# [BIO18] Partial characterisation of ATP-binding cassette encoding genes in *Cryptosporidium parvum*

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## Introduction

*Cryptosporidium parvum* is an intestinal parasitic protozoa which causes self-limited diarrhoea in immunocompetent individuals and devastating and fulminant diarrhoea in immunocompromised patients (Griffiths, 1998).

Despite extensive effort in searching for chemotherapeutic agents against cryptosporidiosis, it remains elusive. The major impediment in the use of a promising chemotherapeutic compound is the intrinsic multidrug resistance exhibited by *C. parvum* towards an array of drugs that is generally effective against similar organisms (Chen *et al.* 2002). It is postulated that the intrinsic multidrug resistance of *C. parvum* is mediated by a general transporting mechanism, possibly by the ATP-binding cassette (ABC) superfamily of transporters.

The ABC superfamily of transporters, which consists of more than 1,000 members, is the largest and most widespread protein family known to date. The vast majority of its members are ATP driven transporters, which is responsible of translocating a wide variety of compounds across biological membranes (Higgins, 1992; Ambudkar *et al.*, 1999).

Most ABC transporters share a similar architecture: a fundamental structure made up of four domains. Two of these domains are the hydrophobic membrane-spanning domains (MSDs), and two other domains are the hydrophilic nucleotide-binding domains (NBDs). NBDs are evolutionally conserved domains, which have considerable sequence homologies across the entire family, and the homologies range from 30% to 50%. Two NBDs within a transporter show higher similarity to each other than to the respective domains from other member of transporter (Higgins, 1992; Ambudkar *et al.*, 1999).

In addition to their physiological functions, ABC proteins have enormous medical relevance. Members of the superfamily, the Multidrug resistance protein (MDR) (Juliano and Ling, 1976) and Multidrug resistance associated protein (MRP) (Cole and Deeley, 1993), are almost always associated with the multidrug resistance phenomenon exhibited by many intrinsic or acquired drug resistance cancer cell lines or human pathogens (Davidson, 2002). Transfection studies and investigation on the drug sensitive partial revertant cell lines clearly elaborated the correlation between MDR, MRP and multidrug resistance phenotypes (Gao *et al.*, 1998).

The present study aims to explore and characterise the possible mechanisms underlying the MDR characteristic of *C. parvum* by detecting the presence of ABC transporter protein encoding genes, especially one that shows high similarity to members belonging to the MDR and MRP subfamilies of transporters.

## Materials and methods

### *Cryptoporidium parvum genomic DNA extraction*

*C. parvum* oocysts (IOWA isolate, bovine genotype) used in this study were purchased from Waterborne Inc, USA. Genomic DNA was extracted from *C. parvum* oocysts by using the QIAamp DNA blood mini kit (QIAGEN, Germany).

### *Degenerate primer for PCR amplification of ABC transporter encoding gen*es

PCR approach using degenerate primers, was used to identify ABC transporter encoding gene in *C. parvum*. The sense and antisense primers corresponding to peptide GCGKST(L/I)(I/L) and (G/A)(V/S)KLSGGQ were selected based on the analysis of the highly conserved NBDs of several ABC transporter proteins (Dallagiovanna *et al*., 1994). The degeneracy of the primers was adjusted to account for the codon bias found for *C. parvum* genes.

***PCR amplification of ABC transporter encoding genes***

The amplification of *C. parvum* ABC transporter protein genes involved two rounds of PCR, namely the primary and secondary amplifications. In primary amplification, degenerate primers were incubated with *C. parvum* genomic DNA, according to standard protocol, in a volume of 20µl. The optimum PCR annealing temperature was 55$^{o}$C. The secondary PCR amplification condition was identical to the first round, except that 1 µl of 50X diluted primary PCR products was used as DNA template.

***PCR product purification and cloning***

The QIAquick gel extraction kit (QIAGEN, Germany) was used to extract DNA bands from agarose gels. Purified PCR products were cloned into pGEM®-T vectors (Promega, USA), and transformed into JM109 *E. coli* (Promega, USA).

