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RESEARCH ARTICLE

Identification and Molecular Characterization of *Eimeria tenella* based on EtMic5 Gene in Pakistan

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A B S T R A C T

The aim of this study was to investigate the molecular characterization and phylogenetic analysis of microneme gene 5 of *Eimeria tenella* (EtMic5) from Pakistan to confirm its evolutionary relationship among different *Eimeria* species. Birds were reared and infected with *Eimeria tenella* oocysts. Postmortem of birds revealed the presence of lesions within intestinal caeca. Oocysts were collected, sporulated and used for RNA extraction. RNA was converted to cDNA and analyzed for EtMic5 gene using polymerase chain reaction (PCR). PCR products were confirmed through gel electrophoresis and the samples positive for EtMic5 gene were cleared using PCR cleanup process. EtMic5 gene was partially sequenced from Macrogen® laboratory Korea. Phylogenetic analysis revealed that the sequence is similar to all those previously reported in other parts of the world. The nucleotide sequence was deposited in GenBank and the assigned accession number is MT684461. The outcomes of this investigation indicate the presence of high frequency of *Eimeria tenella* infection in Pakistan.

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INTRODUCTION

Coccidiosis is a protozoal disease caused by different species of *Eimeria* and considered as most destructive disease within poultry (Shahid *et al*., 2020). It possesses a significant importance in poultry as it is a disease which causes huge economic losses. Poultry is one of the fast growing industries in Pakistan and existing threats like diseases need great attention. Standing as a source of earning for more than 1.5 million people in Pakistan, it shares almost 31% of the total meat production. Seven hundred billion rupees are expected to be the existing speculation of this industry bearing a worthy status (Abbas *et al*., 2015).

Coccidiosis in poultry is caused by species of genus *Eimeria* belongs to phylum Apicomplexa. It is a completely intracellular parasite which possesses great veterinary importance. Huge economic losses were observed due to decrease in egg production, meat production, feed conversion ratio and high mortality in poultry sector (Clark *et al*., 2016). As far as poultry is concerned, one of the most pathogenic and important parasitic protozoa within genus *Eimeria* is *Eimeria tenella*. It effects caecum in birds causing caecal coccidiosis and is responsible for enormous losses to poultry industry (Zhou *et al*., 2017).

Coccidiosis has worldwide prevalence including all important poultry birds like chicken, turkey, geese and ducks (McDougald, 2008). There are seven recognized species in chicken, responsible for coccidiosis (Allen and Fetterer, 2002). These species are *Eimeria mitis*, *Eimeria acervulina*, *Eimeria maxima*, *Eimeria brunetti*, *Eimeria praecox*, *Eimeria necatrix*, and *Eimeria tenella*. Out of these important species in poultry, *Eimeria tenella* is further considered as most pathogenic on the basis of lesions produced and area of gut involved (Williams, 2002).

The oocysts of *Eimeria* species are very resistant towards environmental conditions due to presence of a

cyst around them. These oocysts can survive for years without sporulation at ordinary climatic conditions. There are different ways for the transmission of coccidiosis in host. These include uptake of infected feed ingestion, contaminated water, through the fomites and personnel visiting the houses (Belli *et al*., 2006).

Eimeria infection causes malabsorption of feed, inefficient feed utilization, less feed intake, high morbidity, mortality, inappropriate growth rate in broilers and declined egg production in layers which sometimes leads to secondary bacterial infections causes necrotic enteritis (Chapman *et al*., 2002; Riaz *et al*., 2017). These all circumstances possess an optimizing impact on international poultry production. Loss in international poultry industry caused by *Eimeria* has been estimated to exceed US $3 billion annually (Blake and Tomley, 2014).

Different studies were performed regarding *Eimeria* and coccidiosis in Pakistan (Abbas *et al*., 2008, 2017a, 2017b, 2017c, 2019; Akhter *et al*., 2012; Masood *et al*., 2013, Bachaya *et al*., 2015, Hussain *et al*., 2017; Zhang *et al*., 2020) in which different aspects of disease and count down strategies were discussed but molecular characterization about microneme proteins was never been studied earlier. However, identification of different genes and molecular characterization was exercised in other parts of world for various purposes and novel investigations. Genetic diversity of field isolates related to *Eimeria tenella* was searched by Tan *et al*. (2017) in which extracted RNA and DNA of sporozoites were used. Analysis of isolates by multi sequence alignment and Random Amplification of Polymorphic DNA (RAPD) techniques showed that there is a great genetic diversity among all isolates.

