# Phylogenetic Relationship Among Apicomlexan Parasites Based on *In silico* Analysis of Enzymes of the MEP Pathway

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## Abstract

Apicomplexans such as *P. falciparum* use MEP pathway to synthesize isoprenoids crucial for their survival in the host. Here we report phylogenetic relationship among 11 parasites of phylum apicomplexa based on *in silico* analysis of enzymes of the MEP pathway with reference to *Plasmodium falciparum* 3D7. In addition, structure based homology of DXR of apicomlexans were performed with respect to DXR of *Mycobacterium tuberculosis* in order to gain detail insight of their DXR structure. The study revealed that 9 of 11 apicomlexans showed presence of the MEP pathway and all of its enzymes while the *MEP* pathway was absent in two members namely *Cryptosporidium hominis* and *C. parvum*. In two apicomlexans *Toxoplasma gondi* and *Eimeria tenella* an enzyme MCT and in *P. knowlesi HDR* enzyme of the MEP pathway was absent. Sequence analysis of the MEP pathway enzymes and resultant cladogram indicated that *plasmodium* sp. can be easily distinguished from non-*plasmodium* sp. The cladogram revealed close relationship among plasmodium sp. *viz., P. falciparum* 3D7, *P. berghei* str. ANKA, *P. vivax SaI-1* and *P. knowlesi H.* Results of structure based homology analysis showed presence of amino acids viz., Thr (T) 21, Gly (G) 22, Ser (S) 23, Ile (I) 24, Gly (G) 47, Gly (G) 48, Ala (A) 49 and Glu (E) 129 in the NADPH binding domain; Asp (D)151, Glu (E)153 and Glu in Mn2+ binding domain and Ser (S)152, Ser (S) 177, His (H) 200, Asn (N) 218 and Lys (K)219 in fosmidomycin binding domain in DXRs of apicomlexans. These amino acids were found to be highly conserved. Thus, MEP pathway enzymes served as excellent tools to discern phylogenetic relationship in apicomlexans and an attractive target for development of new anti-parasitic drugs against these parasitic microorganisms.

# 1. Introduction

The Apicomplexa group of protozoa comprises approximately 5000 species of which majority causes infectious diseases in animals (Cavalier-Smith 1993). Important species of this group such as *Plasmodium falciparum*, *P. vivax, P. berghei* and *P. knowlesi* are well known as they are causative agents of malaria in human. Among others, *Toxoplasma gondii* causes toxoplasmosis; *Babesia bovis* Texas Cattle Fever and *Theileria parva* is the causative agent of East Coast fever, corridor disease, and Zimbabwean theileriosis (Wasmuth *et al.,* 2009). Table 1 summarizes host and environment specificity for apicomplexans used in this study. Members of the apicomplexa are characterized by the presence of four-membrane relict plastid called apicoplast (Arora *et al.,* 2010).

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Apicoplast is a 35 KB extra chromosomal DNA in *P. falciparum* which harbor around 551 nucleus encoded apicoplast targeted (NEAT) proteins (Gardner 2002). Many of these NEAT proteins are promising drug targets for killing of the Apicomplexan parasites (Steinbacher *et al.,* 2002). Apicoplast is essential for survival of the apicomlexans as it possess many pathways of formation of fatty acids, heme and isoprenoids (Roos *et al.,* 2002). Isoprenoids play crucial roles in the survival of malarial parasite *P. falciparum*. Isoprenoids are one of the major classes of secondary metabolites which consist of $C_5$ compound called isoprenes or isopentenyl diphosphate (IPP) and its isomer dimethyl allyl diphosphate (DMAPP). In plants and many bacteria isoprenoids are biosynthesized *via* 2C-methyl-D-erythritol-4-phosphate (MEP) pathway (Rohmer *et al.,* 1993; Eisenreich *et al.,* 1998; Ganjewala *et al.,* 2009). Apicomlexans also have the MEP pathway in apicoplasts. However, the enzymes of this pathway are being imported from the nucleus as NEAT proteins/enzymes (Kemp *et al.,* 2002). The genes encoding enzymes of the MEP pathway have been isolated and characterized from *Plasmodium* species (Kemp *et al.,* 2002; Rohdich *et al.,* 2001). The MEP pathway comprises seven enzymatic steps (Fig. 1). The first step is the formation of 1-deoxy-D-xylulose-5-phosphate (DOXP) by condensation of pyruvate and glyceraldehyde (Takahashi *et al.,* 1998) catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS). The second step DXP leads to formation of 2-C-methyl-erythritol-4-phosphate (MEP) from DOXP by an NADPH–dependent enzyme 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR; EC 1.1.1.267) (Argyrou and Blanchard 2004). These two initial steps are the rate limiting or committed steps of the MEP pathway hence enzymes DXS and DXR appears an attractive target for development of enzyme based novel anti-parasitic drugs. The DXR of *P. falciparum* and *P. vinckei* is reported to be inhibited by fosmidomycin thereby arresting the formation of isoprenoids (Henriksson *et al.,* 2007).

