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Histopathology and phylogenetic tree of *Eimeria* species in goat in Al-Diwaniyah Province

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Abstract---The aim of this study which conducted in Al-Diwaniyah province during the period from October (2021) to April (2022) was to study histopathological examination of intestine and molecular identification with phylogenetic analysis of *Eimeria*. A total of 25 tissue samples (small intestine and large intestine) of goat were collected from slaughter houses in Al-Diwaniyah province. Histopathological study of the intestine revealed immature oocyst of the parasite in the colon, and the reproductive stages of the parasite were observed, represented by the presence of female (macrogametes) and male (microgametes), in addition to observing some histopathological changes represented by the presence of infiltration of lymphocyte and eosinophil of villi in goat. Genomic DNA was extracted from 100 goat's fecal samples and 18S rRNA gene of *Eimeria* was amplified by polymerase chain reaction. PCR technique showed that, out of 100 goat's fecal samples 87(87%) were positive for 18S rRNA gene of *Eimeria*. Fifteen PCR positive product of local *Eimeria* isolates were sent to Bioneer company in Korea for detected the DNA sequence and compared it with that of other *Eimeria* published in GenBank. Sequencing and phylogenetic result show four local *Eimeria bovis*, five local *E. arloingi* and six local *E. christenseni* isolates were recorded in GenBank with homology sequence identity with NCBI-Blast of many countries in different rates.

Keywords---histopathology, phylogenetic tree, *Eimeria* species, goat.

Introduction

Coccidiosis is one of the most important parasitic diseases of goats with worldwide distribution (Kimbata et al. 2009; Chartier and Paraud 2012). This disease leads to economic losses due to high mortality and morbidity, poor growth

and treatment costs (Kaya 2004; Temizel *et al.*, 2011) Coccidiosis is caused by intestinal protozoan parasite of the genus *Eimeria* (Rehman *et al.*, 2011; Cavalcante *et al.*, 2012).

Taxonomy genus of *Eimeria* could be classify according to the morphological feature of speculated oocysts and host identify from which the oocysts have been recovered (Ogedengbe *et al.*, 2015). Although goats can be parasitized by 16 different *Eimeria* species, most of them do not cause visible clinical coccidiosis. Therefore, diagnosis must be combined with species identification in order to avoid economic losses. *Eimeria ninakohlyakimovae* and *E. arloingi* are considered to be the most pathogenic species (Ruiz *et al.*, 2006). Goat *Eimeria* species have different reproductive rate and pathogenicity according to the site of infection, which result in different host pathophysiological responses and histopathological lesions (Hoste, 2001; Dai *et al.*, 2006).

The oocysts are passed in the faeces of infected hosts, goats are infected through the ingestion of speculated oocytes. In the small intestinal, speculated oocysts release sporozites and invade the intestinal epithelial cells, resulting in loss of electrolytes and nutrients malabsorption (McGavin and Zachary, 2011 and Temizel *et al.*, 2011).

The disease is more serious in 4–6 months old kids and also when animals of any age are kept in overcrowded houses and under stressor factors such as weaning, dietary changes; transportation, and cold or heat weather (Kaya, 2004; Gül, 2007). Temperature, moisture and oxygen tension are the main factors which determine the survival and development of coccidian oocysts to the infective stage (Ruiz *et al.*, 2012).

The optimum temperature for the sporulation of most *Eimeria* species oocysts of sheep and goats is 28–31°C temperatures below 40 and above 40°C are considered to be lethal (Kheysin, 2013). The main histopathological lesion of coccidiosis is hyperplastic or proliferative enteritis in sheep and goats, but the pathological changes vary in detail according to the species concerned (Hashemnia *et al.*, 2012).

Hyperplasia of the intestinal epithelium and sub-acute to chronic enteritis which characterized by multifocal infiltration of lymphocytes in the small and large intestine are the main pathological changes associated with this *Eimeria* species (Hashemnia *et al.*, 2015; Khodakaram-Tafti and Hashemnia, 2017).

