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Homology modeling and docking studies of novel drug candidate atovaquone on *Babesia bovis* dihydroorotate dehydrogenase (BbDHODH) receptor

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Abstract
Bovine babesiosis is one of most economically important tick borne disease across worldwide which is caused by an intra-erythrocytic apicomplexan parasite i.e. *babesia bovis.* Although different common chemotherapeutic drugs including imidazole, diproprionate and diminazene aceturate have been tried against this disease, but all these compounds do not produce their effect adequately and also there is induction drug resistance. In this work, we studied homology modeling and docking studies of a dihydroorotate dehydrogenase from *bovis babesia,* dimensional structure of the enzyme was built using MODELLER version 9.21 and docking study was performed under AutoDock platform. The protein model with lowest discrete potential energy (DOPE) -41275.21 Kcal/mol, was subjected to energy minimization and showed a good quality model. It has been resulted that the best docked Atovaquone confirmation with lowest binding energy (-9.73 kcal/mol) and lowest inhibition constant 73.57 µM had a number of amino acid residues such as Asp 264, Arg 267, Leu 270, Glu 293, Iso 324 and Gly 327 in close contact. This study was concluded that BbDHODH receptor would be a potential drug target for the novel drug atovaquone against *B. bovis* infection in dairy cattle.

Keywords: *Babesia bovis,* dihydroorotate dehydrogenase, homology modeling, atovaquone, molecular docking

Introduction
Now days, among agriculture sector, the livestock contributes a greater proportion of gross domestic product (GDP) to farmers as well as to our nation [1]. With an annual production of about 130 million tons, India is the highest milk producer in the world contributing 15% of the total world milk production [2], but the occurrence of different infectious as well as non infectious disease causes the major economic loss in dairy herds, which affects 10-30% loss of annual milk production in 3-6% of herd animals of developed countries [3].

Among protozoan diseases, bovine babesiosis is one important tick borne disease which is caused by *babesia bovis,* an intra-erythrocytic apicomplexan parasite of *Babesiidae* family [4]. This disease is transmitted by feeding of larval stages of one-host Rhipicephalus spp tick and more widely distributed in Africa, Asia, Australia, and Central as well as South America. The infected dairy cows are mainly characterized by following clinical manifestations such as high fever, ataxia, in coordination, anorexia, production of dark red or brown-colored urine, signs of general circulatory shock, sometimes nervous signs associated with sequestration of infected erythrocytes in cerebral capillaries with appearance of anemia and haemoglobinuria in later course of the disease [5] which causes huge annual economic losses in India i.e. estimated to be about 57.2 million US dollars due to babesiosis [6]. So keeping this huge economic impact, the advancement of novel therapeutics has been stood as a great hope for control of this disease. Although, use of various chemotherapeutics drugs including imidazole diproprionate and diminazene aceturate has been reported against bovine babesiosis, but these compounds causes adverse side effects and induce drug resistance [7]. So now it prompts for further exploration of new drug targets and development of new chemotherapeutic compounds as strategy for treatment of this disease.

Babesia bovis dihydroorotate dehydrogenase (BbDHODH), one important enzyme in *de novo* pyrimidine biosynthesis pathway for oxidation of dihydroorotate to orotate can be targeted for

novel chemotherapeutics [8]. Reduction of uridine 5′ monophosphate (UMP), blocks the pyrimidine synthesis, an essential for RNA, DNA, glycoproteins and phospholipids biosynthesis in the protozoa that can be achieved by inhibition of DHODH [9]. Previously, DHODHs have also been identified as novel drug targets for malaria, toxoplasmosis and leishmaniasis [10]. As per our knowledge, there have no previous studies about structural and physiochemical characterization of BbDHODH. So this enzyme can be targeted for development of different novel inhibitors for treatment of bovine babesiosis.

Further, different therapeutics such as diminazene diaceturate, imidocarb, amicarbalide, quinuronium, acridine derivatives as well as imidocarb diproprionate have been practiced since long time at field level but they act on protozoa without any specific target for which there is always a risk of residues in milk and meat and induction of drug resistance in animal. So lack of vaccine as well as target oriented drugs prompts intensive searching for development of new chemotherapeutic compounds against this protozoon. To fullfill this research gap, Atovaquone, a hydroxynaphthoquinone compound which showed a tremendous lethal effect on plasmodium species by inhibiting the electron transport chain [11] can be explored as a new inhibitor against BbDHODH enzyme.