Currently, complete genome sequences of several apicomplexan parasites are available which helped to understand pathogenicity of apicomplexans at molecular level (Wasmuth *et al.,* 2009; Ajioka *et al.,* 1998; Howe 2001; Li *et al.,* 2003a; Cui *et al.,* 2005). The present study was undertaken to discern phylogenetic relationship among 11 parasite members of apicomplexan family by *in silico* analysis of the enzymes of the MEP pathway using *P. falciparum* 3D7 as a reference. In addition, structure based homology analysis of DXRs of apicomlexans were performed with respect to DXR of *M. tuberculosis* to gain detail insight in to amino acid residues of DXRs which interacts and binds with NADPH, $Mn^{2+}$ and fosmidomycin.
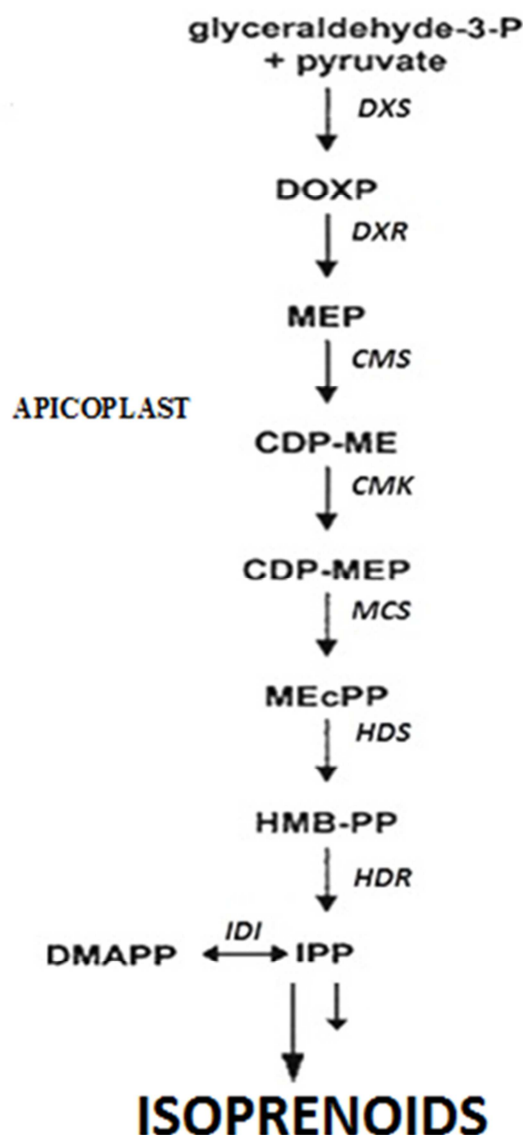


**Figure 1.** MEP pathway of isoprenoid biosynthesis. CDP-ME, 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2Cmethyl-D-erythritol 4-phosphate; DMAPP, dimethylallyl diphosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; GAP, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; HBMPP, 4-hydroxy- 3-methylbut-2-enyl diphosphate; IPP, isopentenyl diphosphate; ME-cPP, 2Cmethyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2C-methylerythritol 4-phosphate;Enzymes are: indicated in italic; *CMK*, 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol kinase; *CMS*, 4-diphosphocytidyl-2Cmethyl- D-erythritol 4-phosphate synthase; *DXR*, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *DXS*, 1-deoxy-D-xylulose 5-phosphate synthase; *HDS*, 4-hydroxy-3-methylbut2-en-yl-diphosphate synthase; *MCS*, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IDI, isopentenyl-diphosphate isomerise.

