ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 2 (2): 133-138, 2003

Characterization of Microneme-2 (EtMIC-2) Gene of *Eimeria tenella* Guangdong Strain

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Abstract: Micronemes are secretary organelles of the invasive stages of all Apicomplascan parasites, and their proteins are crucial for parasite motility, adhesion and host cell invasion. The gene encoding the Microneme-2 protein (EtMIC-2) was isolated from oocysts of the GuangDong strain of *Eimeria tenella* by RT-PCR of extracted RNA using EtMIC-2 primers designed from a published sequence of the gene. The sequence of the EtMIC-2 gene from the GuangDong strain, Houghton and Beijing strains revealed high homology (99.6 and 99.7% respectively), indicating a limited allelic polymorphism within *Eimeria tenella* strains for MIC-2 gene. As far as deduced amino acids sequence is concerned, however, there was a difference of only two amino acids between the GuangDong strain and any of the other two strains.

Key words: *Eimeria tenella*, microneme, GuangDong strain, gene

Introduction

The intestinal disease known as coccidiosis caused by protozoan parasites of genus *Eimeria* is one of the most economically devastating diseases in the poultry industry worldwide. Direct evidence to support this fact, despite prophylactic chemotherapy and vaccination, this disease costs the world's poultry producers, every year, in excess of £2 billion (Williams, 1999). Of the seven species of *Eimeria*, which infect the chicken (Calnek *et al*., 1997; Shirley, 1999), *Eimeria tenella* is one of the most ubiquitous (Quarzane *et al*., 1998) and most pathogenic (Arakawa and Xie, 1993; Yadav and Gupta, 2001).

Natural infection of any of the chicken *Eimeria* induces little or no cross protection (Rose and Mockett, 1983). However, this can be achieved by immunization of chicken with soluble parasite antigens (Murray *et al*., 1986) or recombinant protein (Crane *et al*., 1991; Bhogal *et al*., 1992). The cross-protective nature of the immunity indicates that the response is different from that induced by natural infection. This has encouraged researchers to focus on molecular cloning of genes encoding protecting antigens in order to come out with vaccines protecting against several species. In recent years particular attention has been paid in diverse *Eimeria species* to organelles involved in the invasion of the host cells by extra cellular motile stages of *Eimeria* located within the apical complex (Dubremetz *et al*., 1998; Tomley *et al*., 2001; Striepen *et al*., 2001). Of specific interest are the micronemes and the rhoptries whose contents are thought to be required for invasion of potential host cells and for the formation of parasitophorous vacuole respectively in all apicomplexa. In this paper, we describe the isolation and characterization of the EtMIC-2 gene from GuangDong strain *Eimeria tenella* sporozoites.

Materials and methods

Primer design: From the sequence of *Eimeria tenella* Microneme-2 (EtMIC-2) reported by Tomley *et al*. (1996), two primers were designed by Primer Premier 5.0 software. The sequence of the forward primer was (36-54) 5'GTTGCATTGCATAACCTCA 3' and the sequence of the reverse primer was (1161-1142) 5'CAGTAGACGAACTTGGAG 3.' The primers were synthesized by Shanghai Sangon Biotech. Co. Ltd.

Parasites: Unspoulated oocysts of the GuangDong strain of *Eimeria tenella* were obtained from the caecal contents 7 days post-infection, and allowed to sporulate according to the procedures described by Shirley (1995). Sporulated oocysts were purified from bacteria and fungal contamination by treatment with 5.75% sodium hypochlorite and incubation on ice for 30 min (Zhao *et al*., 2001) followed by extensive washing with sterile cold water. Oocysts were then separated from other debris by centrifugation over a cushion of 0.6 M sucrose in an eppendorf 5804R centrifuge, F34-6-38 rotor at 2600 g for 5 minutes at 4 ^{0}C. The purified oocysts were then collected from the interface of the layer and again washed several times with cold water, rinsed with phosphate buffer saline (PBS) and used immediately.

