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ISOLATION AND MOLECULAR CHARACTERIZATION OF ETMIC2 GENE OF *EIMERIA TENELLA* CAUSING COCCIDIOSIS IN CHICKENS

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Abstract
The purpose of this study was to isolate the gene of *Eimeria tenella* species for molecular characterization and analysis. In this study, the evolutionary relationship of microneme gene2 of *Eimeria tenella* was confirmed by isolation, molecular characterization and phylogenetic analysis. The birds reared for this purpose were infected with oocyst of *Eimeria tenella*. Samples were collected from caecal lesions that appeared at the postmortem of birds. Oocysts were isolated using different egg floatation techniques. Analysis of EtMic gene 2 was performed by polymerase chain reaction (PCR). All the samples collected from the caecal lesion showed positive results for EtMic2 gene. The samples showing the best result for the EtMic2 gene were sent for sequencing at Macrogen ® Laboratory Korea. It was revealed from the phylogenetic analysis that its similarity index was 97.9% to all worldwide related sequences of the EtMic2 gene. It was revealed from the analysis that isolated gene of EtMic 2 will provide better opportunities for the development of DNA vaccine against different strains of *Eimeria tenella* in Pakistan.

Keywords: *Eimeria*, Poultry; Coccidiosis, Polymerase Chain Reaction, Molecular characterization, Phylogeny

Introduction
The poultry industry emerges as a pivotal sector in Pakistan, serving as a significant source of livelihood for over 1.5 million individuals and contributing to 31% of the nation's total meat production (Ali et al., 2014). With an estimated existing investment of approximately Rs. 700 billion (Abbas et al., 2015), this industry plays a vital role in the country's economic landscape. In the realm of disease prevention, various strategies such as chemotherapy, medicated feed, and vaccination are employed to combat coccidiosis (Alexandar et al., 2015). However, challenges arise from the development of drug resistance and escalating medication costs, posing difficulties for poultry producers (Innes et al., 2006). While live immunization and attenuated vaccines offer partial protection against re-infection, they also pose risks of accidental infection under immunosuppressive conditions (Bachaya et al., 2012). Consequently, there is growing interest in

developing recombinant and adjuvant vaccines that stimulate cellular immune responses, as the host immune system's efficacy against avian coccidiosis predominantly hinges on cellular immunity (Dalloul et al., 2007).

Research has identified various concealed or membrane-bound protein molecules as potential targets for immunological interventions, including exotic and enzootic antigens that modulate host-parasite interactions at the cellular and molecular levels (Dexing et al., 2010).

Contrary to previous assumptions, studies indicate that antibody concentrations in blood serum and intestines do not correlate with the level of protection against oral coccidia infection (Blake et al., 2015). Instead, protection primarily relies on cellular immune responses, particularly CD8+ and CD4+ T cells, with antibodies playing a minor role. Consequently, DNA vaccination has gained attention for its ability to elicit cellular immune responses (Song et al., 2017; Shah et al., 2013).

DNA immunization or recombinant antigens have shown promise in stimulating both humoral and cellular immune responses (Carmina et al., 2015). Furthermore, the concurrent delivery of cytokines and chemokines as adjuvant holds potential for enhancing the efficacy and durability of DNA vaccines or recombinant vaccines (Huang et al., 2015).

Recent advancements in DNA vaccination against poultry coccidiosis have spurred interest in evaluating the protective immunity generated by microneme protein 7 of *E. maxima* and antigen-encoding DNA vaccines (Champan et al., 2002). Research endeavors aim to assess the efficacy of these vaccines against experimental challenges, particularly targeting the EtMic7 gene (Shah et al., 2014).

Polymerase Chain Reaction (PCR) has become a cornerstone in detecting parasite genomes due to its ability to amplify minute amounts of genetic material (Clark et al., 2012). In this context, the current study endeavors to achieve several objectives: 1. Isolation and identification of *E. tenella* from various poultry samples, 2. Identification of *Eimeria* using traditional macroscopic and microscopic techniques, 3. Isolation and identification of *Eimeria tenella* through molecular biological techniques, 4. Molecular characterization and phylogenetic analysis of a segment of the EtMic protein gene of *E. tenella*.