# 2. Materials and Methods

## 2.1. Retrieval of Enzymes Sequences

Database searches were performed independently for seven enzymes of the MEP pathway in selected Apicomlexans (Table 1). Completely annotated genomes are available online *P. falciparum* at PlasmoDB (release version 4.4;

http://plasmodb.org/), *T. gondii* at ToxoDB (release version 3.0; http://www.toxodb.org/), *C. parvum* (version 3.4), and *C. hominis* (version 3.4) at ApiDB, *T. parva* and *T. annulata* at GenBank, NCBI (Wasmuth *et al.,* 2009). BLAST algorithm was used for similarity searches in the above databases and sequence homology analysis was performed among seven

enzymes of the MEP pathway retrieved from selected apicomlexans using specialized BLAST (bl2seq) from http://www.ncbi.nlm.nih.gov/blast/Blast.cgi server. Amino acid sequences of enzymes of the MEP pathway were obtained from NCBI, a publicly available database.

**Table 1.** Host and environment specificity of apicomplexa parasites selected for the study.

| Species | Definitive host | Intermediate host | Preferred host environment | Disease |
|---|---|---|---|---|
| *Plasmodium falciparum* | Mosquito | Human | Erythrocyte | Malaria |
| *Plasmodium vivax* | Mosquito | Human | Erythrocyte | recurring (tertian) malaria |
| *Plasmodium berghei* | Mosquito | Rat | Erythrocyte | Malaria |
| *Plasmodium knowlesi* strain H | macaques (monkeys) | humans | Erythrocyte | 70% Malaria in South East Asia |
| *Plasmodium yoelii* | Mosquito | Rat | Erythrocyte | Malaria |
| *Babesia bovis* | Tick | cattle | Erythrocyte | Hemolytic anemia known as Babesiosis |
| *Theileria annulata* | Tick | Bovine | Leukocyte | tropical theileriosis (Mediterranean theileriosis) |
| *Theileria parva* | Tick | Bovine | Leukocyte | East Coast fever ( theileriosis) in cattle |
| *Eimeria tenella* | Poultry | None | Intestinal tract | Hemorrhagic cecal coccidiosis in young poultry |
| *Toxoplasma gondii* | Feline | Warm-blooded animals | Broad range | Toxoplasmosis |
| *Cryptosporidium hominis* | Human | None | Intestinal track | Gastroenteritis and diarrhea characteristic of cryptosporidosis |
| *Cryptosporidium parvum* | Mammal | None | Intestinal track | Gastroenteritis and diarrhea characteristic of cryptosporidosis |

**Table 2.** Distribution of genes encoding enzymes of the MEP pathway in selected Apicomplexan.

| Apicomlexans | DXS | DXR | MCT | CMK | MDS | HDS | HDR |
|---|---|---|---|---|---|---|---|
| *P. falciparum 3D7* | + | + | + | + | + | + | + |
| *Pl. vivax SaI-1* | + | + | + | + | + | + | + |
| *P. berghei ANKA* | + | + | + | + | + | + | + |
| *P. knowlesi strain H* | + | + | + | + | + | + | - |
| *T. gondii ME49* | + | + | - | + | + | + | + |
| *T. annulata strain Ankara* | + | + | + | + | + | + | + |
| *T. parva strain Muguga* | + | + | + | + | + | + | + |
| *B. bovis T2Bo* | + | + | + | + | + | + | + |
| *E. tenella* | + | + | - | + | + | + | + |
| *C. parvum Iowa II* | None of the enzymes of MEP pathway was found | | | | | | |
| *C. hominis* | None of the enzymes of MEP pathway was found | | | | | | |

(+) presence and (-) absence of a gene in the genome

## 2.2. *In silico* Analysis

Multiple sequence alignment was performed with the Clustal W program at EMBL (http://www.ebi.ac.uk/) and GeneDoc. Homology (identity percentage) in amino acid sequences of enzymes of the MEP pathway from selected Apicomplexans parasites were compared with that of *P. falciparum* 3D7 using BLAST algorithms at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). We randomly used an expectation (E-value) cut off of 10-3. The non-homologous entries were then subjected to BLASTP against the DEG for the identification of homologous sequences to essential genes at a cut off score of 10-10.