Total RNA extraction: The total RNA was extracted by combining the method that exploits the ability of the ionic detergent SDS to inhibit RNASE activity described by

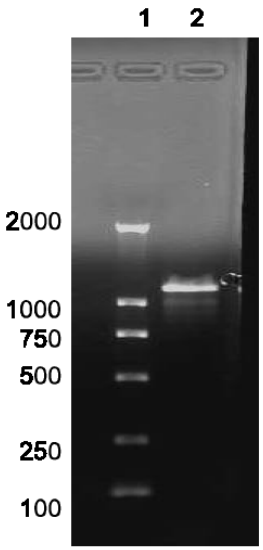


Fig. 1: The electrophoretogram of RT-PCR amplification
1: DL2000 DNA Marker 2: EtMIC-2 (1126 bp) cDNA.

Robert and Farrell (1998) with Pasternak *et al*. (1981) procedure with minor modifications. Thus total RNA was isolated using reagents commonly found in laboratories performing even the most rudimentary molecular biology procedures and without isopycnic centrifugation. Briefly, to approximately two grams pelleted oocysts, 10 ml (5 ml per 1 g wet weight) of lyses buffer containing (2%SDS, 200 mM Tris-cl, pH 7.5, 0.5 mM EDTA) was added. The oocysts were then broken by vigorous shaking with equal volume of sterile Jencon's glass beads number 8. Breakage of the oocysts was monitored microscopically until no intact oocysts, sporocysts, or sporozoites were seen. The resulting mixture of damaged oocysts was mixed thoroughly before adding 3 ml (For each 500μl lyses buffer 150μl) potassium acetate solution (50 g potassium acetate; 11 ml glacial acetic acid; H_2 o to 100 ml). The tube was then inverted quickly several times and incubated on ice for 4 min and centrifuged at 10000 g for 5 min at room temperature. The supernatant solution was recovered by 2 successive extractions with a mixture of chloroform:isoamyl alcohol (24:1). RNA was precipitated with the addition of an equal volume of ice-cold isopropanol and incubation at –20 ^{o}C for 30 min. The RNA was then collected by centrifugation at 10000 g for 5 min at room temperature and washed twice with 70% ethanol and once with 95% ethanol and the sample was air dried and then dissolved in sterile double distilled diethylpyrocarbonate (DPEC) treated water.