Different diagnostic methods are available for specific identification of *Eimeria*, traditional methods are based mainly on oocysts morphological characteristics under microscopic examination, clinical signs and histopathology (Carvalho *et al.*, 2011). However, due to the presence of interspecies variation, the morphological method is not fully reliable since natural infections by *Eimeria* are generally mixed with more than one species and several species have confusing features (Khodakaram-Tafti *et al.*, 2013).

Molecular techniques have been reported as useful for species identification or classification of this genus, and have further demonstrated the phylogenetic

position of each *Eimeria* species and phylogenetic clades (Yang *et al.*, 2014). The 18S rRNA and its genes has been used extensively as a molecular marker in phylogenetic analysis (Ogedengbe *et al.*, 2011). For the purpose of the identification of species belonging to *Eimeria* parasite that effect the goat and to study the sequence variations of this parasite in Al-Diwaniyah province this study was designed.

Materials and Methods

A total of 25 tissue samples (small intestines and large intestines) in Al-Diwaniyah province, respectively were collected from each individual animal after slaughtering. A total of five tissue samples (small intestine and large intestine) from each individual animal during the time of necropsy were collected. Small intestine like duodenum, jejunum and ileum were collected and large intestine like caecum (whole), colon and rectum were collected in 10% formal saline and were kept for 24 hours for proper fixation. The fixed samples were dehydrated in ascending grades of alcohol, hardened, cleared, embedded in paraffin and sectioned at 5 μ m thicknesses for light microscopy and stained with haematoxylin and eosin (H&E) following standard histopathological procedures (Fox, 1985). Special stain such as PAS was used wherever necessary.

DNA Extraction

1. DNA extraction using the kit provided from AddBio, Korea initially, about 20mg of faeces lysed within individual Eppendorf tubes with 200 μ l lysis solution as well as 20 μ l of proteinase k (20mg/ml). These were incubated at 60 °C for 1 hour.
2. The tubes were briefly centrifuged at 5000 rpm for 2 minutes.
3. Subsequently, 200 μ l of binding solution was added to the clear lysates and mixed well by vortexing for 15 seconds.
4. A 200 μ l of absolute ethanol was added into each tube and mix well by vortexing for 15 sec followed by brief centrifugation at 5000 rpm for 2 minutes.
5. The clear lysate was transferred into the upper reservoir of the spin column with 2.0ml collection tube without wetting the rim.
6. The columns were centrifuged at 13000 rpm for 1 minute meanwhile the flow-through was poured off then the spin column was reassembled with the 2 ml collection tube without wetting the rim.
7. A 500 μ l of washing-1 solution was added to the spin column with collection tube and centrifuge at 13000 rpm for 1 min followed by poured off the flow-through then reassembled the spin column with the 2.0ml collection tube.
8. A 500 μ l of Washing-2 solution was added to the spin column with collection tube and centrifuge at 13000 rpm for 1 min followed by poured off the flow-through then reassembled the spin column with the 2.0ml collection tube.
9. The spin columns were dried by additional centrifugation at 13000 rpm for 1 min to remove the residual ethanol in spin column.
10. The spin columns that containing the bound DNA were transferred into new 1.5ml micro-centrifuge tubes.
11. A 50 μ l of elution solution was added into each spin column and was let stand for 3 minutes.

12. Finally, the DNA was eluted by centrifugation at 13,000 rpm for 1 min and kept at -20 until further analysis.

Polymerase Chain Reaction (PCR)

The PCR technique was performed for detection of *Eimeria* spp. that were collected from suspected cases, This method was carried out according to a method described by Kawahara *et al.* (2010) as following:

PCR Master Mix Preparation

PCR master mix was prepared by using (PCR Pre Mix Kit) and this master mix was done according to company instructions. After wards, these PCR master mix components were listed in the Table (1) placed in standard AccuPower PCR PreMix tube and transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (Bio rad /USA) Table (1).

Table (1): Multiplex PCR master mix preparation

Multiplex PCR Master mix	Volume
Master mix	10 μ L
DNA template	2 μ L
Forward primer (10pmol)	1 μ L
Reverse primer (10pmol)	1 μ L
PCR water	6 μ L
Total volume	20 μ L

PCR Thermocycler Conditions

PCR thermocycler conditions were set for DNA amplification by using conventional PCR thermocycler system as shown in Table (2).