# 3. Results and Discussion

## 3.1. Distribution of the MEP Pathway Enzymes

*Plasmodium falciparum* 3D7 use MEP pathway located in apicoplast to form isoprenoids. The genes encoding enzymes of the MEP pathways have been cloned and characterized from *P. falciparum* 3D7. Apart from *P. falciparum* the MEP pathway is present in other members of the apicomlexa which suggests that these members also dependent on isoprenoids for their survival. We performed *In silico* analyses of the seven enzymes of the MEP pathway in 11

members of Apicomplexa with respect to *P. falciparum* 3D7 as a reference. The study revealed the presence of the MEP pathway and its enzymes in 9 of the 11 selected apicomplexans (Table 2). In *C. parvum* Iowa II and *C. hominis* the MEP pathway was absent thereby suggesting no roles of isoprenoids in their survival and/ or pathogenicity. In *E. tenella* an enzyme MCT was absent which may be due to its very low expression beyond the limit of detection. It is reported that the *Isp* encoded enzyme are expressed at relatively low levels in *P. falciparum*, which may affect their representation within the partial genome data sets. This pathway is important in the modification of transfer RNAs, suppressing premature stop codons and frame shifts in coding regions (Petrullo*et al.,* 1983).

## 3.2. Sequence Analysis and Phylogenetic Relationship

Amino acid sequences of enzymes of the MEP were analysed using BLAST and compared to homologous enzymes of *P. falciparum* 3D7. Three BLAST bit score cut offs were used for identity percentage analysis. The results revealed that only two enzymes of the MEP pathway DXR and HDR from apicomlexans displayed significantly higher 50-80% and 50-75% amino acid sequence similarity, respectively with homologous enzymes of *P. falciparum* 3D7 (Fig. 2). Four of the MEP pathway enzymes namely DXR, CMS, MCS and HDR of *P. berghi* showed significant (70%) amino acid sequence similarity whereas DXS, CMK and HDS only 55-70% with homologous enzymes of *P. falciparum* 3D7. The MEP pathway enzymes DXS, DXR, CMS and HDR from *T. annulata* demonstrated comparatively less 50-70% amino acid sequence similarity with those of *P. falciparum* 3D7 enzymes. In *B. bovis*, only two enzymes DXR and HDR showed 58% and 50% amino acid sequence similarity with

enzymes of the reference organisms. The DXRs of *P. vivax* and *T. knowlesi* had 75% sequence similarity with the reference organisms and MCS and HDS 60-65% while the CMK shared only 50% amino acid sequence similarity with the corresponding enzymes of *P. falciparum* 3D7. The MEP pathway enzymes in *T. parva* and *T. gondi* displayed low amino acid sequence similarity with reference organisms *P. falciparum* 3D7. Multiple sequence alignment analysis of enzymes of the MEP pathway performed with the ClustalW revealed close phylogenetic relationship within the *plasmodium* sp. of phylum apicomplexan and they could be easily distinguished from distantly related non *plasmodium* sp. (Fig. 3)