***Sequencing analyses***

The resultant positive clones were sequenced in both directions on an ABI PRISM® 377 DNA sequencer (BST Techlab, Singapore). Sequencing primers used were T7 forward and SP6 reverse promoter primer encoded on the pGEM-T vector.

Nucleotide and amino acid sequences were compared with the sequences deposited in NCBI databases via the Blast server. Multiple alignments of DNA and protein sequences were performed using ClustalW programme. Protein similarities were calculated using the GeneDoc sequence editor. Conservative amino acids are grouped as: (D,E,N,Q,H)(S,A,T)(K,R)(F,Y)(L,I,V,M).

The aligned protein sequences were analysed by distance based neighbour-joining method, using the Njplot programme. Bootstrap replicates of 1000 was set to assess the reliability of the tree. An unrooted tree was also constructed by using the Njplot.

**Results and Discussion**

PCR using ABC-specific degenerate primers successfully amplified two unique fragments from *C. parvum* genomic DNA. Sequencing of the fragments revealed two partial open reading frames (ORFs). ORF1 was constituted by 279bp, which encoded a peptide of 96 amino acids. ORF2 was 303bp in length and encoded a 101-amino acid peptide. Comparisons of ORF1 and ORF2 to the protein sequences in NCBI databases indicated that both fragments exhibited significant high similarities to the NBDs of a variety of ABC transporters. Thus, ORF1 and ORF2 were respectively designated *Cpnbd1* and *Cpnbd2*. For protein sequences, there were designated as Cpnbd1 and Cpnbd2 [Figure 1(a) and (b)].

***Comparisons of Cpnbd1 and Cpnbd2 with the NBDs of C. parvum ABC proteins***

At amino acids level, Cpnbd1 was 100% identical to the $NH_2$-terminal half (_N) of CpABC1, an ABC protein characterised in *C. parvum* KSU-1 isolate (bovine genotype) (Zapata *et al.*, 2002). At nucleotide level, both gene fragments showed 99% identity. The difference was due to a single nucleotide changed. The nucleotide substitution, which was at the third position of the codon-33 was a silent change as both CAG$^{99}$ in *Cpnbd1* and CAA$^{99}$ in *CpABC1*_N codes for the amino acid glutamine (data not showed).

Cpnbd1 also showed high similarity to the NBD at the $NH_2$-terminal of CpABC. CpABC was the gene encoding the ABC protein of *C. parvum* of SFGH1 isolate, an isolate of human genotype (Perkin *et al.*, 1999). Both proteins were 97% and 98% identical at amino acid and nucleotide levels, respectively, and the differences were attributed by four nucleotide substitutions. Two of the nucleotide substitutions resulted in silent changes: CAG$^{99}$ at codon-33 of *Cpnbd1* and CAA$^{99}$ of *CpABC*-_N, which codes for glutamine, and GAT$^{174}$ at codon-58 of *Cpnbd1* and GAC$^{174}$ of *CpABC*_N, which codes for aspartic acid. Two other nucleotide substitutions, which occurred at the first position of the corresponding codons, resulted in amino acid changes. These were the $^{64}$ATT at codon-22 of *Cpnbd1*, which codes for isoleucine, and the corresponding codon $^{64}$GGT at *CpABC*_N, which codes for valine. Whereas at codon-42, $^{124}$GTT of *Cpnbd1* coding for valine while $^{124}$ATT of *CpABC*_N coding for isoleucine (data not showed).

(a) *Cpnbd1*/Cpnbd1

ggatgtggtaaatcaacattgattgaacttatattacaagaacttaaaccaagattaggaacaattcaatca
 G C G K S T L I E L I L Q E L K P R L G T I Q S
aatggttcagtttttattgttcacagtcatcatggattattaatggtacagttagaagtaatattatactt
 N G S V F Y C S Q S S W I I N G T V R S N I I L
gatttacctttgatcaagcctggtatgatattgttattaatgcttgttcattagtttatgatttaaaagct
 D L P F D Q A W Y D I V I N A C S L V Y D L K A
atgccaaatggggatttaacagaaattggtgaaaatggtgttaaactttctggaggtcaa
 M P N G D L T E I G E N G V K L S G G Q