Table (2): PCR Thermocycler Constructs reaction Conditions

PCR step	Temp.	Time	repeat
Initial Denaturation	94°C	5min	1
Denaturation	94°C	35sec.	39 cycle
Annealing	60°C	30sec	
Extension	72°C	45sec	
Final extension	72°C	5min	1

PCR Product analysis

The PCR product were analyzed by agarose gel electrophoresis as following steps:

1. 1 % (1gm) agarose powder was dissolved in 1x TBE buffer (100ml) and placed in microwave till reach 95°C for 2 minutes, after that was left to cool down at 60°C.

2. Then one drop of Ethidium bromide stain was added to the melted agarose. This was poured into the gel-tray after insertion of the comb in proper position which then left to solidify for 15 minutes at room temperature. After complete solidification, the comb was removed from the tray gently.
3. The gel tray was placed in the electrophoresis tank and filled with 1x TBE buffer.
4. Then 5 µl of PCR products were added into each comb's well.
5. Then the electric current was set at 100 volt and 80 AM for 30 min.
6. Finally, PCR products were visualized by the gel documentation system.

Amplicon sequencing and analysis

DNA sequencing technique was performed for species typing of some positive local *Eimeria* species isolated from goat, as following step:

1. The PCR products were sent to Macrogen Company in Korea in ice bag by DHL to carry out the DNA sequencing by Sanger sequencing system.
2. Once the sequences obtained that were then submitted into NCBI-GenBank to get Genbank accession numbers.
3. The DNA sequencing analysis (Phylogenetic tree analysis) was conducted using Molecular Evolutionary Genetics Analysis version 10 (Mega x) and multiple sequence alignment analysis based on Clustal W alignment analysis.

The identified *Eimeria* species analysis was done by phylogenetic tree analysis in comparison with NCBI-Blast known sequenc

Results

Histopathological examination of the intestine revealed immature oocyst of the parasite in the colon (Figure 1) and the reproductive stages of the parasite were observed, represented by the presence of female (macrogametes) and male (microgametes), as shown in Figures (2 and 3 respectively), in addition to observing some histopathological changes represented by the presence of infiltration of lymphocyte and eosinophils of villi in goat as shown in Figure (4).

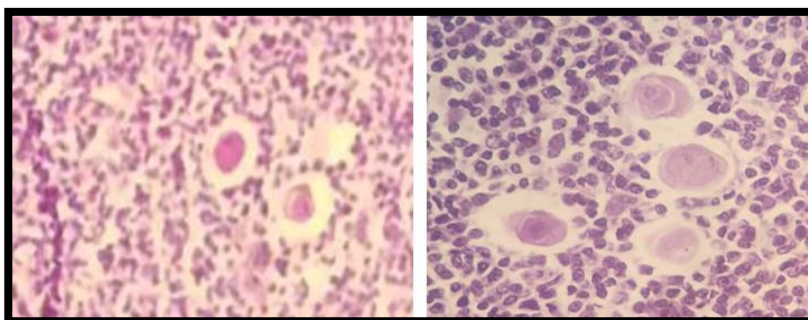


Figure (1): Immature oocyst of *Eimeria* in goat intestine (colon) are seen with H&E, 200 X

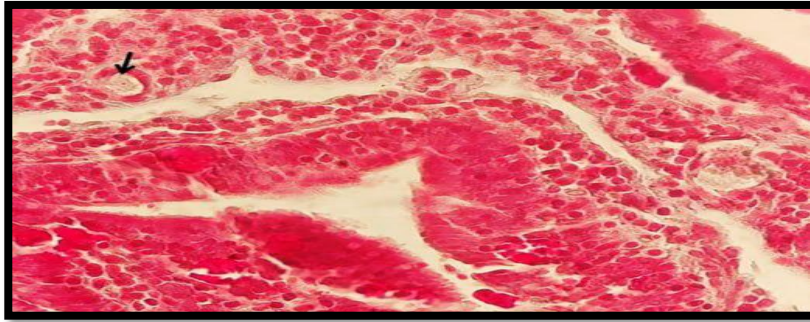


Figure (2): macrogamete of *Eimeria* in goat intestine (duodenum) are seen with masson trichrom stain 400X