## 3.3. Structure Based Homology Analysis

Structure based homology analysis of DXR enzyme of the MEP pathway performed using DXR of *M. tuberculosis* Hominis revealed the amino acid residues present in the NADPH and $Mn^{2+}$ binding domains as well as those interacts with fosmidomycin. The essentiality of DXR in *M. tuberculosis* Hominis has been demonstrated recently (Goble *et al.,* 2007). *M. tuberculosis* DXR provides the most complete picture of interactions in the active site of enzyme. The DXR is a NADPH dependent enzyme catalyse rearrangement and reduction of 1-deoxy-D-xylulose 5-phosphate (DXP) to form 2-C-methyl-D-erythritol 4-phosphate (MEP). The reaction requires the presence of a divalent cations such as $Mg^{2+,}$ $Co^{2+}$or $Mn^{2+}$ (Takahashi *et al.,* 1998). Fosmidomycin is a potent inhibitor of DXR and hence it can block the MEP pathway (Henriksson *et al.,* 2007). It is reported to be active against the protozoan parasite *P. falciparum* in humans (Missinou *et al.,* 2002; Lell *et al.,* 2003) and *P. vinckei* in mice (Jomma *et al.,* 1999).
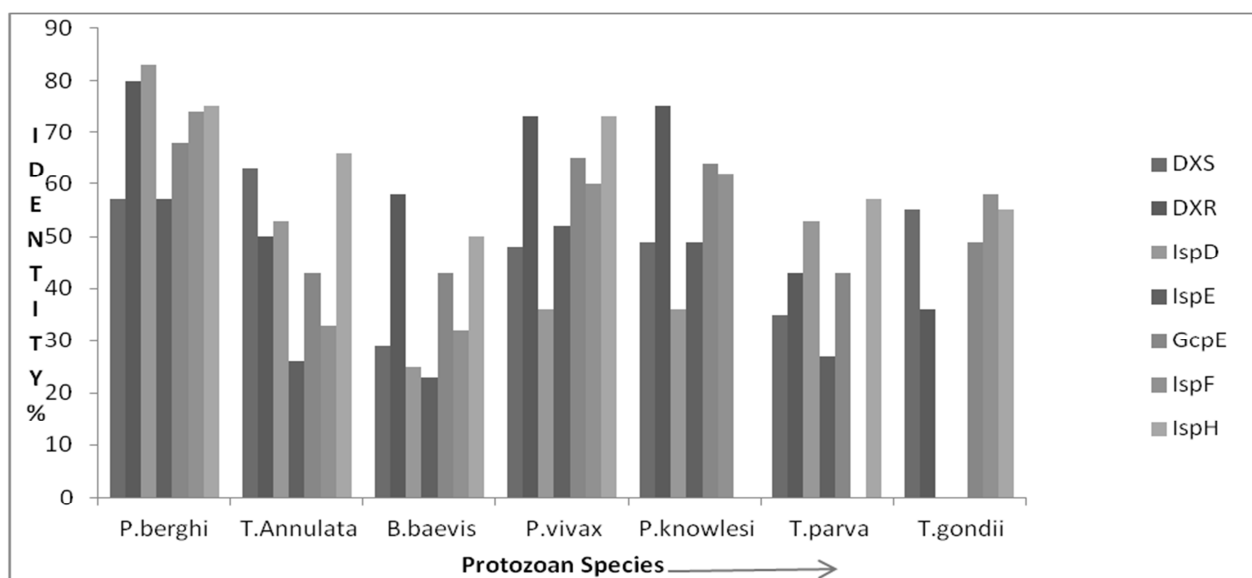