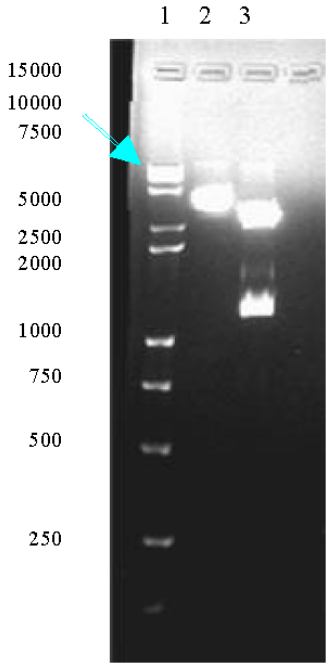
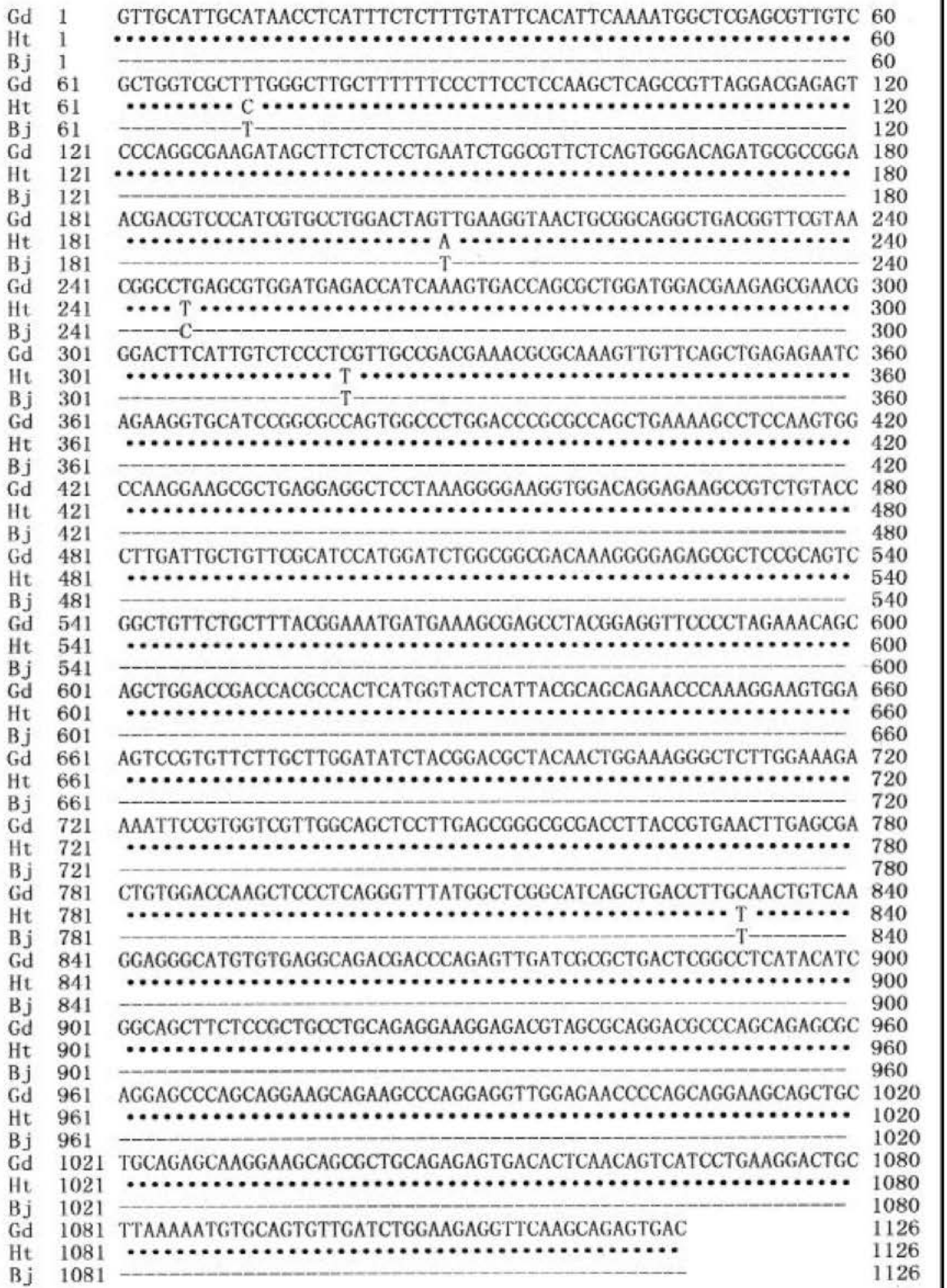


Fig. 2: Electrophoretic results of plasmid PGEM-T easy-MIC-2 after EcoR1 and Sac1 digestion
1: DL2000 DNA Marker +DL1500 DNA Marker
2: Plasmid PGEM-T-Easy-MIC-2 SacI digested product (4141 bp)
3: Plasmid PGEM-T- Easy-MIC-2 EcoRI digested products (2995+1146 bp)

Reverse transcription and amplification of cDNA (RT-PCR): Total RNA was directly subjected to a reverse transcriptase polymerase chain reaction (RT-PCR) using the Access RT-PCR system (Promega). The primers were used through 45 min at 48 ^{o}C, 2 min at 94 ^{o}C, 40 cycles (30 sec at 94 ^{o}C, 1 min at 54.8 ^{o}C, 2 min at 68 ^{o}C), 7 min at 68 ^{o}C using a Gene Amp PCR System 2700 (BIO MEN Instruments Ltd). The PCR product was purified by electrophoresis in a 1% agarose gel and extracted using EZNA gel extraction kit (Omega) according to manufacturer's instructions.