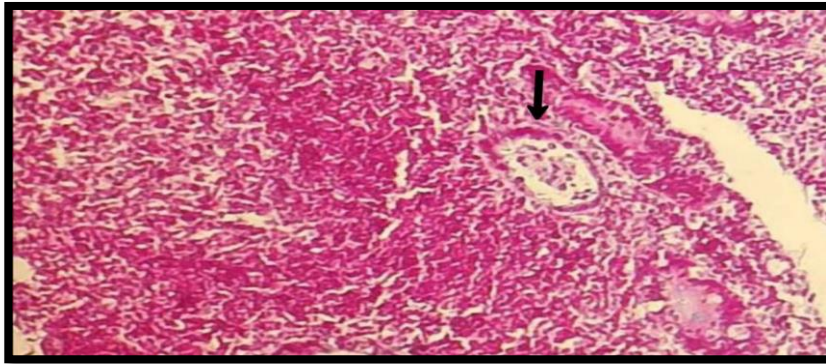


Figure (3): microgamete of *Eimeria* in goat intestine (duodenum) are seen with combined AB-PAS stain 10x

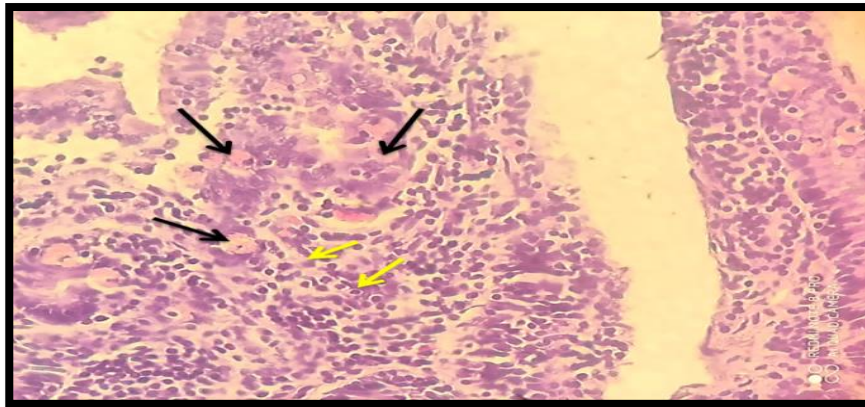


Figure (4): Infiltration of lymphocyte (yellow arrows) and eosinophils (black arrows) of villi in goat intestine (jejunum) are seen with H&E, 200 X

DNA sequencing and phylogenetic tree construction

The result of nucleotide sets of local *Eimeria* species in the present study checked and confirmed by using National Center for Biotechnology Information (NCBI).

The local *Eimeria* species isolates were submitted in NCBI-Genbank data base and Genbank accession numbers were obtained. Based on Clustal W alignment tool of (MEGA 6.0), Sequences of local strains alignment with references strains for *Eimeria* species which previously recorded in GenBank. Four local *E. bovis* isolates (No.1, No.5, No.7 and No.11) were recorded. No.1 and No.11 were homology sequence (99.75%) and (99.23%) identity with tow NCBI-Blast *Eimeria bovis* of Poland. While No.5 and No.7 were closed related to two NCBI-Blast *E. bovis* of Iraq with identity 100%. Five local *E. arloingi* isolates (No.2, No.4, No.8, No.9 and No.10) were recorded. Isolates No.2 and No.9 were homology sequence (99.74%) identity with tow NCBI-Blast *Eimeria arloingi* of Iraq. While isolates No.4, No.8 and No.10 were homology to three NCBI-Blast *E. arloingi* of Portugal with identity (99.75%), (99.24%) and (99.49%) respectively. Six local *E. christenseni* isolates (No.3, No.6, No.12, No.13, No.14 and No.15) were recorded. Isolates No.3 and No.14 were showed closed related to NCBI-Blast to *E. christenseni* of Iraq with identity (100%), also isolate No.13 was (99.74%) identity with NCBI-Blast to *E. christenseni* of Iraq. While No.6 and No.15 were homology to two NCBI-Blast *E. christenseni* of Myanmar with identity (99.23%) and (99.49%) respectively, whereas isolate No.12 was homology to NCBI-Blast *E. christenseni* of Australia with identity (99.79%). Phylogenetic tree construction was performed by using MEGA 6.0 version. Most *Eimeria* species isolates were close related to NCBI-Blast *Eimeria* of some countries as shown in Figures (5), (6)