**Figure 2.** Reletive amino acid sequence similarity in enzymes of the MEP pathway with referene to *P. falciparum* 3D7**.**
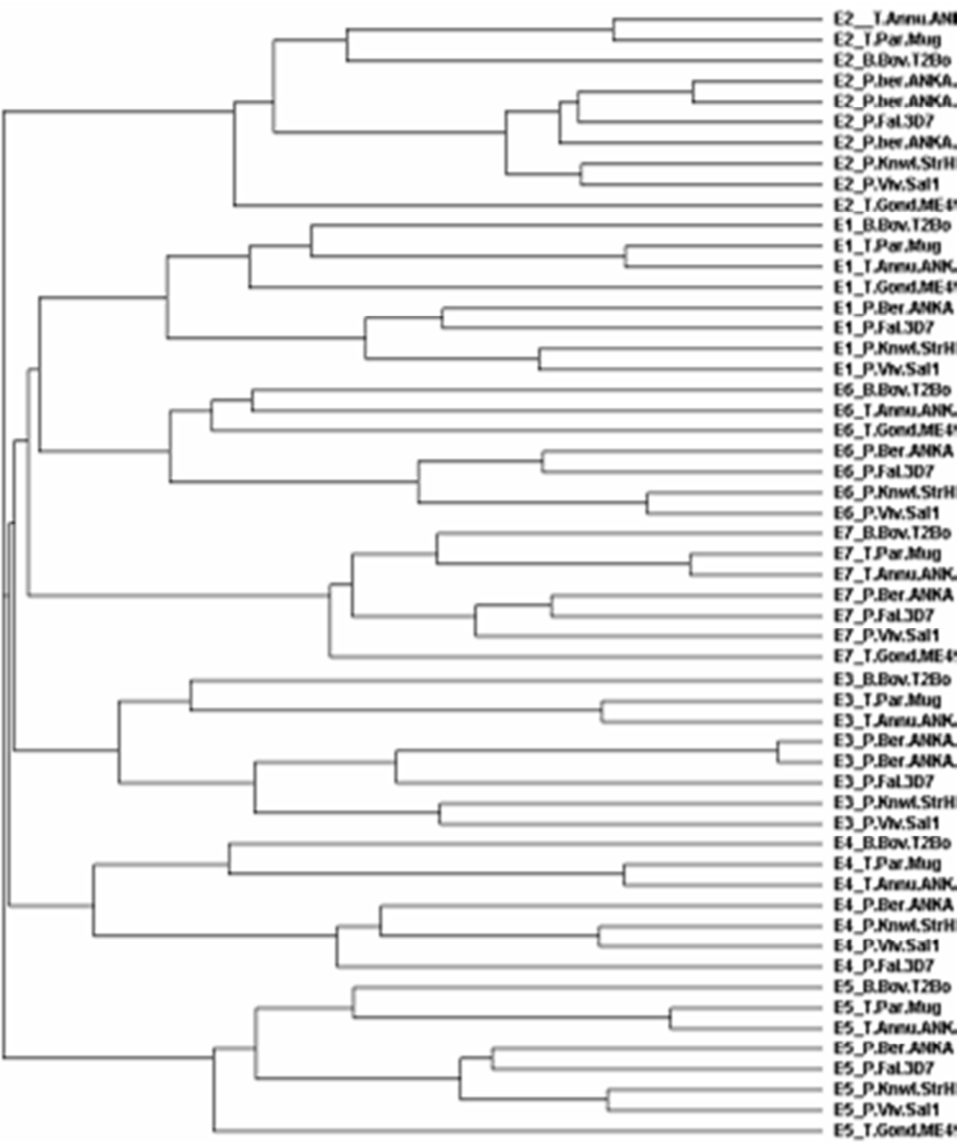
**Figure 3.** Cladogram depicting phylogenetic relationship among selected Apicomplexans. E1:DXS; E2: DXR; E3: CMS; E4:CMK; E5: HDS; E6: MCS; and E7: HDR.

**Table 3.** Conserved amino acid residues of NADPH and Mn$^{+2}$ binding domains in crystal structure of DXR of *M. tuberculosis* and DXRs of Apicomplexans.

| Organisms/Apicomplexan | NADPH Binding Residues | | | | | | | | Mn$^{+2}$ Binding Residues | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | T21 | G22 | S23 | I24 | G47 | G48 | A49 | E129 | D151 | E153 | E222 |
| *M. tuberculosis* | T21 | G22 | S23 | I24 | G47 | G48 | A49 | E129 | D151 | E153 | E222 |
| *P. falciparum* 3D7 | T | G | S | I | N | K | S | E | D | *D* | *D* |
| *P. vivax* SaI-1 | T | G | S | I | N | K | S | E | D | E | E |
| P. berghei str. ANKA | T | G | S | I | N | - | K | E | D | E | E |
| *P. knowlesi* strain H | T | G | S | I | N | K | S | E | D | E | E |
| *T. gondii* ME49 | T | G | S | I | G | G | S | E | D | E | E |
| *T. annulata* strain Ankara | T | G | S | I | K | S | N | E | D | E | E |
| *T. parva* strain Muguga | T | G | S | I | N | S | N | E | D | E | E |
| *B. bovis* T2Bo | T | | | | G | S | I | N | D | E | E |
| E. tenella | T | | | | G | S | I | G | D | E | E |

Earlier reports have revealed that in *M. tuberculosis* DXR, NADPH is bound in the N-terminal domain and comes in close contact with fosmidomycin and the Mn$^{2+}$ ions that are positioned underneath the active site flap. Amino acid residues Thr (T) 21, Gly (G) 22, Ser (S) 23, Ile (I) 24, Gly (G) 47, Gly (G) 48, Ala (A) 49 and Glu (E) 129 of the N-terminal domain of *M. tuberculosis* DXR interact with NADPH. Amino acid residues Gly (G) 206 and Asn (N) 209 from the active site flap in the catalytic domain also interact with NADPH (Levitt and Perutz 1988). Structure based homology analysis performed here also revealed the presence of similar amino acid residues in the DXRs of apicomplexans which