(b) *Cpnbd2*/Cpnbd2

ggatgtggtaaatctacacttataaagcaaatgattggttttattaagcctgatcagggagatgttcactac
 G C G K S T L I K Q M I G F I K P D Q G D V H Y
ggagatatttcttttatacataaccgtaaacaagctcgaacaatgatgagttatatgtcacaacagtatgca
 G D I S F I H N R K Q A R T M M S Y M S Q Q Y A
cctcttgaaaagttaactgttgagcaaaatttagagatgattggaagaatgagagggttaagtacctcagaa
 P L E K L T V E Q N L E M I G R M R G L S T S E
ctccaaaaagaaattgagcaccttctagcagatctggaaatagtagaataccgtgataaaaaaggaagtaaa
 L Q K E I E H L L A D L E I V E Y R D K K G S K
ctttcaggaggacaa
 L S G G Q

FIGURE 1 Nucleotide and deduced amino acid sequences of (a) *Cpnbd1*/Cpnbd1 and (b) *Cpnbd2*/Cpnbd2. Nucleotide sequences are indicated by small letters. Whereas the putative amino acid sequences are indicated by capital letters and are represented by single-letter codes. The regions of which the primers correspond are underlined.

The high degree of similarities between Cpnbd1, CpABC and CpABC1 implied that they could be encoded by homologous genes of a type of ABC transporter protein found in different *C. parvum* isolates.

As for Cpnbd2, it showed moderate similarities to the NBDs at both $NH_2$- and COOH-terminal halves of ABC proteins characterised in *C. parvum* to date (Table 1). Therefore, Cpnbd2 could be a novel member of an ABC superfamily of proteins in *C. parvum.*

***Comparisons of Cpnbd1 and Cpnbd2 with the NBDs of ABC proteins characterised in drug resistance cells***

Phylogenetic analysis on a list of ABC transporters known to associate with MDR phenotype (EhPGP 5: *Entamoeba histolytica* P-gp-like transporters 5; EhPGP 6: *E. histolytica* P-gp-like transporters 6; HuABCB1: *Homo sapien* ATP-binding cassette B1; HuABCC1: *H. sapien* ATP-binding cassette C1; HuABCC2: *H. sapien* ATP-binding cassette C2; LaMDR1: *Leishmania amazonensis* Multidrug resistance protein 1; Ldmdr1: *L. donovan*i Multidrug resistance protein 1; LeMDR1: *L. enrietti* Multidrug resistance protein-like 1; TbMRPA: *Trypanosoma brucei* Multidrug resistance protein A; and TbMRPE: *T. brucei* Multidrug resistance protein E) has strongly related Cpnbd1 to HuABCC1 and HuABCC2.

HuABCC1 was a human MRP transporter identified in the H69AR doxorubicin-selected human lung carcinoma cell line (Mirski *et al.*, 1987). In normal tissue, HuABCC1 was found ubiquitously expressed at low levels. Certain normal tissues, however, do highly express HuABCC1 at cellular levels. In human cancer cells, HuABCC1 was found overexpressed in various cancer types. In certain cancer types, HuABCC1 expression is an important predictor of treatment outcome. The expression of HuABCC1 is usually highest in tumours that derived from tissues that normally express the transporter protein, and tumours arising from these tissues are known to be intrinsically resistant against chemotherapeutic treatment (Cole *et al.*, 1994; Hipfner *et al*., 1999).

