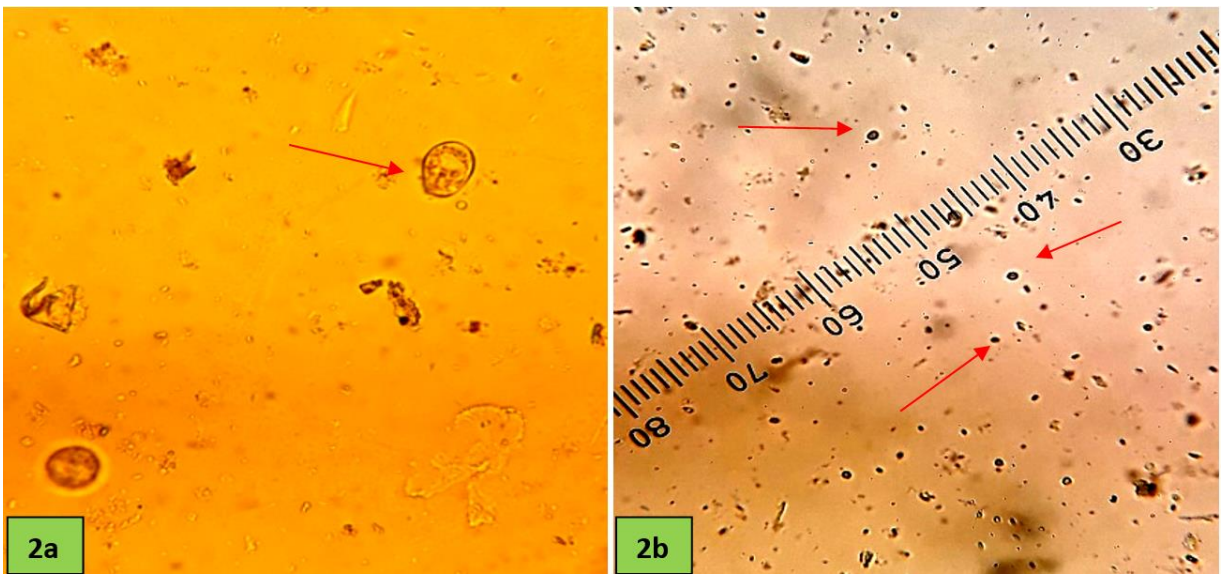

Fig 2a: (Emptied *E. alabamensis* suspect oocyst with inner mass out).
Fig 2b: (Black residual and inner sporocyst mass)

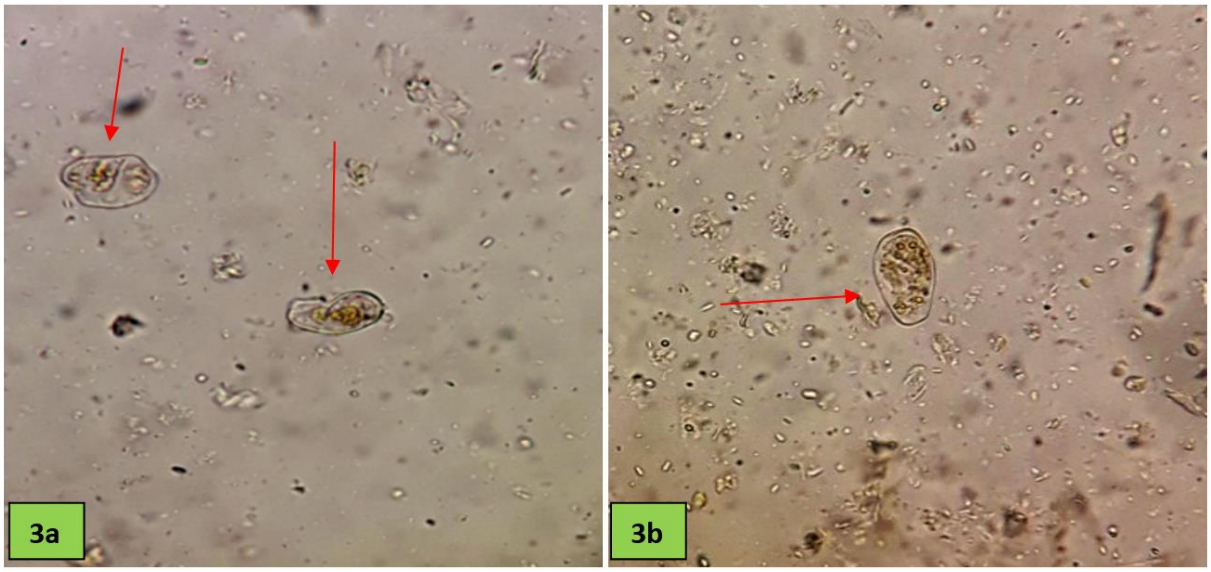

Fig 3a: (Enlarged amoeboid and paramecium shaped *E. bovis* suspect oocysts).
Fig 3b: (Four dots like structures visible inside *E.bovis/E.alabamensis* suspect and vibrating white dotted internal mass out in the field)