interacts with NADPH (Table 3A). Further the amino acid residues, Asp (D)151, Glu (E)153 and Glu (E)222 of DXR in *M. tuberculosis* were also present in the DXRs of apicomplexans. These amino acid residues interact with $Mn^{2+}$ ion and fosmidomycin (Table 3). Fosmidomycin also interacts with amino acid residues, Ser (S)152, Ser (S) 177, His (H) 200, Asn (N) 218 and Lys (K)219. These amino acid residues found in the catalytic domain DXR of *M. tuberculosis* also elucidated in the DXRs of all apicomplexans studied (Table 4). Amino acids interacting with NADPH, $Mn^{+2}$ and fosmidomycin deciphered from the crystal structure of DXR of *M. tuberculosis* Hominis are depicted in Fig. 4.
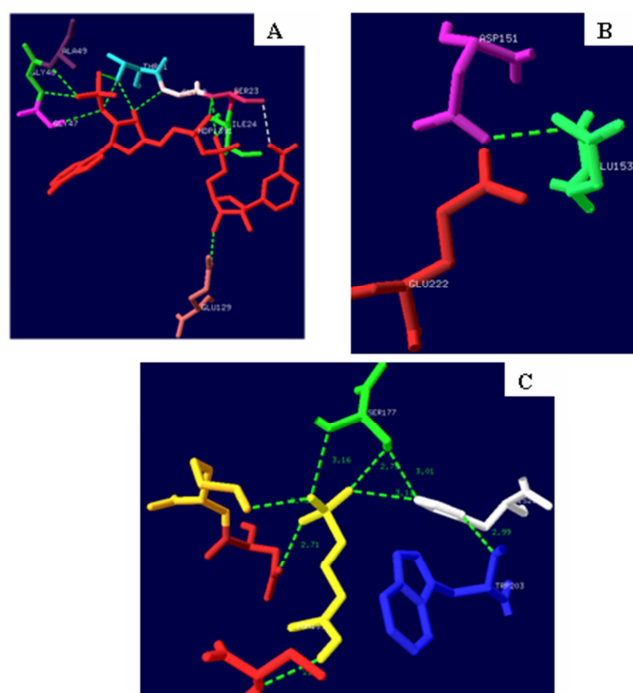


**Figure 4.** Amino acids interacting with [A] NADPH, [B] $Mn^{+2}$ and [C] fosmidomycin deciphered from the crystal structure of DXR of *M. tuberculosis* Hominis.

**Table 4.** Conserved amino acid residues in crystal structure of DXR of *M. tuberculosis* and DXRs of Apicomplexans interacting with fosmidomycin.

| Organisms | Fosmidomycin Binding Pocket Residues in DXR | | | | | |
|---|---|---|---|---|---|---|
| *2JCV (M. Tuberculosis)* | *S152* | *S177* | *H200* | *W203* | *N218* | *K219* |
| *P. falciparum* 3D7 | S | S | H | W | N | K |
| *P. vivax* SaI-1 | S | S | H | W | N | K |
| P. berghei ANKA | S | S | H | W | N | K |
| *P. knowlesi* H | S | S | H | W | N | K |
| *T. gondii* ME49 | S | S | H | W | N | K |
| *T. annulata* Ankara | S | S | H | W | N | K |
| *T. parva* Muguga | S | S | H | W | N | K |
| *B. bovis* T2Bo | S | S | H | W | N | K |
| E. tenella | S | S | H | W | N | K |

# 4. Conclusion

Apicomlexans parasites such as *P. falciparum* use MEP pathway to produce isoprenoids which are crucial for their survival and pathogenesis. Therefore the MEP pathway enzymes can be attractive targets for development of newer anti-parasite drugs. The MEP pathway enzymes may also serve as biochemical marker to elucidate phylogenetic relationship among species. In the present study, MEP pathway enzymes have been used as biochemical tools to discern phylogenetic relationship among the members of the apicomplexa family. A key enzyme DXR of the MEP pathway was subjected to structure based homology analyses with respect to DXR of *M. tuberculosis*. The results of these analyses have provided detail insight into amino acid composition of the NADPH, $Mn^{2+}$ and fosmidomycin binding domains of the DXR. Most the amino acid residues were highly conserved. Although the outcome of the present study is preliminary but they may be useful for developing single anti-parasitic drug against broad spectrum of apicomlexan parasites rely on the MEP pathway for their survival.

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