Miska *et al*. (2010) studied the molecular characterization and phylogenetic analysis of different *Eimeria* species from game birds and turkeys along with its implications for evolutionary relationships in other galliform birds. Similar study was performed by Zhang *et al*. (2014) in which he identified and molecularly characterized a microneme 5 gene of *Eimeria acervulina* (EaMic5) and suggested that it might be used as a good candidate for further immunogenic processes.

Saouros *et al*. (2012) studied that the microneme 5 is a glycoprotein and have 11 different receptors which are rich in cysteine with the most resemblance with a domains of plasma pre-kallikrein and blood coagulation factor XI. While the course of infection, the sporozoites of *Eimeria acervulina* came in contact with bird's immune cells. Microneme (EaMic5) was veiled by the parasitic cells to vague the immune system. In a study, functions of EtMic genes during host cell invasion were evaluated and suggested that they might be used as candidates for vaccines development against *Eimeria* infections as functional analysis of EtMic genes were implicated in parasite motility, recognition, migration, and invasion of host cells (Han *et al*., 2016).

The objective of this study was to identify EtMic5 gene from Pakistan and to characterize it for further molecular studies along with confirmation of its evolutionary relationship with different *Eimeria* species. This study might help to investigate further studies as well as to check immunogenic potential of EtMic5 gene belongs to *Eimeria tenella* strain of Pakistan in future.

MATERIALS AND METHODS

Ethics: All the sampling was done by Pakistan Veterinary Medical Council (PVMC) certified DVM clinicians under strict ethical conditions by taking proper permissions wherever required.

Experimental Birds/Oocysts Sporulation: One hundred and twenty birds were reared at poultry experimental sheds PMAS Arid Agriculture University Rawalpindi Pakistan and provided with feed and water ad libitum, without mixing of anticoccidial drugs. Birds were challenged by preparing already preserved sporulated oocyst samples to achieve fresh oocysts in a bulk quantity which may complete whole experiment satisfactorily as it is necessary to passage *Eimeria* oocysts from birds at least every six months (Song *et al*., 2013). After 4 days of infection, faecal material and bedding were collected for separation of oocysts. Faecal material was put into super saturated salt solution and was subjected to centrifugation at 1500-2000 rpm. Supernatant was collected and oocysts were separated. Oocysts were put into potassium dichromate solution (2.5%) and placed in shaker incubator for sporulation at 27°C for 48 hours. Sporulated oocysts were stored at 4°C for further analysis.

Extraction of Ribonucleic acid (RNA): RNA from the samples was extracted using TRIzole LS Reagent kit (Ambion, Life technologies®) following manufacturer instructions. Extracted RNA was immediately stored at -20°C after nano-drop quantification. This analysis gave the Optical Density (OD) value, 260/280 ratios and 260/230 values for each sample.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): RT-PCR was used to synthesize complementary deoxyribo nucleic acid (cDNA) from extracted RNA samples. Maxime RT Premix Kit® containing Oligo dT primer was used. One µg concentration of the RNA template was added in the RT pre-mix tube. RNase free water was added to complete the reaction volume up to 20 µL. A cDNA was prepared using PCR machine with specific conditions i.e. for sixty minutes at 45°C and five minutes at 95°C. cDNA samples were immediately used for PCR. Remaining cDNAs and all other samples were stored at -20°C for further procedures.

Polymerase Chain Reaction (PCR): cDNA was used to amplify the segment of EtMic5 gene using One Taq 2X Master mix (Biolabs®). The sequence for forward primer was (5'-TTCCGTCAGGGCGTTGGATAC-3'), and reverse primer was (5'-ACTTCGTAGGCCGAAGGGC TG-3') obtained from (Ryan *et al*. 2000). A product of 399bp was considered positive. The reaction mixture included 1µl of cDNA sample, forward primer and reverse primers each, while 25µl of Taq 2x Master Mix along with Nuclease Free water up to 50 µl. The PCR amplification was carried out in thermo cycler (2720 thermo cycler by life technologies®) by implementing following conditions. One cycle of 94°C for four minutes (initial denaturation) followed by 35 cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute and finally 72°C for 7 minutes.