Construction of the recombinant plasmid PGEM-T-Easy-MIC-2: The purified PCR amplified fragment was ligated into the PGEM-T-Easy cloning vector (Promega) following the manufacturer's recommendations and the ligation mixture was used to transform *Escherichia coli* DH5α strain using standard method (Wang, 1995). Recombinants were selected as white colonies on plates containing 100 μg/ml ampicillin, 20mM IPTG and 0.2mM X-gal.

Identification of the EtMIC-2 cDNA: EZNA plasmid miniprep kit (Omega) preparations of the recombinant


Gd 1 GTTGCATTGCATAACCTCATTTCTCTTTGTATTCACATTCAAAATGGCTCGAGCGTTGTC 60
Ht 1 60
Bj 1 60
Gd 61 GCTGGTCGCTTTGGGCTTGCTTTTTTCCCTTCCTCCAAGCTCAGCCGTTAGGACGAGAGT 120
Ht 61 C 120
Bj 61 T 120
Gd 121 CCCAGGCGAAGATAGCTTCTCTCCTGAATCTGGCGTTCTCAGTGGGACAGATGCGCCGGA 180
Ht 121 180
Bj 121 180
Gd 181 ACGACGTCCCATCGTGCCTGGACTAGTTGAAGGTAACTGCGGCAGGCTGACGGTTCGTAA 240
Ht 181 A 240
Bj 181 T 240
Gd 241 CGGCCTGAGCGTGGATGAGACCATCAAAGTGACCAGCGCTGGATGGACGAAGAGCGAACG 300
Ht 241 T 300
Bj 241 C 300
Gd 301 GGACTTCATTGTCTCCCTCGTTGCCGACGAAACGCGCAAAGTTGTTCAGCTGAGAGAATC 360
Ht 301 T 360
Bj 301 T 360
Gd 361 AGAAGGTGCATCCGGCGCCAGTGGCCCTGGACCCGCGCCAGCTGAAAAGCCTCCAAGTGG 420
Ht 361 420
Bj 361 420
Gd 421 CCAAGGAAGCGCTGAGGAGGCTCCTAAAGGGGAAGGTGGACAGGAGAAGCCGTCTGTACC 480
Ht 421 480
Bj 421 480
Gd 481 CTTGATTGCTGTTCGCATCCATGGATCTGGCGGCGACAAAGGGGAGAGCGCTCCGCAGTC 540
Ht 481 540
Bj 481 540
Gd 541 GGCTGTTCTGCTTTACGGAAATGATGAAAGCGAGCCTACGGAGGTTCCCCTAGAAACAGC 600
Ht 541 600
Bj 541 600
Gd 601 AGCTGGACCGACCACGCCACTCATGGTACTCATTACGCAGCAGAACCCAAAGGAAGTGGA 660
Ht 601 660
Bj 601 660
Gd 661 AGTCCGTGTTCTTGCTTGGATATCTACGGACGCTACAACTGGAAAGGGCTCTTGGAAAGA 720
Ht 661 720
Bj 661 720
Gd 721 AAATTCCGTGGTCGTTGGCAGCTCCTTGAGCGGGCGCGACCTTACCGTGAACTTGAGCGA 780
Ht 721 780
Bj 721 780
Gd 781 CTGTGGACCAAGCTCCCTCAGGGTTTATGGCTCGGCATCAGCTGACCTTGCAACTGTCAA 840
Ht 781 T 840
Bj 781 T 840
Gd 841 GGAGGGCATGTGTGAGGCAGACGACCCAGAGTTGATCGCGCTGACTCGGCCTCATACATC 900
Ht 841 900
Bj 841 900
Gd 901 GGCAGCTTCTCCGCTGCCTGCAGAGGAAGGAGACGTAGCGCAGGACGCCCAGCAGAGCGC 960
Ht 901 960
Bj 901 960
Gd 961 AGGAGCCCAGCAGGAAGCAGAAGCCCAGGAGGTTGGAGAACCCCAGCAGGAAGCAGCTGC 1020
Ht 961 1020
Bj 961 1020
Gd 1021 TGCAGAGCAAGGAAGCAGCGCTGCAGAGAGTGACACTCAACAGTCATCCTGAAGGACTGC 1080
Ht 1021 1080
Bj 1021 1080
Gd 1081 TTAAAAATGTGCAGTGTTGATCTGGAAGAGGTTCAAGCAGAGTGAC 1126
Ht 1081 1126
Bj 1081 1126