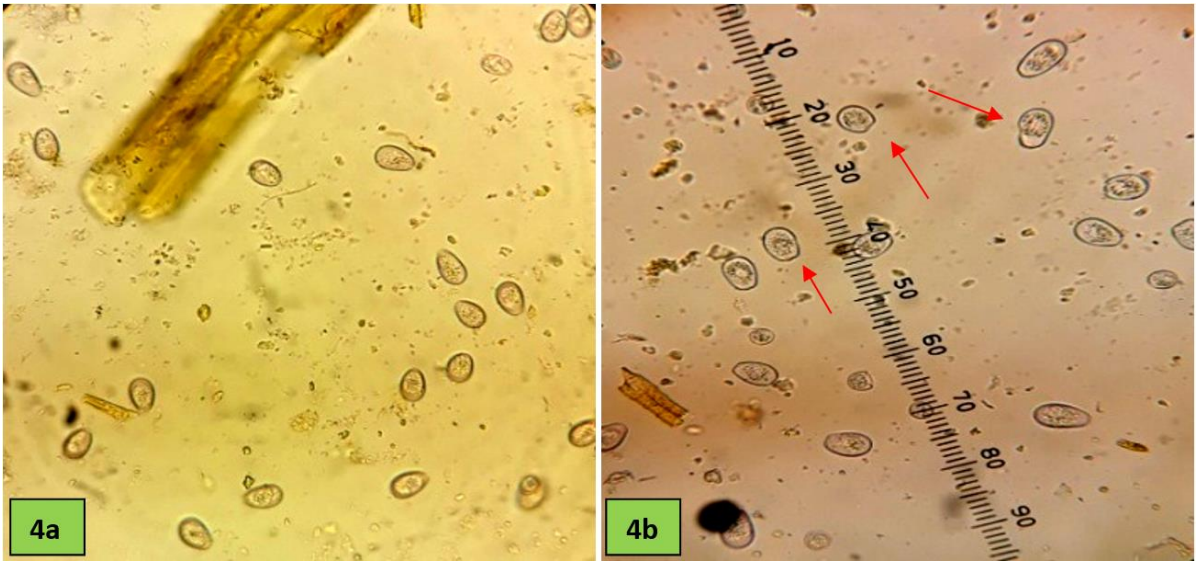

Fig 4a: (Intact oocysts before 2 months).
Fig 4b: (Oocysts after 2 months from same sample with development of more clear micropyle and dent in some oocysts)