Materials and Methods

Ethics

The study was conducted at the Department of Parasitology & Microbiology, Faculty of Veterinary and Animal Sciences, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi. It was authenticated by PSF-NSLP and received full funding for all research-related expenses. Ethical considerations were meticulously adhered to during sampling, and permissions were obtained as needed.

Plastic wares and consumables

For this study following plastic ware were used as falcon tubes, Pipette tips, PCR tubes, graduated cylinder, Eppendorf tubes, Para film, U-bottom micro-titer plates, cotton swabs, latex gloves, aluminum foil and gloves ware racks.

Soft wares

NCBI blast, and MEGA7® was used for the alignment, editing and analysis of EtMic genes DNA sequences

Eimeria isolates

One hundred fifty poultry birds were reared at PMAS-AAUR experimental sheds. Oocysts of *E. tenella* (non sporulated) were obtained from clinically infected birds using standard procedures and protocols mentioned bellow at Arid Agriculture university Rawalpindi. Day-old layer birds were obtained from Poultry Research Institute Rawalpindi (PRI).The birds were raised in a coccidia-free setting until they were ready for experimentation. At 40 days of age (N=75), they were manually inoculated into the crop with freshly prepared sporulated oocysts. Group 1, the infected birds group,

received a single dose of 5000 sporulated oocysts, while Group 2 served as non-infected controls. Seven days after infection, the birds were euthanized, and their ceacal tonsils were collected for analysis.

Primers

Specific primers for EtMic (*Eimeria tenella* microneme) protein gene were used to amplify the fragment of EtMic protein gene. RNA was extracted by Trizol extraction. cDNA was synthesized using gene specific primers; reverse Trascriptive PCR was carried out on the extracted RNA fragment (Song et al., 2009). For PCR a pair of primers was used as

- F- 5'-GAGCGAACGGGACTTCATTG-3'
- R- 5'-ACTCTGCTTGAACCTCTTCC-3'

The working solutions were prepared for each primer in a dilution of 1:10 from stock solution. The primers were properly labelled and kept at -20^{o}C conditions.

RNA Extraction

The RNA was extracted from the samples using Trizol LS Reagent (Ambion, Life Technologies®). Seven hundred and 50µL of Trizol were combined with 250µL of allantoic fluid in an Eppendorf tube, maintaining a ratio of 3:1 to the sample. After incubating for five minutes at room temperature to ensure complete dissociation of the nucleoprotein complex, 200µL of chloroform was added to the tube and vortexed for fifteen seconds. Following 15 minute incubation at room temperature, the samples were centrifuged at 12000 x g for fifteen minutes at 4°C (Farooq et al., 2014). The resulting mixture separated into three layers: the lower red phenol-chloroform phase containing DNA and proteins, the central interphase, and the upper colorless aqueous layer containing the RNA. The aqueous phase was carefully removed using a micropipette by tilting the tube at a 45-degree angle into another clean Eppendorf tube, and the remaining mixture was discarded. 500µL of 100% isopropanol were added to the separated aqueous phase and incubated at room temperature for ten minutes. After centrifugation at 12000 x g for 10 minutes at 4°C, an RNA pellet formed at the bottom of the tube (Lai et al., 2011). The supernatant was meticulously removed without disturbing the pellet using a sterile pipette tip. The RNA pellet was then washed by adding 1000 µL of 75% ethanol to the tube and centrifuging at 7500 x g for five minutes. After removing the supernatant fluid, the pellet was allowed to air dry (Kumar et al., 2014). The air-dried pellet was resuspended in 30µL of RNase-free water and incubated at 55-65°C in a water bath for ten to fifteen minutes to dissolve the pellet. Finally, the extracted RNA was immediately stored at -20°C after quantification using a nano-drop (Jamil et al., 2016).

Nano-drop quantification

The nano-drop quantification of the samples was done in Animal Breeding and Genetics lab, Department of Livestock Production and Management (LPM), using a Nano-drop quantifier (Quawell® UV-vis Spectrophotometer Q5000). Initially, the machine underwent calibration using one µL of water as the 'blank' sample. Subsequently, 1 µL of each sample was applied to the pedestal, with the pedestal being cleaned after each sample. This analysis provided the Optical Density (OD) value, as well as the 260/280 and 260/230 ratios for each sample (Lilihoj et al., 2009).

