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

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Abstract: Micronemes are secretory organelles of the invasive stages of all Apicomplexan 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 (30-54) 5’GTGGCATGCAAGCCTGCA 3’ and the sequence of the reverse primer was (1161-1142) 5’CAGTAGACGAATCTGGAG 3’. The primers were synthesized by Shanghai Sangon Biotech. Co. Ltd.

Parasites: Unsporulated 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 appendix 5804R centrifuge, F34-6-38 rotor at 2600 g for 5 minutes at 4 ºC. 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
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 isopropyl alcohol:centrifugation. Briefly, to approximately two grams pelleted oocysts, 10 ml (5 ml per 1 g wet weight) of lysis 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 0. Breakage of the oocysts was monitored microscopically until no intact oocysts, sporozoites, or sporozoites were seen. The resulting mixture of damaged oocysts was mixed thoroughly before adding 3 ml (For each 500 μl lyses buffer 150μl potassium acetate solution (50 g potassium acetate; 11 ml glacial acetic acid; H2O 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°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.

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 ºC, 2 min at 94 ºC, 40 cycles (30 sec at 94 ºC, 1 min at 54.8 ºC, 2 min at 68 ºC), 7 min at 68 ºC 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
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 2996 bp (PGEM-T-Easy), 1146 bp (inserted DNA fragment) and one band of 4141 bp (linear recombinant P-SEG-T-Easy) were obtained (Fig. 2). The sequence results revealed that the entire GuangDong strain EIMIC-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 sporozoite 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
I nvasion of the host cells by extra cellular molie zoites of Eimeria is associated with secretion of proteins from characteristic three sets of secretory 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 EIMIC-1 (Tomley et al., 1991), EIMIC-4 (Brown et al., 2000), EIMIC-5 (Tomley et al., 2001) and EIMIC-2 (Tomley et al., 1996) have been cloned and characterized. Our strategy for isolating EIMIC-2 from sporozoites was successful and a high degree of similarities (99.6 and 99.7%) were revealed by comparing the sequence of EIMIC-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 88 (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 micronemeres 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, DNAassist 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.

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References