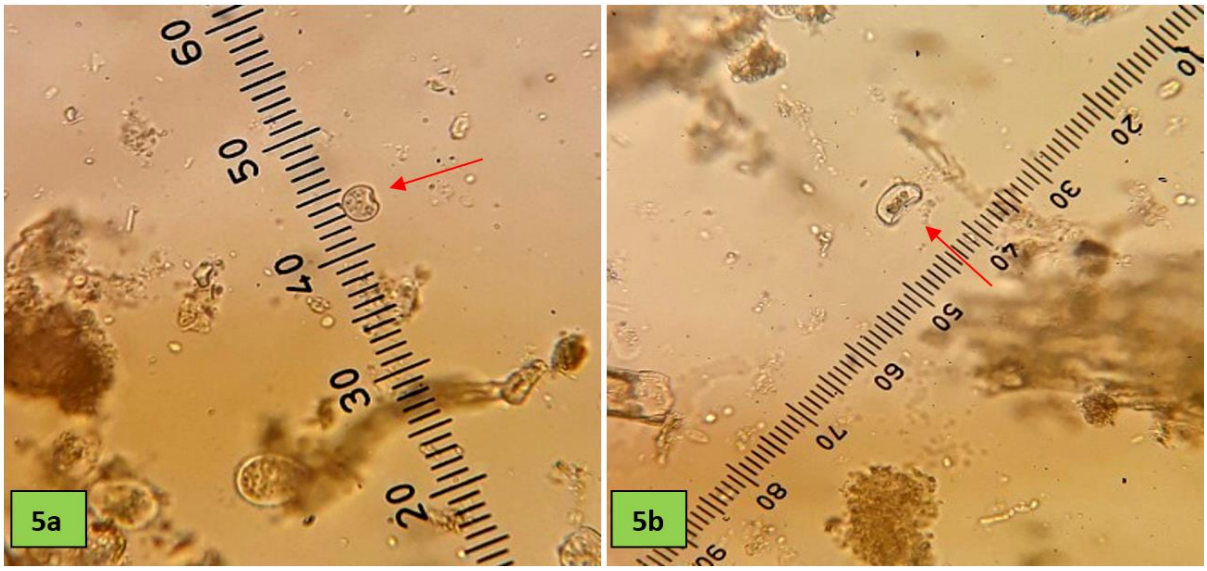

Fig 5a: (*E. subspherica* like oocyst with micropyle).
Fig 5b: (Bowl or china cup shaped oocyst with a wide micropyle possibly due to dent in one side).

Table 3: Summary of changes in individual *Eimeria* oocysts

Species	Change in morphology and opg count
E. alabamensis	Maximum change observed. Some empty and deformed oocysts resembling in shape and dimensions of the same observed. Maximum decrease in opg count of the same observed (fig 1a, fig 1b, fig 1c, and fig 2a). Decrease in size was observed in some oocysts.
E. bovis	Same changes observed as above with more conspicuous micropyle in some oocysts (fig 3a, fig 3b).
E. zuernii	Minimum or almost no changes observed using the said protocols except a partial change in spherical shape and increase in dimension observed using magnetic stirring technique for more than 3 minutes. A distorted spherical shaped oocyst was also observed in the faeces in stored sample (fig 1d).
E. subspherica	Micropyle observed in some oocysts in stored sample (fig 5a).
E. auburnensis	One velvety dotted oocyst observed in stored sample.
E. ellipsoidalis/E. cylindrica	Changes could not be ascertained owing to their very low count.

DNA amplification

On using six primers of cattle (*E. alabamensis*, *E. bovis*, *E. zuernii*, *E. cylindrica*, *E. ellipsoidalis*, and *E. auburnensis*) individually with all the four descriptive samples, genus primer showed amplification in all the four samples (Fig 6a), *E. alabamensis* and *E. bovis* in first (S1), second (S2) and fourth sample (S4) at 184 bp length for *E. alabamensis* and 238 bp length for *E. bovis* (Fig 6a, 6c), *E. zuernii* in third sample (S3) only at 344 bp length (Fig 6b) while *E. cylindrica*, *E. ellipsoidalis* and *E. auburnensis* showed in none of the samples. The results of sequenced PCR products using ITS-1gene when compared with already published gene sequences using BLAST search of NCBI (14) showed 98.5% similarity with *E. alabamensis*, 94% similarity with *E. bovis* and 96% similarity with *E. zuernii.*

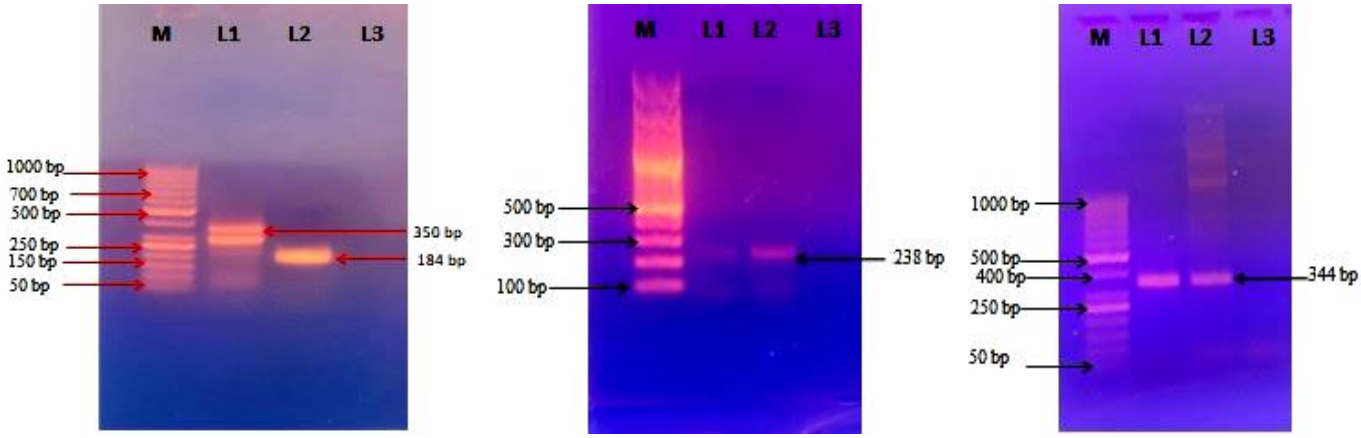

Fig 6a: M indicates 50 bp ladder, L1 indicates –*Eimeria* (genus), L2 indicate *Eimeria alabamensis* and L3 indicates negative control
Fig 6b: M indicates 100 bp ladder, L1and L2 indicate *Eimeria bovis* and L3 indicates negative control
Fig 6c: M indicates 50 bp ladder, L1 and L2 indicate –*Eimeria zuernii* and L3 indicates negative control