Reverse transcriptase PCR for cDNA synthesis

Complementary DNA (cDNA) synthesis was performed via Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) utilizing extracted RNA (Mehran et al., 2011). The Maxime RT Premix Kit® containing Oligo (dT) primer and a random primer was employed for this purpose. A concentration of 1µg of RNA template was added to the RT pre-mix tube, and DNase-free water was added to bring the reaction volume to 20µL (Munir et al., 2012). cDNA synthesis was carried out using a PCR machine under the following conditions: 45°C for sixty minutes followed by 95°C for five minutes. The resulting cDNA samples were promptly utilized for PCR, while any remaining cDNA was stored at -20°C (Zaman et al., 2012).

Polymerase Chain Reaction (PCR)

The cDNA synthesized in the preceding step (Section 3.9) was employed for amplifying the EtMic gene segment utilizing One Taq 2X Master Mix (Biolabs®). The reaction mixture comprised the following components: 1µL of cDNA, 1µL of Forward primer, 1µL of Reverse Primer, 1-2.5 µL of Taq 2x Master Mix, and Nuclease-Free water up to a total volume of 50 µL. PCR amplification was conducted using a thermal cycler (2720 Thermo cycler by Life Technologies®) under the following conditions: an initial denaturation step at 94°C for four minutes, followed by 35 cycles of denaturation at 95°C for one minute, annealing at 62°C for one minute, extension at 72°C for one minute, and a final extension step at 72°C for seven minutes (Pederson et al., 2004).

Gel electrophoresis

The products of PCR were analyzed through gel electrophoresis using one point five per cent (w/v) agarose gel. The agarose gel was dissolved by heating in a microwave oven. The TAE (Tris base, Acetic acid and EDTA) buffer was prepared in a ratio of 1:10. Six grams of agarose was dissolved in 400ml of TAE buffer and heated in oven till boiling. 20 µL of Ethidium bromide was added in the solution (Saouros et al., 2012). The gel was poured into the gel caster. The combs were placed in the gel at the correct positions carefully to create wells for sample loading. The gel is then given time to solidify and set in the caster. Once the gel was solidified, the combs are removed and transferred to the electrophoresis tank which is filled with 1x TAE buffer. 10 µL of sample was prepared in a separate PCR tube with 3 µL dye. A ladder is loaded in a well as a standard. 100 bp ladders from BioLabs® was prepared by adding 5 µL ladder solution, 5 µL water and 3 µL dye (Wang et al.,2004). These samples and ladder were carefully loaded in the wells. The machine (Bio-Rad®) was run at 110 Volts for ninety minutes. The samples along with the dye move along the electric charge according to the size of base pairs.

Gene sequencing

For sequencing the EtMic gene, the samples were sent to Macrogen® Korea. The gene sequence results were analyzed by ABI3730XL, using Standard sequencing.

Phylogenetic analysis

Out of all the positive samples most confirmed positive sample of (EtMic2_Pak) was sent to Macrogen® laboratory Korea for authentic and confirmed sequences of EtMic2 gene. The NCBI BLAST was used to analyze the sequenced data. The Clustal0 method (software Seaview®) was exercised for nucleotide sequences and deduced amino acid sequences of EtMic gene. The MEGA7® software was used compute the distances overall and mean-wise. The Seaview® software was used for gene sequences translation.

To acquire the genetic relativity and the phylogenetic similarities of the coccidial isolates from the infected bird group, phylogenetic trees were constructed using MEGA7®. Every single detailed genotype around the globe, just as, in Pakistan was examined and their successions were given to the MEGA7® programming. The tree was developed utilizing Maximum likelihood technique in MEGA7®.