Gel Electrophoresis: Agarose gel was used (1.5% w/v) to analyze the products of PCR. A 100bp ladder was used as a DNA marker (BioLabs®). Samples and ladder were carefully loaded and was allowed to run at 110 Volts for 90 minutes. After that gel was carefully examined in a gel documentation system.

Gene Sequencing and Phylogenetic Analysis: The positive PCR Product was further subjected to sequencing and phylogenetic analyses. For gene sequencing, the sample was sent to Macrogen® Korea using ABI 3730xL, standard DNA sequencer. Sequence derived from this study and those obtained from the GenBank database were aligned by the CLUSTAL_W method in the software Seaview®. The distances were computed mean-wise and overall using MEGA7®.The gene sequences were translated using Seaview®. Sequences were subsequently analyzed with neighbor joining to construct the phylogenetic tree (Kumar *et al*., 2016). The statistical significance of the relationships obtained was determined by bootstrap re-sampling analysis with 1000 repetitions. The sequence was deposited to GenBank database. A comparison was made among the sequence of EtMic5 gene from this study with sequences of other studies (AJ245536.1, JN987489.1, EU335049.1, XM 013578697.1, KX377352.1 and XM_013372919.1). (Table 1).

RESULTS

The birds were examined on daily basis post infection. After 4 days of challenge, few birds were found off feed and lethargic. A few droppings with blood contamination were also observed (Fig. 1). From 6th day to 9th day post infection, droppings with blood contamination were found in higher concentration. Some weak birds were slaughtered for post mortem examination and intestinal investigations clearly revealed blood deposition especially in caecum. Faecal samples were examined under microscope which showed the presence of *Eimeria* oocysts. The diagnosis of coccidiosis was made on gross lesions observed during postmortem examination while confirmation of oocysts was done after microscopic examination. These oocysts were subjected for counting by using Mcmaster counting chamber. *Eimeria tenella* oocysts were clearly seen within Mcmaster counting chamber (Fig. 2). These oocysts were used to extract RNA by TRIzole method. A clear pellet of RNA was observed which was later used to synthesize cDNA in a PCR machine. These cDNA samples were stored at -80°C for further usage.

Detection of EtMic5 gene by RT-PCR: With the help of reverse and forward primers, EtMic5 gene fragments from coccidial isolates were amplified. A band of 399bp of EtMic5 gene in all the tested samples confirmed the presence of *Eimeria tenella* (Fig. 3). Purification of positive samples was done and PCR product was sent for sequencing of DNA.

Sequencing and phylogenetic analysis of EtMic5 gene of *Eimeria tenella:* The sequence was compared with 6 other sequences which were reported by other researches worldwide (Table 1) using MEGA10® software. Results have shown that sequence (EtMic5_Pak2) from present study was 98 to 100% identical to the sequences of EtMic5 reported by other studies including AJ245536.1 (United Kingdom), JN987489.1 (Malaysia), EU335049.1 and KX377352.1 (China), ; XM_013578697.1 and XM_013372919.1 (USA). It has been observed through sequencing analysis that only 6 sequences were retrieved from the NCBI against (EtMic5_Pak2) and out of those, three sequences were of *Eimeria tenella*, two were of *Eimeria necatrix* and one from *Eimeria steidai* (Table 1). It has also been observed that the *Eimeria necatrix* sequence (EU335049.1 showed only two base pairs and two amino acids difference from our sequence (Fig. 4). Another sequence (KX377352.1) of *Eimeria stiedai* have shown 100% similarity with the sequence of present study however the query cover was only 159bp.

Fig. 1: Faecal samples of experimental birds showing clear signs of blood (Typical sign of *Eimeria tenella*).

