Molecular Characterisation of *Eimeria tenella* Porin, a Potential Anticoccidial Drug Target

(Pencirian Molekul Porin *Eimeria tenella*, Sasaran Dadah Antikoksidia yang Berpotensi)

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**ABSTRACT**

*Eimeria tenella* is an apicomplexan parasite that causes the economically important disease coccidiosis in chickens. An estimated loss over $3 billion USD per annum has been reported. Control of coccidiosis relies on chemotherapy and vaccination, but drug resistance is common and live vaccines are relatively expensive. Therefore, there is an urgent need to develop new drugs to control *Eimeria* infections. Recent studies have shown that the pore forming structures of porin play important roles in many eukaryotic organisms. In this study, we generated and characterised a putative porin cDNA sequence from *E. tenella* that we have named Etporin. Sequence alignments showed that Etporin is 47% similar to the putative porin sequence of *Toxoplasma gondii*, while a search against the Conserved Domain Database (CDD) shows that Etporin contains the Porin3 superfamily domain. Multiple sequence alignment with porin sequences from various eukaryotic organisms showed that the conserved VKXKX and GLK/STK motifs are present in Etporin. Analysis of the predicted Etporin 3D structure showed a classic beta barrel structure consisting of 19 beta-strands. Taken together, these results suggested Etporin has the potential to be developed into an anticoccidial drug target.

**Keywords:** Coccidiosis; drug target; protein structure

**INTRODUCTION**

*Eimeria* species are intracellular protozoan parasites that cause coccidiosis in animals including chickens, cows, pigs, rabbits, and ducks. Seven different species of *Eimeria*, namely *E. acervulina*, *E. maxima*, *E. tenella*, *E. brunetti*, *E. necatrix*, *E. mitis*, and *E. praecox* have been recognised to infect chickens (Chapman 2014). Of these, *E. tenella* is the most studied and widely used as a standard reference in many laboratories because of its high replication rate, and it can be easily isolated, recovered and handled in the laboratory (Blake et al. 2015; Reid et al. 2014). Each *Eimeria* species has its own specificity where different species will invade different locations and possess different pathogenicity, immunological specificity, pre-patent period and sporulation time. The pathology of coccidiosis is caused by the destruction of epithelial cells and blood capillaries due to the release of merozoites from schizonts that in turn result in debris and blood clots causing blockages that lead to necrosis. In serious cases, necrosis can result in the deaths of infected chickens. It has been estimated that losses due to coccidiosis in the chicken industry are over $3 billion USD per annum (Dalloul & Lillehoj 2006; Williams 1999).

To reduce the occurrences of coccidiosis in chickens, prophylactic anticoccidial drugs and vaccines are being used with considerable success (Blake & Tomley 2014). Prophylactic chemotherapy which involves in-feed anticoccidial drugs has been the main method of controlling...
chicken coccidiosis. Generally, anticoccidial drugs such as monensin, lasalocid, salinomycin, narasin and maduramycin are chemicals that act on parasite metabolism by altering ion transport and disrupting osmotic balance (Noack et al. 2019). Vaccination is an alternative way to control coccidiosis in chickens. Protective immunity is induced when chickens are primarily infected with low numbers of *Eimeria* parasites (Chapman & Jeffers 2014). However, the emergence of drug resistance and relatively high production costs of vaccines emphasise the need for the development of more efficient methods of coccidiosis control.

A number of proteins have been previously assessed for their potential as anticoccidial drug targets including glucose-6-phosphate isomerase (Loo et al. 2010) and glycogen synthase kinase-3 (Yao et al. 2016). Recent studies have shown that the porin protein may also be a good candidate as it plays an important role in cell adherence to eukaryotic host cells and pathogen/symbiont recognition (Goo et al. 2006; Hejair et al. 2017; Nyholm et al. 2009). In addition, porin has also been demonstrated as one of the most-heavily lysine acylated proteins, indicating that lysine deacetylase inhibitors such as apicidin can be used to prevent the acetylation of porin and subsequently attenuate the host-pathogen interaction and the infection process (Jeffers & Sullivan Jr. 2012). Porin has been reported to be conserved across all known apicomplexan parasites and its unique properties as protein transport complexes could be exploited for possible drug targeting (Gajdács 2019; Mather et al. 2006; Pusnik et al. 2009). In addition, porin has also been demonstrated as one of the most-heavily lysine acylated proteins, indicating that lysine deacetylase inhibitors such as apicidin can be used to prevent the acetylation of porin and subsequently attenuate the host-pathogen interaction and the infection process (Jeffers & Sullivan Jr. 2012). Porin has been reported to be conserved across all known apicomplexan parasites and its unique properties as protein transport complexes could be exploited for possible drug targeting (Gajdács 2019; Mather et al. 2006; Pusnik et al. 2008). Here, we report the sequencing and characterisation of a putative porin cDNA sequence for *E. tenella*.