Results

Nano-drop results

After the extraction of RNA from the oocysts collected from faecal samples were subjected to a nano-drop test. Nano drop quantification was performed to arrive at the point to analyze the RNA concentration values of the samples. The values are as follows:

Table 1: Nano-drop concentration of samples in ng/µL

Eimeria Sample	Concentration
1	119.7
2	39.9

3	22.1
4	655.200
5	552.100
6	1024.554

RT-PCR

A fragment of microneme 2 gene from coccidial isolated were subjected to amplified by Reverse Trascriptive PCR with the help of a sets of reverse and forward primers with the band size of 980 bp. Results shows that gel documentation acquired from running the coccidial RNA samples for amplified segments of microneme glycoprotein in agarose gel electrophoresis. The band size is 980 bp confirm the incidence of *Eimeria tenella*. The negative control was also used and subjected to run to attain an expected negative result which enables us to build a logical ground for the assurance to obtain a specific result that only amplifies the required segment and to ensure absence of any contaminant. To measure the size of our expected band a known molecular-weight size marker (DNA ladder) was also run simultaneously on the gel. This ladder set a standard which helped us to identify and analyze the approximate size of a molecule run on the gel.

Phylogenetic analysis

PK/1 and PK/2 shows great resemblance with a variety of isolates found throughout the world, including China, India, USA, Italy, Iran, Russia, Bangladesh and existing isolates of Pakistan as shown in black color while have dissimilarities with the Japan and United Kingdom isolates as shown in figure 1.

When comparing the EtMic-2 nucleotide sequence with known microneme sequences in the NCBI database, it exhibited a homology of 97-99% with *Eimeria tenella* microneme protein, complete cds, and 92% with *Eimeria necatrix* microneme 2. Conversely, *Eimeria brunette* and *E. acervulina* displayed lower homology and similarities with our isolates, at 71% and 78%, respectively. The phylogenetic analysis of amino acid sequences, constructed using MEGA7, depicted a close relationship of the EtMic-2 gene within *Eimeria* species compared to other apicomplexans parasites. Ancestral states were determined utilizing the Maximum Likelihood method with the Tamura-Nei model (Tamura & Nei, 1993). The cladogram resulting from this analysis illustrated the potential nucleotide states at each ancestral node, with ambiguous states omitted. The initial tree was constructed using uniform rates among sites. This analysis incorporated 14 nucleotide sequences, including 1st+2nd+3rd+Noncoding codon positions, total 1957 positions in the final dataset.