Discussion

Boughton (1930) [3] working with sparrow coccidia, for the first time cast doubt on the constancy of oocyst size limits when he found a great deal of variation in the size and volume of oocysts of Isospora lacazei over a 2-month period. Fish (1931) [10] reported the first documented change of oocyst size during patency when he noted that oocysts of *E. tenella* from chickens became longer and broader as the infection developed. Cheissin (1947) [4] studied the variability of shape and size of the oocysts of *E. magna* from rabbits. He found that oocysts appearing earliest in the infection were smaller and lighter in color than those observed later during patency. In contrast to the results of Jones (1932) [12], he noticed that size of the oocysts of *E. magna* decreased when he increased the size of the inoculum. Donald (1971) [8] in a study on *Eimeria separata* in rats has found change in the dimensions of oocyst during the patent period.

In the present study changes in the morphology (shape and size) of some oocysts in cattle were reported under different conditions. The changes were supposedly to be more drastically reported in *E. alabamensis* and *E. bovis* suspect. Little or almost no effect was seen in *E. zuernii*. Changes in *E. auburnensis*, *E. ellipsoidalis* and *E. cylindrica* could not be ascertained. Certain oocysts of *E. subspherica* like were also seen with a micropyle. The demorphing and breaking of certain oocysts like *E. alabamensis* and *E. bovis* can primarily be assumed to their thin, weak and less resistant oocyst wall present in them and almost no or little change in *E. zuernii* can be assumed to its thick and resistant wall. The factors like affect of prolonged storage in salt water needs to be ascertained further since demorphing has been reported in the sample that was harvested and processed in a single day also and further almost no deforming was reported in *E. zuernii* in the same time that was stored in salt water. In the present study more conspicuous micropyle was reported in certain oocysts with time that earlier was not reported in the same sample under study. It is primarily assumed the micropyle starts developing in *E. bovis*, *E. alabamensis* and *E. subspherica* from their relatively weak walls by the changes occurring during the storage of these oocysts. The decrease in the number of oocysts on pooling can be attributed to the fact that a good number of oocysts break down during centrifugation, magnetic stirring and also with time in certain cases because of their weak walls. The presence of black rounded and cylindrical mass is supposed to be internal mass of sporocysts/sporozoites, residual bodies and other sub structures that have come out due to complete or partial breakage of oocysts as it was confirmed by PCR

amplification. The vibratile motion is supposed to be due to the early release of sporocysts/sporozoites from the damaged/ broken oocysts. The amplification of genus and species specific bands validates the identification of *Eimeria* at genus and species level. The results of the present findings also validate that DNA is not much affected by prolonged storage in salt water in refrigerator and DNA remains intact in sporocyst/sporozoite even if oocysts have broken. However such an assumption needs further validation.

Conclusion

The present study primarily concludes that *E. alabamensis* and *E. bovis* are relatively more fragile species compared to other *Eimeria* species present in cattle. *E. zuernii* however is relatively more resistant. The resistant nature of *E. zuernii* due to its stronger wall may contribute to its tiding over unfavorable conditions and thus its global abundance and pathogenicity. It is because of the weak and less resistant nature of oocyst wall in some oocysts that changes in morphologies have been reported. Micropyle also develops or becomes more conspicuous with time in some oocysts. Magnetic stirring for varying times changes both the morphology of oocysts and also helps in breaking the oocyst wall. Magnetic stirring thus also can be a promising methodology of oocyst wall weakening or breaking for helping in obtaining of DNA along with bead method. The study also primarily concludes that these morphological changes like development of micropyle and dent in body wall on prolonged standing which if not validated properly can lead to misidentification of species with time. It may be because of these facts that there is high misunderstanding in morphological identification of *Eimeria* species by different authors. Black mass is supposed to be internal mass of sporocysts/ sporozoites, residual bodies and other sub-structures.

Declaration of Competing Interest

The authors have no conflict of interest.

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