**MATERIALS AND METHODS**

**VECTORS AND CLONES**

The recombinant clone used in this study was isolated from a cDNA library of *E. tenella* obtained from the Institute of Medical Sciences, University of Tokyo, Japan. The cDNA library was constructed by cloning *E. tenella* merozoites into pME18S-FL3 vector using an oligo-capping approach (Amiruddin et al. 2012).

**BACTERIA GROWTH**

Stock cells of *E. coli* carrying pME18S-FL3 vector were streaked onto Luria-Bertani (LB) agar and incubated overnight at 37 °C. A single colony was inoculated into 5 mL LB Broth medium containing 50 µg/mL ampicillin. The inoculums were grown 18 h at 37 °C with agitation. Overnight at 37 °C. A single colony was inoculated into 5 mL LB Broth medium containing 50 µg/mL ampicillin.

**PLASMID DNA EXTRACTION**

The pME18S-FL3 plasmid DNA was extracted with a QIAprep® Miniprep kit (QIAGEN, USA), according to the manufacturer’s protocol. The extracted plasmid was eluted out using 50 µL sterilised water.

**DNA CYCLE SEQUENCING**

Sequencing of extracted DNA plasmids was performed using the ABI PRISM® BigDye® Terminator V3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystem Inc., USA) according to the manufacturer’s protocol. The extracted plasmids were subsequently digested with EcoR1 (Promega, USA) and the digested products were analysed using agarose gel electrophoresis to characterise the Etporin gene.

**PLASMID CHARACTERISATION**

The quality and quantity of DNA samples obtained were determined by Spectrophotometer ND-1000 (NanoDrop, USA). Agarose gel electrophoresis was carried out to determine the presence of the plasmid. DNA supercoiled ladder (Promega, USA) was used as a DNA size marker. The extracted plasmids were subsequently digested with EcoR1 (Promega, USA) and the digested products were analysed using agarose gel electrophoresis to characterise the Etporin gene.

**PRIMER DESIGN**

Primers were designed using Primer3 (frodo.wi.mit.edu/primer3/). Oligo cap linker (5′ GGA TGT TGC CTT TAC TTC TA 3′) and Oligo-dT-adapter (5′ TTT GGT TGG AGG TTT TTT CTC TA 3′) were used as outer primers of cDNA in cycle sequencing reactions. PwF1 (5′ TTT TCT CGA CTT CTC CAC AGA AT 3′) and PwR3 (5′ ATT GGG CCT TGC ATG CGT TTT G 3′) were designed as inner primers.

**PRIMER DESIGN AND DATABASE SEARCHES**

The sequences generated were quality-clipped using Phred (Machado et al. 2011). Vector and host contamination were identified and masked using the sequence comparison program Crossmatch (Gordon et al. 1998). Vector trimming excised the longest non-masked sequence and further trimming removed low quality bases (Phred score less than 30) at both ends of a read. Rearrangement and assembly of the sequences generated were done by Biededt (Hall 1999). Open reading frames in the sequences obtained were identified by ORF Finder (www.ncbi.nlm.nih.gov/orf/ orf.cgi) at the National Centre for Biotechnology Information (NCBI) website (Sayers et al. 2018).

Sequence database similarity searches were carried out using BLAST suites (Camacho et al. 2009) for similarity with known porin proteins. Conserved protein
domains were analysed using the Conserved Domain Database (CDD) (Marchler-Bauer et al. 2014), CATH protein domain database (Dawson et al. 2017) and InterPro domain database (Mitchell et al. 2018). Multiple sequence alignments were produced using MAFFT (Katoh & Standley 2013). Protein function prediction was done using ARGOT (Falda et al. 2012), PANDA (Wang et al. 2018) and PANTHER (Mi et al. 2019).