Table 1: Names and accession numbers of highly similar genes with query sequence

Sr. No.	Name of gene	Accession No	Countries
1.	*Eimeria tenella* mRNA for microneme protein 5 (mic-5 gene)	AJ245536.1	United Kingdom
2.	*Eimeria tenella* clone Etm094A02 hypothetical protein mRNA	JN987489.1	Malaysia
3.	*Eimeria necatrix* strain LZ microneme protein 5 (MIC5) gene	EU335049.1	China
4.	*Eimeria necatrix* PAN domain-containing protein	XM_013578697.1	USA
5.	Microneme protein 5 (mic-5) of *Eimeria steidai*	KX377352.1	China
6.	*Eimeria tenella* Micronemal protein MIC4, related partial mRNA	XM_013372919.1	USA

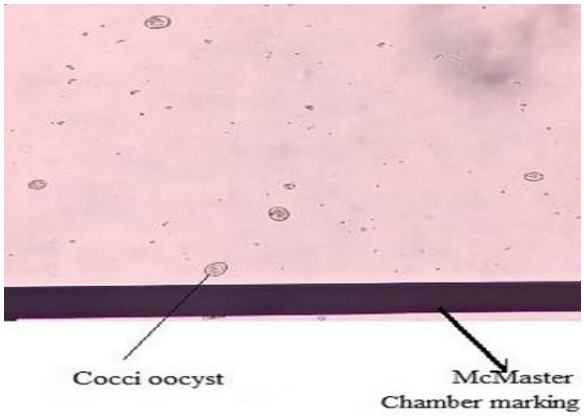

Fig. 2: *Eimeria tenella* oocysts clearly seen in samples. Observed under 10X lense.

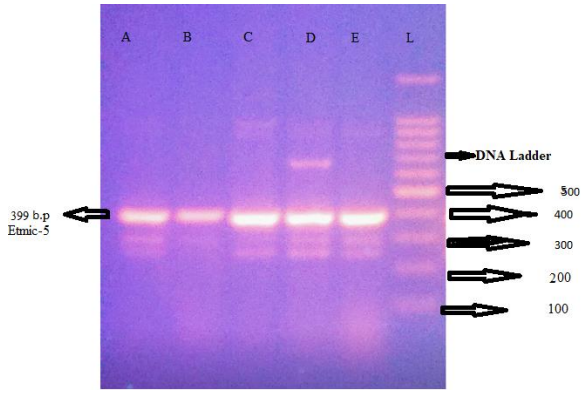

Fig 3: PCR products of 5 positive samples as A, B, C, D, and E from *Eimeria* infected birds. L stands for ladder. A PCR product of 399bp was considered as positive for EtMic5.

Eimeria necatrix strain LZ microneme protein 5 (MIC5) gene, partial cds

Sequence ID: EU335049.1 Length: 1470 Number of Matches: 1

Range 1: 637 to 1006 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
667 bits(361)	0.0	367/370(99%)	0/370(0%)	Plus/Plus

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CDS: Putative 1       1     E A N L L W T L P S E N A E E C R Q R C
Query                 2     GAGGCGAACTTGCTATGGACACTCCCAAGCGAAAACGCAGAGGAATGCAGACAGAGGTGC  61
Sbjct                 637   .......................T.....................................  696
CDS:microneme protei  213    E A N L L W T L P S E N A E E C R Q R C

CDS: Putative 1       21    E V M E S C G R F S Y D A A S K A C S M
Query                 62    GAAGTGATGGAGAGCTGCGGGCGATTCAGCTACGACGCGGCGAGCAAAGCCTGCTCAATG  121
Sbjct                 697   .....................................................  756
CDS:microneme protei  233    E V M E S C G R F S Y D A A S K A C S M

CDS: Putative 1       41    L S G E G E E V Q G E N L V S G P P R C
Query                 122   CTCTCTGGTGAGGGCGAGGAAGTACAGGGCGAAAACCTCGTGTCAGGGCCCCCTCGGTGC  181
Sbjct                 757   .....................................................  816
CDS:microneme protei  253    L S G E G E E V Q G E N L V S G P P R C

CDS: Putative 1       61    T R R D T C Y Q N G V S F T G G K A I S
Query                 182   ACCCGTCGCGACACTTGCTACCAGAACGGGGTGTCATTCACAGGTGGCAAAGCGATTTCT  241
Sbjct                 817   G.............C.....................................  876
CDS:microneme protei  273    A R R D T R Y Q N G V S F T G G K A I S

CDS: Putative 1       81    E A K A A S S Q A C Q E L C E K D A K C
Query                 242   GAGGCAAAGGCAGCTTCTTCTCAGGCGTGCCAGGAGCTCTGCGAGAAGGATGCCAAGTGC  301
Sbjct                 877   .....................................................  936
CDS:microneme protei  293    E A K A A S S Q A C Q E L C E K D A K C