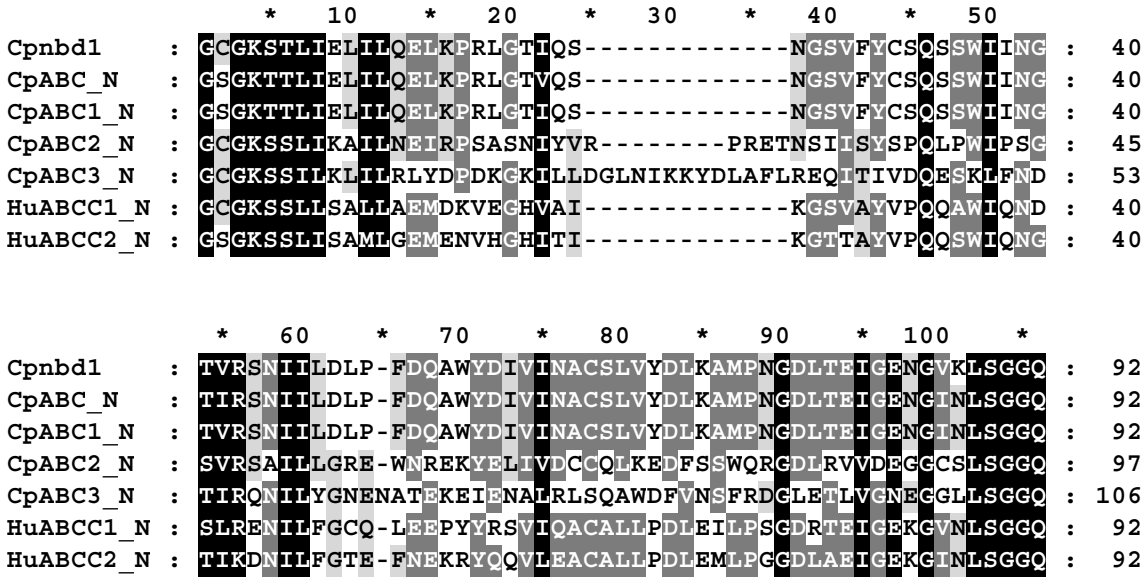


FIGURE 2 Comparisons of the amino acid sequences of Cpnbd1 and the NBDs of ABC proteins from *Cryptosporidium parvum* and from *Homo sapiens.* Similar amino acids shared by all, 70% and 50% of the aligned sequences, are boxed by black, dark grey and light grey, respectively.


FIGURE 3 Comparisons of the amino acid sequences of Cpnbd2 and the NBDs of ABC proteins from *Cryptosporidium parvum*, *Homo sapiens* and *Trypanosoma cruzi.* Similar amino acids shared by all and two of the aligned sequences, are boxed by black and dark grey, respectively.

HuABCC2 is the second member identified in the now nine-member MRP subfamily of transporter in human. Taniguchi *et al.* (1996) successfully isolated the HuABCC2 encoding gene from a cisplatin-resistant human cancer cell line. The research group also reported a good correlation between cisplatin resistance and the up-regulation expression of HuABCC2. HuABCC2 has been detected in the liver, kidneys, intestine, placenta and the brain at normal level. HuABCC2 is believed to play an important role in eliminating endogenous toxic compounds and xenobiotics from the body. In hepatocytes, the expression of HuABCC2 is markedly increased by various chemical carcinogen and chemopreventive agents (Payen *et al.*, 2002). Meanwhile, intestinal HuABCC2 could play a role in reducing the level of 2-amino-1-methyl-6-phenylimidazo [4,5-6] pyridine; the most abundant food derived carcinogen, which is formed during cooking, frying and grilling of meat (Dietrich *et al.*, 2001).

The relationship of Cpnbd2 with the same group of transporters was less distinct. It was clustered in the clade, which consisted of the NBDs of various ABC transporter proteins (Figure 4). The similarities of Cpnbd2 to these NBDs were generally

moderate (Table 1). Interestingly, Cpnbd2 did show significant high similarity to the NBDs at the $NH_2$-terminal halves of two ABC proteins [the *H. sapien* ATP-binding cassette A5 (HuABCA5) and *T. cruzi* ABCA1 transporter (TcABCA1)]. It was 65% similar to HuABCA5_N and 59% similar to TcABCA1_N. However, neither the substrate nor the function of HuABCA5 and TcABCA1 is known (Dean *et al.*, 2001; Torres, 2000). The possible function of Cpnbd2, therefore, cannot be inferred from the phylogenetic analyses.