**FOLD RECOGNITION AND 3D MODELLING**

Secondary structures were predicted using PSIPRED (Buchan & Jones 2019). Different protein structure modelling servers i.e. I-TASSER (Yang et al. 2015), Phyre2 (Kelley et al. 2015), Raptor-X (Peng & Xu 2011) and IntFOLD5 (McGuffin et al. 2019) were utilised to generate 3D models of Etporin. MolProbity (Williams et al. 2018) were used to check the stereochemical quality of the protein structure. ProQ3 (Uziela et al. 2016), a machine learning based model quality assessment method in CASP12 (Kryshtafovych et al. 2018), was also used to determine the model quality.

**RESULTS AND DISCUSSION**

*Eimeria tenella* is an apicomplexan parasite that causes coccidiosis, an economically important disease that affects chicken production worldwide. This disease is dominantly controlled by prophylactic chemotherapy but the widespread occurrence of drug resistant strains requires the development of new drug targets. Identification and validation of a potential drug target are the beginning steps in the drug discovery pipeline. Before investing more resources in the target, it is crucial to have collective corroborative evidence that can support the choice of a target to be further explored. A good drug target must be involved in a crucial, ideally an essential biological pathway that has been functionally and structurally characterised as well as being distinguishable from any previously known targets (Bakheet & Doig 2009).

Studies have shown that porin, a transmembrane protein located in the outer membrane of mitochondria, is a major interface between the mitochondrial and the cellular metabolisms (Shoshan-Barmatz et al. 2006). It has been recognised as a key protein in mitochondria-mediated apoptosis, thus making it a potential target for the rational development of new therapeutics. In this study, a putative porin cDNA sequence in *E. tenella* was generated and characterised for further understanding of this protein.

A recombinant plasmid containing the cDNA of interest was sequenced by primer walking and resulted in a contig of 1368 bp in size (Figure 1). Analysis with ORF Finder showed the most probable open reading frame of the sequence is located in the +2 reading frame with a start codon at position 104 encoding methionine and ending with a stop codon at position 982 for a total length of 879 bp and encoding for 292 amino acids.

A BLASTx search showed the sequence to be similar to only other eukaryotic porin sequences (Table 1). We thus annotated this sequence as a putative porin and named it as Etporin. The database matches included highly significant matches to other apicomplexan sequences from *Toxoplasma gondii*, *Plasmodium vivax*, *P. falciparum*, *P. knowlesi*, and *Cryptosporidium parvum*. The Etporin sequence showed the highest similarity with the putative porin protein from *T. gondii* (XP_002365430.1). The Etporin sequence length of 292 aa fitted well into the average sequence length for a single porin unit which averages ~290 aa and therefore indicative of a complete sequence.

The Etporin sequence search against CDD showed that Etporin contains the Porin 3 superfamily domain (E-value: 5.72e-29). The Porin 3 superfamily has two subfamilies - the Voltage Determinant Anion Channel (VDAC) subfamily and the Tom40 subfamily. Etporin is believed to be a VDAC subfamily member because it possesses a sequence with motifs consistent to other VDAC members. Etporin is also mapped to CATH mitochondrion outer membrane porin functional family (2.40.160.160.FF9682) and eukaryotic porin family (IPR001925). These families composed of VDAC that behaves as diffusion pores for small hydrophilic molecules.

Multiple sequence alignment with porin sequences from various eukaryotic organisms identified the conservation of two glycine residues (G145 and G285) and two lysine residues (K94 and K283) (Figure 2). The conserved lysine residues may function by funneling anions toward the binding site and may be required for the formation of the inorganic phosphate-specific binding site of porin (Sukhan & Hancock 1996).

However, a GLK motif found to be present in other porin sequences is absent in Etporin and in its place at the aligned position is an STK sequence (Figure 2). The GLK motif was originally suggested to be an ATP binding site because the replacement of LYS with GLU will lead to the impaired binding of ATP thus resulting in cation selectivity but not anion selectivity (Runke et al. 2000). In Etporin, the GLK motif is not present. Recent studies showed that porin sequences that lack this motif were scattered throughout the phylogenetic tree, and there is no clear relationship between this motif and eukaryotic porin signature motif. However, in plants, there are two clusters of porin sequences where GLK is replaced by a neutral event or it may have been selected due to a specific function.
FIGURE 1. Generation of the full-length sequence of *Etoporin* via the primer walking strategy

Dark bar indicates generated full-length sequence. Forward primers 5' 3' are represented by PwF1, PwF2 and PwF3. Reverse primers 3' 5' are represented by PwR1, PwR2 and PwR3. Number below primer name represents the location of each primer.