CDS: Putative 1       101   R F F T L A S G K C S L F A D D A A L R
Query                 302   AGATTCTTCACCTTGGCTTCTGGGAAGTGCAGCCTGTTCGCAGACGACGCAGCCCTTCGG  361
Sbjct                 937   .....................................................  996
CDS:microneme protei  313    R F F T L A S G K C S L F A D D A A L R

CDS: Putative 1       121   P T K
Query                 362   CCTACGAAGT  371
Sbjct                 997   ..........  1006
CDS:microneme protei  333    P T K
```

Fig. 4: Alignment of sequences also showed considerable similarity to *Eimeria necatrix* microneme protein 5 (Acc. No EU335049.1). The Sequence difference is of 2 base pairs and two amino acids.

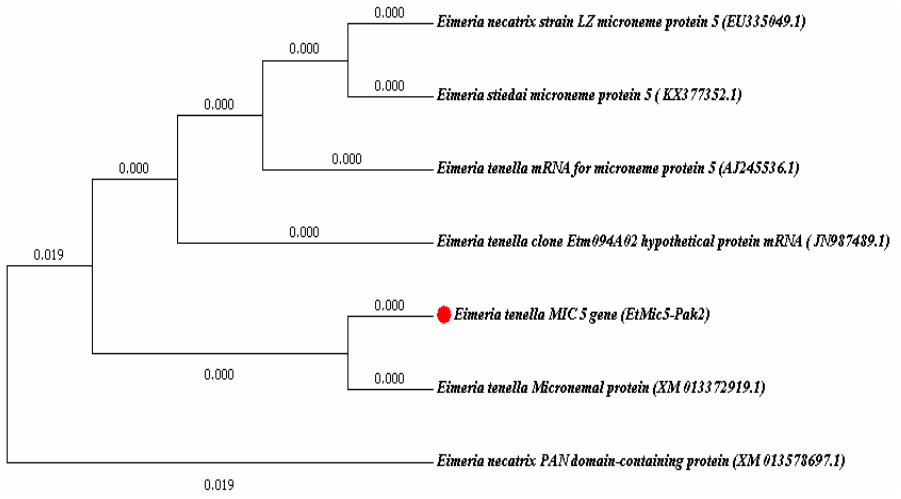

Fig. 5: Phylogenetic Analysis using maximum likelihood method. Red dot or gene sequence is our sample (EtMic5_Pak2).

A phylogenetic tree was also constructed (Fig. 5) based on the alignment with 6 sequences retrieved from NCBI database showing high homology with our sequence. Phylogenetic analysis has shown that this sequence is almost 98-100% percent similar to EtMic5 gene sequences. Tree was constructed using maximum likelihood method, the analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Two big branches were found; one branch was showing only one sequence (XM013578697.1) whereas other branch was further divided into two branches. EtMic5_Pak2 was found in the small branch showing close similarity with *Eimeria tenella* sequence XM013372919. Branches of phylogenetic tree showed high bootstrap values. The nucleotide sequence was deposited in GenBank and the assigned accession number is MT684461.

DISCUSSION

Gastrointestinal parasitic infections including coccidiosis have been a major threat in all animals especially in poultry (Saddiqi *et al*., 2006; Bachaya *et al*., 2015). Coccidiosis is caused by various species of genus *Eimeria. Eimeria tenella* is the most hazardous specie within all 7 species belongs to poultry specifically (Zhou *et al*., 2017).

Identification and molecular characterization of genes is the primary step to proceed further advanced studies and trials. It is necessary to discover and study novel genes encoding immunogenic proteins. After successful molecular studies, the role of different genes will be cleared and these genes might be used as a potential immunogenic candidate and might adopt as successful vaccine against *Eimeria* in future. Molecular characterization and phylogenetic analysis of different *Eimeria* species as done by Miska *et al*. (2010) who revealed the evolutionary relationship of *Eimeria* species and genes with other related ones. Similarly, Zhang *et al*. (2014) also identified and molecularly characterized a microneme 5 gene of *Eimeria acervulina* (EaMic5) and disclosed its evolutionary relationship along with an assumption that it might be used as a good candidate to produce immunogenicity.

All apicomplexan protozoa possess microneme organelles which contain proteins critical and multifunctional for host cell invasion and parasite motility (Bansal *et al*., 2013). Nine microneme proteins have been reported in *Eimeria* so far. These proteins are microneme protein 1-7 (MIC1-7) and two apical membrane antigen 1, 2 also known as AMA1-2 (Carruthers and Tomley, 2008). Functions of MICs during host cell invasion were evaluated and it suggested that they might be used as candidates for vaccines development (Han *et al*., 2016).

Molecular study of AMAI of *Eimeria tenella* by investigating full length cDNA using two techniques i.e. rapid amplification cDNA technique and expressed sequence technique showed that EtAMA1 is 1608bp open reading frame encoding 535 amino acids proteins (Jiang *et al*., 2012). The study also revealed that EtAMA1 might play a critical role in invasion and development of sporozoites according to results obtained from immunohistochemistry and immunofluorescence analysis.