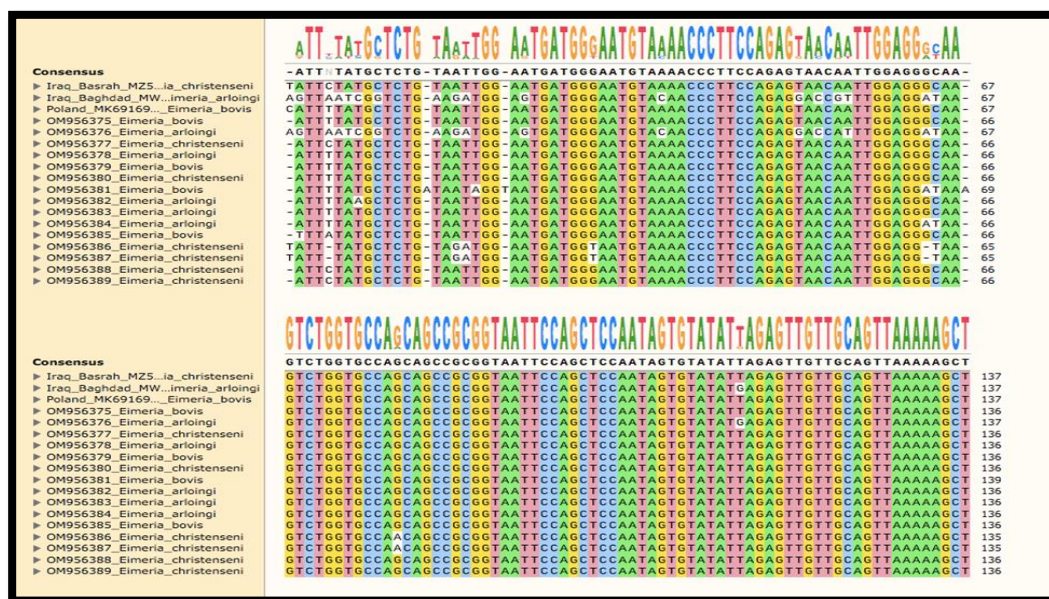


Figure (5): Multiple sequence alignment of the identified *Eimeria* sp. in comparison with homologues global strains. Highlighting with four colours to indicate the similarity