TABLE 1. Quantitative analysis of amino acid similarities between Cpnbd1, Cpnbd2 and the NBD at the $NH_2$-terminal half and COOH-terminal half of the ABC proteins of several drug resistance parasitic protozoan and *Homo sapiens*. Chemically similar amino acids were defined as: (D,E,N,Q,H) (S,A,T) (K,R) (F,Y) (L,I,V,M).

| | Similarity (%) | |
|---|---|---|
| | Cpnbd1 | Cpnbd2 |
| CpABC_N | 100 | 30 |
| CpABC_C | 46 | 43 |
| CpABC1_N | 100 | 30 |
| CpABC1_C | 46 | 46 |
| CpABC2_N | 58 | 28 |
| CpABC2_C | 40 | 49 |
| CpABC3_N | 37 | 36 |
| CpABC3_C | 41 | 35 |
| EhPGP5_N | 43 | 36 |
| EhPGP5_C | 37 | 36 |
| EhPGP6_N | 30 | 37 |
| EhPGP6_C | 40 | 32 |
| HuABCA5_N | Nil | 65 |
| HuABCB1_N | 35 | 39 |
| HuABCB1_C | 36 | 33 |
| HuABCC1_N | 68 | 32 |
| HuABCC1_C | 36 | 41 |
| HuABCC2_N | 68 | 32 |
| HuABCC2_C | 39 | 34 |
| LaMDR1_N | 37 | 36 |
| LaMDR1_C | 41 | 30 |
| Ldmdr1_N | 37 | 36 |
| Ldmdr1_C | 42 | 30 |
| LeMDR1_N | 36 | 35 |
| LeMDR1_C | 40 | 31 |
| TbMRPA_N | 59 | 39 |
| TbMRPA_C | 36 | 37 |
| TbMRPE_N | 60 | 38 |
| TbMRPE_C | 39 | 39 |
| TcABCA1_N | Nil | 54 |

The study of ABC protein family in *C. parvum* is considered at its infancy as no functional study was thus far been conducted on any of the structural characterised ABC proteins of the parasite. In intracellular stages, CpABC1 was found exclusively at the host-parasite boundary of mature meronts.

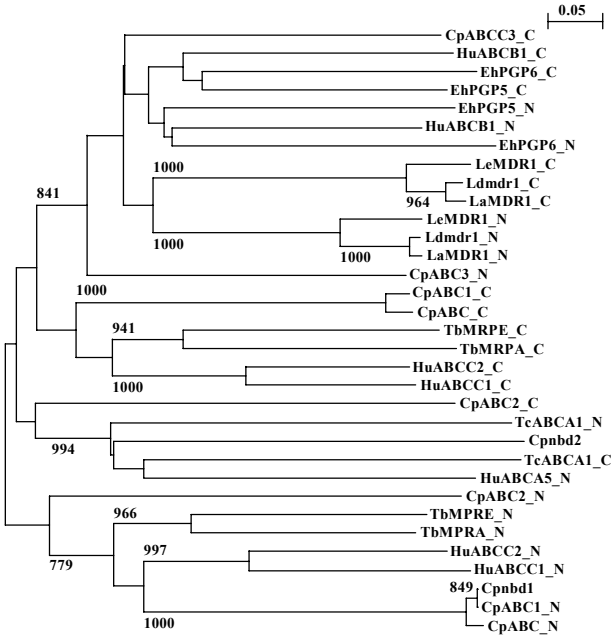


FIGURE 4 The phylogenetic relationship of NBDs among various ABC superfamily of proteins.

The location of CpABC1 suggested that it could be a component of one or more of the membranes (the meront plasma membrane, the parasitophorous vacuole membrane, and the feeder organelle) at the host-parasite boundary (Zapata *et al.*, 2002). Back in 1999, similar observation was reported by Perkin *et al.* on CpABC. The peculiar location of CpABC and CpABC1, suggested that they could play a role in metabolic interaction between the parasites and the infected host. The localisation of both proteins also correlates well and support a role as drug efflux pumps that could transport endogenous and xenobiotic away from the parasites.

Since some genes of the P-gp and MRP subfamilies of transporters have been associated with MDR phenomenon in cancer patients and certain drug resistance pathogens, the presence of such homologous

protein in *C. parvum* could also attribute to the intrinsic MDR phenotype of the parasite.

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