Evolutionary analyses were performed using MEGA 7

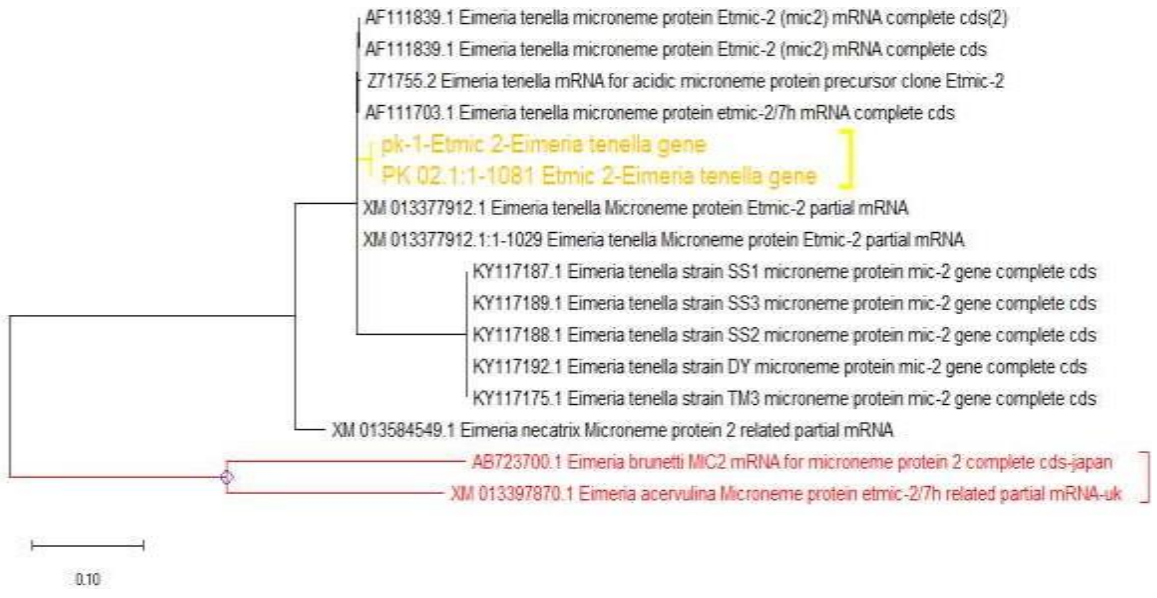
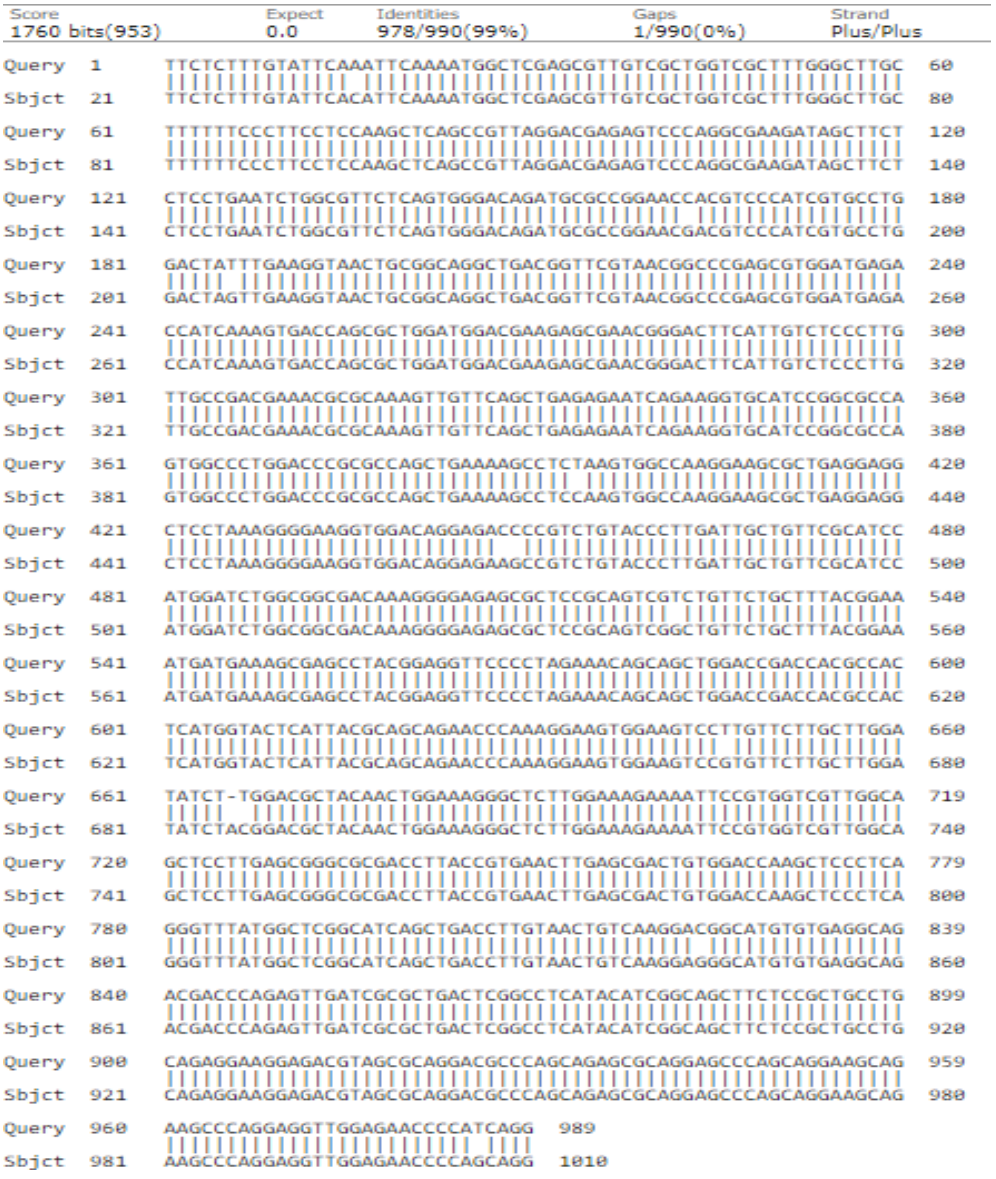


Figure 1: Phylogenetic analysis of isolated E. tenella by Maximum Likelihood method Gene sequence alignment

The gene sequences were organized to identify regions of likeness that could be an outcome to impact auxiliary, utilitarian or developmental association between the groupings.