Dark grey arrows indicate nucleotide sequences generated by clone 1 while light grey arrows indicate nucleotide sequences generated by clone 2.

| α1 | β1 | β2 |
|----|----|----|
| 2D | --- | --- |
| Eimeria | --- | --- |
| Gallus | --- | --- |
| Homo | --- | --- |
| Mus | --- | --- |
| Yeast | --- | --- |
| Neurospora | --- | --- |
| Saccharomyces | --- | --- |
| Coccidioides | --- | --- |
| Meleagris | --- | --- |
| Zebra | --- | --- |
| Arabidopsis | --- | --- |
| Toxoplasma | --- | --- |
| Chlamydomonas | --- | --- |
| consensus/90% | --- | --- |

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| β3 | β4 | β5 | β6 |
|----|----|----|----|
| 2D | --- | --- | --- |
| Eimeria | --- | --- | --- |
| Gallus | --- | --- | --- |
| Homo | --- | --- | --- |
| Mus | --- | --- | --- |
| Yeast | --- | --- | --- |
| Neurospora | --- | --- | --- |
| Saccharomyces | --- | --- | --- |
| Coccidioides | --- | --- | --- |
| Meleagris | --- | --- | --- |
| Zebra | --- | --- | --- |
| Arabidopsis | --- | --- | --- |
| Toxoplasma | --- | --- | --- |
| Chlamydomonas | --- | --- | --- |
| consensus/90% | --- | --- | --- |
been proven to be important in porin assembly. Predicted by JS conservation analysis to be the conserved cluster which may involve in catalytic activity, oxidoreductase activity and oxidation reduction. This motif has residues (KRHEDQNST) are shown as ‘p’; turn-like residues (ACDEGKNQRST) are ‘t’; bulky hydrophobic residues (ACLIVMHYFW) are ‘h’; the aliphatic subset of these type residues (LIVM) are ‘l’; aromatic residues (FHWY) are ‘a’; small residues (ACDGNPSTV) are ‘s’; and tiny residues (AGS) are shown as ‘u’. Dark grey highlighted VKSKI is consensus/90%. Residues with single underline ( ) are predicted site of pocket 1 while residues with double underline ( ) are predicted site of pocket 2. Residues marked (*) are highly conserved residues; (.) are partially conserved residues; and (.) are less conserved residues. For 2D topology analysis, E are beta strands, C are coils and H are helices. Polar residues (KRHEDQNST) are shown as ‘p’; turn-like residues (ACDEGKNQRST) are ‘t’; bulky hydrophobic residues (ACLIVMHYFW) are ‘h’; the aliphatic subset of these type residues (LIVM) are ‘l’; aromatic residues (FHWY) are ‘a’; small residues (ACDGNPSTV) are ‘s’; and tiny residues (AGS) are shown as ‘u’. Dark grey highlighted VKSKI is predicted by JS conservation analysis to be the conserved cluster which may involve in catalytic activity, oxidoreductase activity and oxidation reduction. This motif has been proven to be important in porin assembly.

| Accession | Organism | Protein | Max score | Query coverage | E value | Identity |
|-----------|----------|---------|-----------|----------------|---------|----------|
| XP_002365430.1 | Toxoplasma gondii | Porin, putative | 302 | 63% | 8e-80 | 47% |
| XP_001616654.1 | Plasmodium vivax | Hypothetical protein | 128 | 62% | 2e-27 | 28% |
| XP_001348475.1 | Plasmodium falciparum 3D7 | Conserved protein | 122 | 62% | 1e-25 | 28% |
| XP_002140850.1 | Cryptosporidium muris RN66 | Hypothetical protein | 103 | 64% | 4e-20 | 26% |
| XP_002260703.1 | Plasmodium knowlesi strain H | Hypothetical protein | 103 | 58% | 4e-20 | 26% |
| XP_676234.1 | Plasmodium berghei strain ANKA | Hypothetical protein | 98.2 | 55% | 2e-18 | 27% |
| XP_746288.1 | Plasmodium chabaudi chabaudi | Hypothetical protein | 82.4 | 32% | 1e-13 | 29% |
| XP_001611333.1 | Babesia bovis | Hypothetical protein | 80.9 | 61% | 4e-13 | 26% |
| XP_955278.1 | Theileria annulata strain Ankara | Hypothetical protein | 66.2 | 62% | 9e-09 | 21% |
| XP_724308.1 | Plasmodium yoelii yoelii | Hypothetical protein | 63.5 | 28% | 6e-08 | 26% |
One signature motif of eukaryotic porins reported by Smith et al. (1995) is that a VKAKV sequence is available in all VDAC sequences that have been studied. Lys-234 and Lys-236 exist in this internal pentapeptide (VKAKV) of yeast porin, and are suggested to have an important role in the membrane insertion of this protein. Besides this, Lys-234 and Lys-236 in yeast have also been shown to be involved in porin assembly. Substitution of these sites by two other polar amino acid residues, glutamate and glutamine, will cause impaired assembly (Smith et al. 1995). In Etporin, we found that VKS5K is present at amino acids 240 to 245 instead of VKAKV, which has been detected in all porin sequences. However, Etporin still possesses the two conserved lysine residues (Lys-241 and Lys-244), which are important for porin assembly. This region VKS5K also has been predicted by Jensen-Shannon Divergence to be involved in catalytic activity, oxidoreductive activity and oxidation activity.