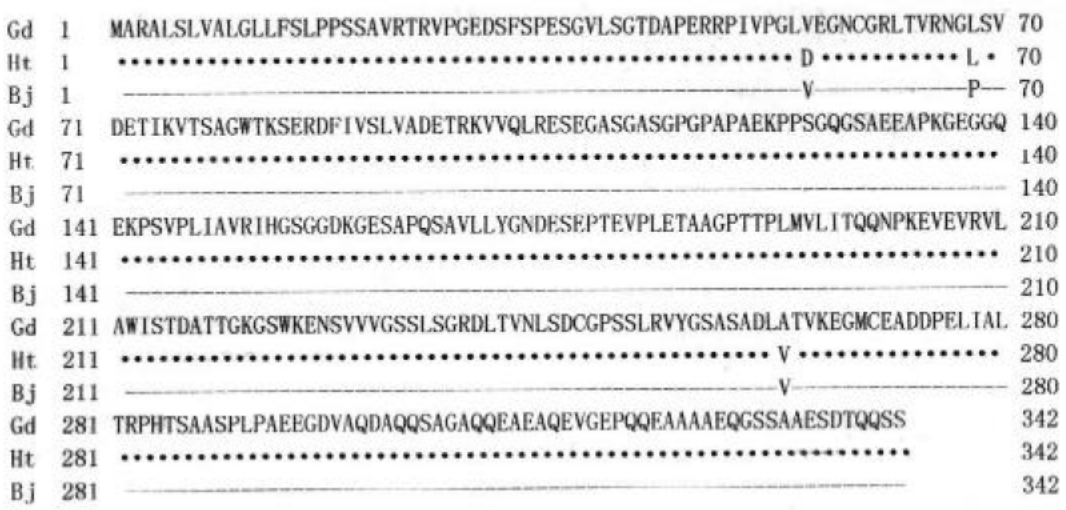


Fig. 3: Nucleotide sequence of MIC-2 cDNA and corresponding amino acids sequence for GuangDong (G.D.), Houghton (Ht), and Beijing (Bj) strains of *Eimeria tenella*. Dashes (---) for (Ht) strain and dots (...) for (Bj) strain. Letters indicated substituted bases.

plasmid were analyzed by EcoRI and SacI enzymes digestion and electrophoresis. Selected recombinant clones were sequenced by Shanghai Genecore Co. Ltd. and sequence data were assembled and analyzed by DNAssist software (version 1.02)

Results

The products of direct subjection of the extracted RNA to coupled reverse transcriptase and polymerase chain reaction amplification (RT-PCR) were analyzed on a 1% agarose gel, stained by ethidium bromide and a fragment of the correct size (1126 bp) was obtained (Fig. 1).

After digestion with EcoRI and SacI the two predicted DNA bands of 2995 bp (PGEM-T-Easy), 1146 bp (inserted DNA fragment) and one band of 4141 bp (linear recombinant PGEM-T-Easy) were obtained (Fig. 2).

The sequence results revealed that the entire GuangDong strain EtMIC-2 nucleotide sequence is 1126 bp. The open reading frame starts at base 44 and ends with a TGA stop codon at base 1069 encoding a polypeptide of 342 amino acids. The identified reading frame predicts a mature polypeptide of around 32 KDa. The sequence contains a 43 bp 5' non-coding region and a 57 bp 3' non-coding region. The ATG codon at position 44 of the sequence shown in Fig. 3 is located within a region homologous to the consensus translation site of sporozoa defined by Yamauchi (1991). The deduced protein contains 26 basic aa residues (15 arginines and 11 lysines), and 49 acidic aa residues (glutamic and aspartic acids). The total net charge is positive.