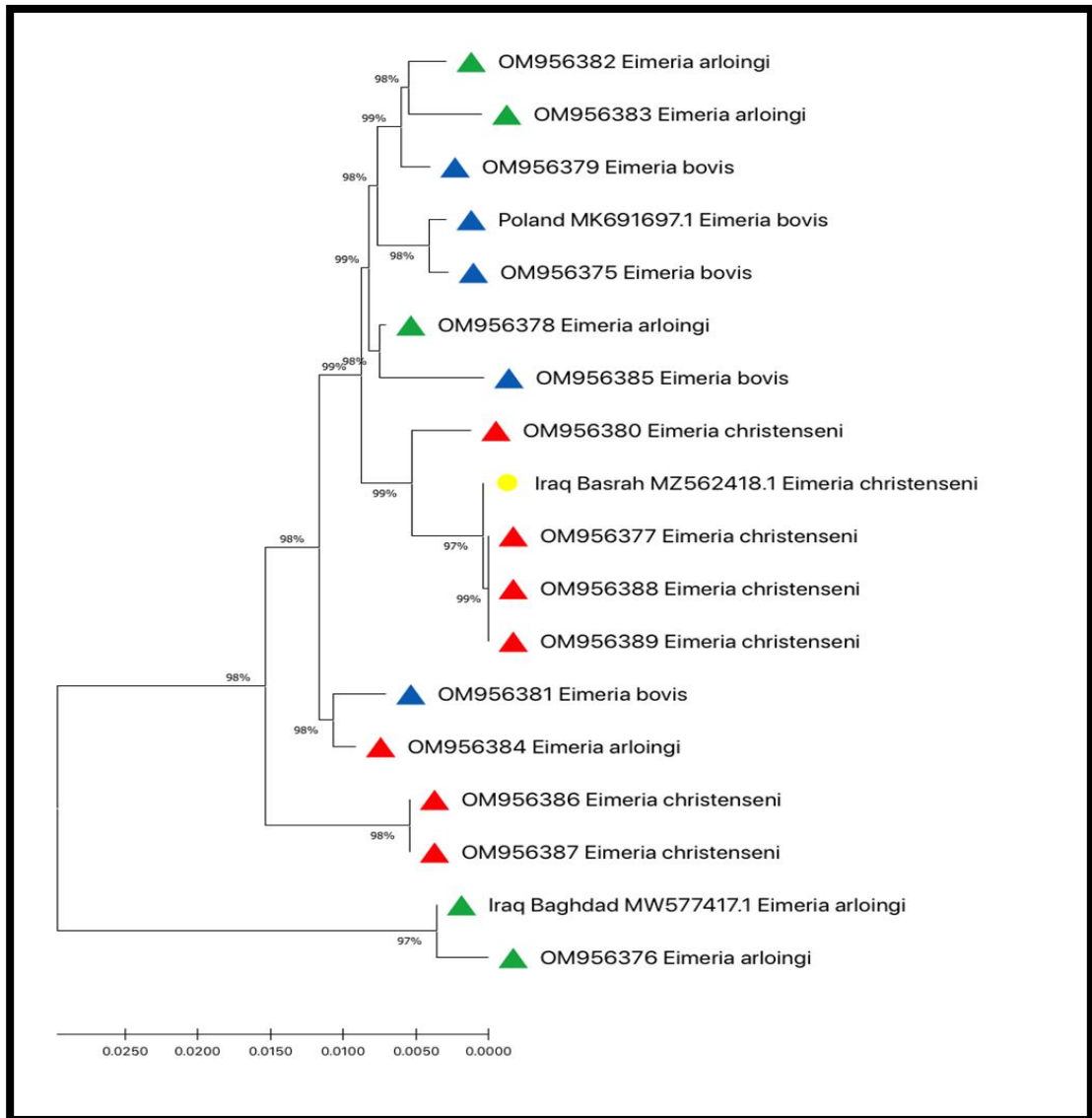


Figure (6): Phylogenetic tree analysis of *Eimeria* spp. targeting (18S rRNA) gene of the currently identified sequences referred triangles (green= *E. arloingi*, red= *E. christensenii*, blue= *E. bovis*). These were deposited in the global gene bank as can be seen as accession numbers followed by the *Eimeria* sp. These were analyzed by Mega 6.0 version.

Discussion

Histopathological examination of the intestine revealed immature oocyst of the parasite in the colon and the reproductive stages of the parasite were observed, represented by the presence of female (macrogametes) and male (microgametes) in duodenum, in addition to observing some histopathological changes represented by the presence of infiltration of lymphocyte and eosinophils of villi in goat.

The results were similar to those reported by Khodakaram Tafti and Mansourian (2008) in which lesions were mainly observed in the jejunum and ileum, and often in cecum. According to Gaboriaud *et al.* (2021), who found that, damages reflect epithelial cell loss, absorptive epithelium atrophy, enteritis from weak to sub-acute with multifocal infiltration of lymphocytes in the small and large intestines, as well as lymphoid hyperplasia in mesenteric lymphatic nodules. Those lesions may destroy huge areas of intestinal epithelium, causing loss of blood and secondary infections that finally might provoke death of goats (Cepeda-Palacios *et al.*, 2015). Lesions caused by *Eimeria* were in close association with the appearance of developing oocysts in the intestinal epithelium. These developing oocysts could be easily observed under the light microscope.