Secondary structure predictions by PSIPRED showed that Etporin consists of an alpha helix at the N-terminal and the expected series of 19 beta strands that would be necessary for forming the beta barrel arrangement. This is therefore in agreement with existing porin structures. The only region of the sequence predicted to have an alpha-helical secondary structure (4th to 15th aa) is partially conserved between porin sequences from various eukaryotic organisms. This segment is usually located less than 10 residues from the amino-terminus (Young et al. 2007). Most of the beta strands in Etporin are surrounded by two parallel partners. This is also the major arrangement of beta strands that have been characterised in other porin structures. Although the 16 beta strands pore structure of porin are commonly seen, porins from different organisms may fold into barrels with different numbers of strands. The predicted secondary structures for Etporin strongly supports a common structural framework that is associated with porin where the main beta-barrel feature is well-conserved structure across all phyla investigated (Young et al. 2007).

Various structural modelling methods were employed to model the 3D structures of the Etporin subunit. All the modelling methods predicted the structures to be composed of 19 anti-parallel beta strands arranged to form a barrel shape. This arrangement is consistent with other known porins. The quality of models was assessed using MolProbity and ProQ3. The best structural model was generated using the IntFOLD5 server (Table 2). Ramachandran plot and result showed that 92.8% of total residues located at most favoured regions, 5.2% of them located at additional allowed regions, 1.6% at generously allowed regions and 0.4% residues is in disallowed regions. A good quality of 3D model would be expected to have over 90% residues in the most favoured regions. The model has a good ProQ3 score of 0.514 (the score ranges from 0 to 1, with 1 being the best score).

The best predicted Etporin structural model was compared with the human porin structure (PDB id: 2JK4, Figure 3). In spite of low sequence similarity (14.3%) shown between two sequences, their 3D structures are highly similar (TM-score 0.8533) where they have the same number of beta strands forming a barrel with an N-terminal alpha-helical region located inside the pore. We speculate that extensive amino acid variations do not bring substantial alterations in the beta-barrel structure of membrane channels and its functions (Shoshan-Barmatz et al. 2010).

The function of Etporin was further investigated using a few protein function prediction web servers (ARGOT, PANDA, PANTHER). Most of the servers provide the most probable annotations for a query sequence in each of the three branches of the Gene Ontology i.e. biological process, molecular function, and cellular component. Its molecular function is described by ion channel activity and voltage-gated ion channel activity, it is thought to be involved in the biological processes associated with transmembrane transport and anion transport, and it can be found in mitochondria outer membrane. Taken together, Etporin has been shown as the porin protein in *E. tenella* and the presence of conserved motifs further suggests that it is an important protein for biological processes.

| Methods     | Template used          | Percentage of residues located at most favoured region | ProQ3 global model quality score |
|-------------|------------------------|--------------------------------------------------------|---------------------------------|
| I-TASSER    | 2JK4A, 3EMNX, 4BUX     | 69.1%                                                  | 0.448                           |
| Phyre2      | 2K4TA                  | 81.5%                                                  | 0.399                           |
| RaptorX     | 3EMNX                  | 88%                                                    | 0.498                           |
| IntFOLD5    | 3EMNX                  | 92.8%                                                  | 0.514                           |
**CONCLUSION**

*In silico* analyses on Etporin has shed light on the putative identity of the protein and showed novel essentials, uncovering its potential as a drug target in *E. tenella*. To increase the value of our findings, additional research, such as gene disruption studies, will be required to confirm the importance of Etporin and to verify the reliability of the data.

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