Discussion

Invasion of the host cells by extra cellular motile zoites of *Eimeria* is associated with secretion of proteins from characteristic three sets of secretary organelles (the micronemes, rhoptries, and dense granules) (Carruthers and Sibley, 1997). Micronemes are the smallest of the apical organelles, structurally and functionally conserved in all apicomplexans (Bumstead and Tomley, 2000). Proteins they secrete promote the attachment of parasites to the potential host cells (Tomley *et al.*, 1996; Tomley and Soldati, 2001) and motility (Sultan *et al.*, 1997) and thus play a crucial role in the invasion process of apicomplexan parasites. Microneme proteins are preformed and stored (Wan *et al.*, 1997; Ryan *et al.*, 2000) and released rapidly at the appropriate time for internalization. Their secretion is regulated by calcium (Carruthers and Sibley, 1999; Striepen *et al.*, 2001) and triggered by contact of the parasite with target host cells (Tomley *et al.*, 1996; Carruthers and Sibley, 1997; Bumstead and Tomley, 2000). In *Eimeria tenella*, it has been shown that the secretion of these proteins can be stimulated by the exposure of sporozoites to albumin (Bumstead and Tomley, 2000). Micronemes contain around 10 abundant proteins (Kawazoe *et al.*, 1992), and a number of the genes that encode micronemal proteins including EtMIC-1 (Tomley *et al.*, 1991), EtMIC-4 (Brown *et al.*, 2000), EtMIC-5 (Tomley *et al.*, 2001) and EtMIC-2 (Tomley *et al.*, 1996) have been cloned and characterized. Our strategy for isolating EtMIC-2 from sporozoites was successful and a high degree of similarities (99.6 and 99.7%) were revealed by comparing the sequence of EtMIC-2 for GuangDong strain with those reported for Houghton and Beijing

strains respectively (Tomley *et al*., 1996; Jianlin and Jinshu, 2002). A sequence comparison against that described for Houghton strain, revealed 4 base substitutions and two amino acid changes at positions 55 (D to V) and 283 (V to A) (Fig. 3). In comparison to EtMIC-2 gene reported by Jianlin and Jinshu (2002) for the Beijing strain, which belongs to second generation merozoites, a difference in only three bases was detected which also resulted in two amino acid changes at positions 68 (P to L), and 263 (V to A) (Fig. 3). This is in agreement with Kawazoe *et al*. (1992) results that showed strong conservation of micronemes epitopes between sporozoites and second-generation merozoites. These amino acid changes were due to the fact that all base substitutions took place within the open reading frame. Although the reasons for these substitutions are not clear, they may be due to strain differences or mutations. Moreover, DNAssist software (version 1.02) analysis of predicted amino acid sequences of EtMIC-2 for the GuangDong, Houghton, and Beijing strains showed amino acid changes in three places. (Fig. 3). However, it appeared that the first and second amino acid changes have impact on antigenicity, while the third one has no effect. Further studies are required to establish the significance of these changes. We observed a genomic restriction pattern similar to those described by Tomley *et al*. (1996). As the profile observed are consistent with a single copy gene, we conclude that we have isolated the GuangDong allele of the gene described by Tomley *et al*. (1996) and Jianlin and Jinshu (2002). The very high sequence homology between our clone and those described by Tomley *et al*. (1996); Jianlin and Jinshu (2002) indicates a limited allelic polymorphism within *E. tenella* strains or different developmental zoites for MIC-2 gene. In conclusion, this strong sequence conservation indicates that, if recombinant protein coded by this gene is found to be immunogenic, it might be an interesting vaccine candidate and warrants further investigation.

Acknowledgments

GuangDong Provincial Natural Science Foundation (No: 000145) supported this work.

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