The arrangements were adjusted utilizing Seaview® by Clustal-W technique. The gene sequences from our subject samples were contrasted with the quality groupings from GenBank. No impressive changes were seen among the gene sequence demonstrating high similarity with the secludes seen all through the world.



Score	Expect	Identities	Gaps	Strand
1760 bits(953)	0.0	978/990(99%)	1/990(0%)	Plus/Plus

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Query  1     TTCTCTTTGTATTCAAATTCAAAATGGCTCGAGCGTTGTCGCTGGTCGCTTTGGGCTTGC  60
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  21    TTCTCTTTGTATTCACATTCAAAATGGCTCGAGCGTTGTCGCTGGTCGCTTTGGGCTTGC  80

Query  61    TTTTTTCCCTTCCTCCAAGCTCAGCCGTTAGGACGAGAGTCCCAGGCGAAGATAGCTTCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81    TTTTTTCCCTTCCTCCAAGCTCAGCCGTTAGGACGAGAGTCCCAGGCGAAGATAGCTTCT  140

Query  121   CTCCTGAATCTGGCGTTCTCAGTGGGACAGATGCGCCGGAACCACGTCCCATCGTGCCTG  180
             ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  141   CTCCTGAATCTGGCGTTCTCAGTGGGACAGATGCGCCGGAACGACGTCCCATCGTGCCTG  200

Query  181   GACTATTTGAAGGTAACTGCGGCAGGCTGACGGTTCGTAACGGCCCGAGCGTGGATGAGA  240
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201   GACTAGTTGAAGGTAACTGCGGCAGGCTGACGGTTCGTAACGGCCCGAGCGTGGATGAGA  260

Query  241   CCATCAAAGTGACCAGCGCTGGATGGACGAAGAGCGAACGGGACTTCATTGTCTCCCTTG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261   CCATCAAAGTGACCAGCGCTGGATGGACGAAGAGCGAACGGGACTTCATTGTCTCCCTTG  320

Query  301   TTGCCGACGAAACGCGCAAAGTTGTTCAGCTGAGAGAATCAGAAGGTGCATCCGGCGCCA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321   TTGCCGACGAAACGCGCAAAGTTGTTCAGCTGAGAGAATCAGAAGGTGCATCCGGCGCCA  380

Query  361   GTGGCCCTGGACCCGCGCCAGCTGAAAAGCCTCTAAGTGGCCAAGGAAGCGCTGAGGAGG  420
             |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  381   GTGGCCCTGGACCCGCGCCAGCTGAAAAGCCTCCAAGTGGCCAAGGAAGCGCTGAGGAGG  440

Query  421   CTCCTAAAGGGGAAGGTGGACAGGAGACCCCGTCTGTACCCTTGATTGCTGTTCGCATCC  480
             |||||||||||||||||||||||||||||  ||||||||||||||||||||||||||||||
Sbjct  441   CTCCTAAAGGGGAAGGTGGACAGGAGAAGCCGTCTGTACCCTTGATTGCTGTTCGCATCC  500

Query  481   ATGGATCTGGCGGCGACAAAGGGGAGAGCGCTCCGCAGTCGTCTGTTCTGCTTTACGGAA  540
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  501   ATGGATCTGGCGGCGACAAAGGGGAGAGCGCTCCGCAGTCGGCTGTTCTGCTTTACGGAA  560

Query  541   ATGATGAAAGCGAGCCTACGGAGGTTCCCCTAGAAACAGCAGCTGGACCGACCACGCCAC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  561   ATGATGAAAGCGAGCCTACGGAGGTTCCCCTAGAAACAGCAGCTGGACCGACCACGCCAC  620

Query  601   TCATGGTACTCATTACGCAGCAGAACCCAAAGGAAGTGGAAGTCCTTGTTCTTGCTTGGA  660
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  621   TCATGGTACTCATTACGCAGCAGAACCCAAAGGAAGTGGAAGTCCGTGTTCTTGCTTGGA  680

Query  661   TATCT-TGGACGCTACAACTGGAAAGGGCTCTTGGAAAGAAAATTCCGTGGTCGTTGGCA  719
             |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  681   TATCTACGGACGCTACAACTGGAAAGGGCTCTTGGAAAGAAAATTCCGTGGTCGTTGGCA  740