Similarly, molecular characterization of microneme 2 protein was performed in which it was investigated that it is a 35 KDa protein. Incubation of gene with specific antibodies against EtMic2 depicts that it reduces the ability of sporozoites to invade hosts cells (Yan *et al*., 2018).

Different hypothetical proteins regarding *Eimeria tenella* have been sequenced earlier i.e. a hypothetical protein of *Eimeria tenella* EtCHP559 was studied by Zhai *et al*. (2016) and found that open reading frame was of 1224bp. The full length cDNA was 1746bp encoded 407 Amino acids with a molecular weight of 46.04 KDa (predicted). The study showed its existence more in sporozoite stage along with its role in invasion of sporozoite stage within hosts cells.

Present study was performed to investigate the molecular characterization of genes encoding proteins (microneme 5) of *Eimeria tenella* (EtMic5) from Pakistan to confirm its evolutionary relationship among different *Eimeria* species. Samples were taken from caecum of birds, infected with *Eimeria* and were clearly examined under microscope by standard procedures, confirmed them as *Eimeria tenella* oocysts (Shah, 2013).

After adaptation of proper protocols discussed above, the results revealed that our gene sequence (EtMic5_Pak2) has no geographic separation and is present worldwide including China, Malaysia and United Kingdom. The most identical sequences to gene sequence (100% with EtMic5_Pak2) were *Eimeria tenella* Micronemal protein MIC4, related partial mRNA with accession number (Sequence ID: XM_013372919.1) from USA, *Eimeria tenella* mRNA for microneme protein 5 (mic-5 gene) from United Kingdom with accession number AJ245536.1 along with *Eimeria tenella* clone Etm094A02 hypothetical protein mRNA (Accession number; JN987489.1 Malaysia). These results solidified the confirmation of gene as microneme 5 of *Eimeria tenella*.

Resemblance with microneme 5 gene of other *Eimeria* species is also considerable like *Eimeria necatrix* strain LZ microneme protein 5 (MIC-5) gene, partial (Accession number; EU335049.1 China) and *Eimeria necatrix* PAN domain-containing protein, related partial mRNA (Accession number; XM_013578697.1 United Kingdom).

This examination demonstrated minor hereditary variety among the arrangement of EtMic5 protein found and indicated phylogenetic likeness with different strains as the gene is highly conserved for different *Eimeria* species. To the best of our knowledge, this gene was isolated, identified and sequenced for the first time in Pakistan same as many other genes were identified and characterized for first time in Pakistan (Riaz *et al*., 2019). This study helped a lot to study evolutionary relationship of EtMic5_Pak2 gene sequence. It will further help in the investigations regarding further molecular studies.

Conclusions: It was concluded that identification and molecular characterization of novel genes for poultry diseases especially in Pakistan is vital. Our research was a first step to identify and characterize *Eimeria tenella*

microneme protein 5 gene. It will helpful further to investigate whole genome sequencing of *Eimeria* species. Furthermore, it will also help to find novel molecular prophylactic techniques by determining immunogenic regions within EtMic5 sequence. Its similarity with microneme proteins 5 of other *Eimeria* species might be helpful to induce cross protection against different *Eimeria* species.

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Authors contribution: SRAS performed experiments and wrote manuscript, MAAS supervised the work, MAAS, LX, MH, AMM and AR provided technical support and helped in manuscript writing, SKAS and BW performed sampling.

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