Palacios *et al.* (2015) observed, lesions caused by *Eimeria* found at necropsy examinations were more frequent in duodenum, jejunum and ileum, while in a lesser extent in cecum and colon. Histopathological findings including proliferation of intestinal villi as well as different stage of coccidial life cycle in epithelial cells were in agreement with previous studies (Khodakaram Tafti and Mansourian 2008; Kaur *et al.* 2019). A result of Satish *et al.* (2019) found goat infected with coccidiosis showed, mature first generation schizonts in more advanced stage of merozoite formation with mild haemorrhage, mononuclear cell (MNC) infiltration, and it was found that, Jejunum of infected goat showed eosinophilic circular to irregular structures of developmental stages of *Eimeria* spp. occupying a portion of intestine completely parasitising mucosal epithelial cell layers. It also showed the, first generation macromeronts without vacuoles in lacteal of villus and distending with severe necrosis, fusion and stunting of villi. Severe lymphocytolysis in Peyer's patches with decreased lymphocytes in the medulla and cortex with necrosis of villi and severe infiltration of MNCs were noticed in jejunum (Kheirandish *et al.*, 2014).

Radad and Khalil (2011) mentioned that the jejunum of goat infected with coccidiosis showed early first generation schizonts with several nuclei, young first generation schizonts and ileum of goat showed intermediate first generation schizonts in the early stage of blastophore formation, and in the early stage of compartmentalization characterized by eosinophilic schizonts with thin eosinophilic irregular border severely infiltrated by MNC. Om *et al.* (2010) were showed intermediate first generation schizonts in the more advanced stage of compartmentalization characterized by multiple round to irregular eosinophilic compartments with peripherally arranged basophilic nuclei with congestion, fusion and severe necrosis of intestinal villi. Bakunzi *et al.* (2010) were noticed developmental stages like intermediate microgametocyte with randomly distributed nuclei, more advanced intermediate microgametocytes with peripheral layer of nuclei and residual body with infiltration of lymphocytes and eosinophils with necrosis.

Hashemnia *et al.* (2012) were showed in ileum of goat mature first generation schizonts in early stage of merozoite formation, with moderate destruction of crypts with necrosis, desquamation of villi and severe infiltration of MNC. Kheirandish *et al.* (2014) were showed in ileum of goat PAS negative basophilic micromeront in the duct of crypt with moderate number of PAS positive goblet cells, intermediate microgametocytes with randomly distributed nuclei in the

crypt's lumen with infiltration of lymphocytes and eosinophils, more advanced intermediate microgametocytes with peripheral layer of nuclei and residual body in the crypt's duct with a few PAS positive mucin producing goblet cells and inflammatory cells infiltration, and mature microgametocyte in the crypt's duct with randomly dispersed basophilic nuclei without residual body. Similar findings were also observed by Khillare and Narladkar (2013). Bakunzi *et al.* (2010) were noticed in ileum of goat PAS positive eosinophilic macrogamonts and PAS negative basophilic microgamonts infecting the intestinal villi with severe infiltration of lymphocytes and eosinophils into the ducts of crypts. Similar to Khillare and Narladkar (2013) were showed, mature macrogametes in the lumen of crypts are characterized by hundreds of basophilic nuclei with infiltration of inflammatory cells such as eosinophils and lymphocytes between the glands.

PCR based techniques, have been developed and used for accurate identification and diagnosis of *Eimeria* spp. because of their high sensitivity, specificity, rapidity and utility (Yang *et al.*, 2014). The total results of PCR technique showed that, out of 100 goat fecal samples 87(87%) were positive for (18S rRNA) gene. Study in Baghdad by Hasson (2022) was recorded 93 out of 100 (93%) of samples were positive for *Eimeria* spp. using the 18sr RNA gen. There is little Molecular studies on the infection rate of *Eimeria* species in goats in world and according to our information the first in Al-Dwiniyah province. In Myanmar, Bawm *et al.* (2020) was recorded 93 out of 100 (93%) of samples were positive for *Eimeria* spp. The difference in results of infection rate may be related to the study sample size which examined.