Query  720   GCTCCTTGAGCGGGCGCGACCTTACCGTGAACTTGAGCGACTGTGGACCAAGCTCCCTCA  779
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  741   GCTCCTTGAGCGGGCGCGACCTTACCGTGAACTTGAGCGACTGTGGACCAAGCTCCCTCA  800

Query  780   GGGTTTATGGCTCGGCATCAGCTGACCTTGTAACTGTCAAGGACGGCATGTGTGAGGCAG  839
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  801   GGGTTTATGGCTCGGCATCAGCTGACCTTGTAACTGTCAAGGAGGGCATGTGTGAGGCAG  860

Query  840   ACGACCCAGAGTTGATCGCGCTGACTCGGCCTCATACATCGGCAGCTTCTCCGCTGCCTG  899
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  861   ACGACCCAGAGTTGATCGCGCTGACTCGGCCTCATACATCGGCAGCTTCTCCGCTGCCTG  920

Query  900   CAGAGGAAGGAGACGTAGCGCAGGACGCCCAGCAGAGCGCAGGAGCCCAGCAGGAAGCAG  959
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  921   CAGAGGAAGGAGACGTAGCGCAGGACGCCCAGCAGAGCGCAGGAGCCCAGCAGGAAGCAG  980

Query  960   AAGCCCAGGAGGTTGGAGAACCCCATCAGG  989
             ||||||||||||||||||||||||||| ||||
Sbjct  981   AAGCCCAGGAGGTTGGAGAACCCCAGCAGG  1010
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Figure 2: Showing sequence similarity between to Pk-2 isolate with DY microneme protein mic-2 gene, complete cds

Discussion

Eimeria was successfully identified in feacal samples from infected poultry. The study indicated that there were no significant genetic variations among the isolates' sequences. It was noted that secretary proteins like microneme play a crucial role in the invasion of extracellular motile sporozoites of *Eimeria* within the host (Carruthers & Sibley, 2008). Microneme, being the smallest apical organelles in apicomplexans both structurally and functionally, facilitate the attachment of the parasite to the host's intestinal lining. The production, storage, and release of microneme proteins

are timed appropriately, with calcium playing a pivotal role in their secretion, triggered by contact with the host (Bumstead and Tomley, 2000). Observations revealed that the secretion of microneme proteins can be induced in *Eimeria tenella*. Microneme harbor approximately ten abundant proteins, some of which have been cloned and characterized, such as EtMic-1, EtMic-4, EtMic-5, and EtMic-2. Our successful strategy for isolating EtMic-2 from oocysts showed high degrees of similarity (99%, 98%, 91%, and 90%) when compared with DY microneme protein mic-2 gene, TM microneme protein-2 gene, EtMic-2 mRNA, and *E. necatrix* microneme-2 gene, respectively. Sequence comparison with the isolated gene revealed slight dissimilarities in bases and gaps, indicating potential strain differences or mutations.

A similar study conducted in China in 2016 found minor differences in the EtMic-2 gene compared to the Nanjing strain reported by Jianlin and Jinshu (2002), resulting in two amino acid changes. Further investigation at the protein level is warranted to evaluate any amino acid or protein alterations corresponding to the observed dissimilarities in our study. Although the reasons behind these substitutions remain unclear, they could stem from strain variations or mutations.

Furthermore, Seaview® software was employed for the analysis of predicted amino acid sequences of EtMic-2 in our study, whereas Lei et al. (2016) utilized DNA assist software (version 1.02) for their analysis, both revealing amino acid changes at multiple sites. Subsequent studies should aim to elucidate the significance of these alterations.

Future research endeavors should prioritize the development of DNA vaccines utilizing microneme gene sequences and investigating the impact of microneme proteins on the avian immune system to combat coccidiosis.

Conclusion

It was concluded from the study that immunogenic EtMic 2 gene characterization and phylogenetic analysis was the first basic step for the affective control of coccidiosis. There were seven different species of *Eimeria* which were responsible for coccidiosis. The immunogenic gene was used and provided humoral as well as cell mediated response for the control of coccidiosis especially in Pakistan. It was first time in Pakistan identification and phylogenetic analysis was performed. It was a great achievement for further research and development of DNA vaccine against chicken coccidiosis.

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