In molecular phylogenetic studies, one of the attractive genomic DNA targets is the internal transcribed spacer1 (ITS1) region derived from the ribosomal RNA (rRNA) genes, the ITS1 region belonging to a multiple copy gene family provides a large number of targets for PCR assays (Kawahara *et al.*, 2010). Recently, 18S rDNA gene sequences have also been used to define the phylogenetic relationship, inter- and intra-species variation existing among some *Eimeria* isolates (Matsubayashi *et al.*, 2005). The 18S rDNA sequences are considered highly species specific and were used widely in differentiating various closely related species of *Eimeria* (Ruttkowski *et al.*, 2001; Matsubayashi *et al.*, 2005).

In this investigation, Identification of *Eimeria* species based on morphological characteristics was consistent with identification by sequencing. The combined use of morphological and molecular tools offers advantages in confirming *Eimeria* spp. identification (Kawahara *et al.*, 2010), particularly with respect to speciation for morphologically similar *Eimeria* spp. oocysts in faecal samples. For example, *E. crandallis* and *E. weybridgeensis* share similar morphological characteristics, with only minor differences evident following sporulation. Without the need for cross infection studies, molecular tools can be used to confirm the identification of morphologically similar oocysts from different hosts to determine if they are genetically identical or different species that are morphologically similar (Al-Habsi *et al.*, 2017). Four local *Eimeria bovis*, five local *Eimeria arloingi*, and six local *Eimeria christensenii* isolates were documented in GenBank with homology sequence identity using NCBI-Blast of numerous coteries at various rates in this investigation. Al-Habsi *et al.* (2017) in Australia reported different species including *E. arloingi*, *E. christensenii* in Austria, and Bawm *et al.* (2020) in

Myanmar recorded different species including *E. arloingi*, *E. christenseni* in Myanmar. Sequences of local strains alignment with references strains for *Eimeria* species which previously recorded in GenBank. Four local *E. bovis* isolates (accession no. OM 956375, OM956379, OM95381 and OM956385) were recorded. No.1 and No.11 were homology sequence (99.75%) and (99.23%) identity with tow NCBI-Blast *Eimeria bovis* of Poland. While No.5 and No.7 were closed related to two NCBI-Blast *E. bovis* of Iraq with identity 100%. Five local *E. arloingi* isolates (accession no. OM956376, OM956378, OM956382, OM956383 and OM956384) were recorded. Isolates No.2 and No.9 were homology sequence (99.74%) identity with tow NCBI-Blast *Eimeria arloingi* of Iraq. While isolates No.4, No.8 and No.10 were homology to three NCBI-Blast *E. arloingi* of Portugal with identity (99.75%), (99.24%) and (99.49%) respectively. Six local *E. christenseni* isolates (accession no. OM956377, OM956380, OM956386, OM956387, OM956388 and OM956389) were recorded. Isolates No.3 and No.14 were showed closed related to NCBI-Blast to *E. christenseni* of Iraq with identity (100%), also isolate No.13 was (99.74%) identity with NCBI-Blast to *E. christenseni* of Iraq. While No.6 and No.15 were homology to two NCBI-Blast *E. christenseni* of Myanmar with identity (99.23%) and (99.49%) respectively, whereas isolate No.12 was homology to NCBI-Blast *E. christenseni* of Australia with identity (99.79%). The phylogenetic tree constructed in this work reveals that *Eimeria* in goats (*E. arlongi*, *E. christenseni*, and *E. bovis*) have a common evolutionary history and hence may have arisen from a single ancestor. In Baghdad, Hasson (2022) was mentioned that seven samples were *E. arloingi*, two samples *E. chirstenseni* and four samples as *E. bovis*. Positive isolates were recorded in NCBI with accession no. *E. arloing* isolates (MW577416, MW577417, MW577419, MW577421, MW577423, MW577424, MW577425) were similar to the Portuguese isolate, *E. christenseni* isolates (MW577418, MW577427) were similar to Myanmar isolate, *E. bovis* isolatse (MW577420, MW577422, MW577426, MW577428) were similar to Polish isolate. In the present study, *E. bovis* was recorded in goat, it might be caused by goats and cattle sharing pasture grazing, resulting in certain hosts being infected with non-pathogenic or pathogenic *Eimeria* species from other hosts. As a result, the sequencing data generated in this work will aid in understanding the genetic diversity and geographic distribution of *Eimeria* species that infect small ruminants across the world.

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