Molecular docking, one advanced bioinformatics tool is used to predict the binding-conformation of small molecule ligands to the appropriate target binding site and the binding behavior has been exploited for rational design of new drugs against different diseases [12]. So the interaction study between BbDHODH and atovaquone would provide a new hope for treatment of this protozoon.

In this study, we constructed a three-dimensional (3D) model for the *Babesia bovis* dihydroorotate dehydrogenase (BbDHODH) to understand its binding mechanism for development of novel drug Atovaquone. We expect that the results of this study would provide a better platform for the researchers as well as farmers to develop an accurate therapeutic regimen against bovine babesiosis.

Materials and method

The work was conducted in the Department of Veterinary Biochemistry, College of Veterinary Science and Animal Husbandry and Department of Bioinformatics, Centre for Post Graduate Studies, OUAT, Bhubaneswar, Odisha from 1st December 2019 to 31st May 2020.

Dihydroorotate dehydrogenase (DHODH) protein sequence of *Babesia bovis*

Babesia bovis dihydroorotate dehydrogenase (BbDHODH) protein sequence was retrived from UniPort KB database which is a freely accessible resource of protein sequence with their complete functional annotations. The accession no for this selected protein was S6B413

Physicochemical characterization

The physiochemical parameters such as molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half life, instability index, aliphatic index, and Grand Average of Hydropathicity (GRAVY) of the BbDHODH protein were estimated under ProtParam characterization tools on the Expert Protein Analysis System (Expasy) server. The solubility and sub cellular localization of this protein was predicted by SOSUI and WoLF PSORT software respectively and Hydropathy-plot analysis was performed using the ProtScale server.

Secondary structure predictions of BbDHODH protein

The secondary structure (alpha helix, beta bridge, beta turn, extended strand and random coil) of BbDHODH protein were estimated by using GORIV secondary structure prediction method.

Template selection and Tertiary structure Prediction

To build the tertiary structure of BbDHODH protein, BLAST of the target protein was performed at the NCBI by using BLASTp algorithm [13] which showed 42.48% and 42.74% similarities to *Eimeria tenella* dihydrorate dehydrogenase (PDB ID: 6AJ5) and human dihydrorate dehydrogenase (PDB ID: 4OQV). However, the *Eimeria tenella* dihydrorate dehydrogenase (PDB ID: 6AJ5) was chosen as a template (based on its high resolution) to predict the three dimensional structure of BbDHODH. The modeling of the protein was performed by homology modeling programs such as Modeller version 9.21 with python script [14].

Energy minimization and quality verification

The protein models generated by the MODELLER was ranked and scored using discrete optimized protein energy (DOPE) score. The best model out of 10 models with the lowest DOPE score was selected. Further, the structural refinement and energy minimization was done through ModRefiner algorithm of Zhang Lab web server (http://zhanglab.ccmb.med.umich.edu/ ModRefiner/) and the structural superimposition of refined model with the template (6AJ5) was performed using Pymol version 2.3 graphical user interface (GUI). The stereochemical quality and accuracy of the refined protein model was verified through RAMPAGE, ERRAT, Verify3D server and validation of the model quality was performed by ProQ and ProSA server for further docking studies.

Ligand selection

The chemical structure of atovaquone was retrieved from pubchem database of NCBI server with accession number DB01117. The correction of structural geometry and energy minimization were carried out using pymol2.0 graphics.

Docking Studies

Molecular docking between the best protein model of BbDHODH with atovaquone was performed using AutoDock-4.2 algorithm by considering the protein structure as rigid whereas ligand were kept as flexible. MGL 1.5.6 tools software was used for preparation of both macromolecule and the ligand. The macromolecule was prepared by removing of water molecules and adding the hydrogen atoms and the ligand was prepared with giving torsion to make it flexible. Blind docking of the atovaquone onto the modelled structure was performed using a pre-set simulation grid box size of 120 × 120 × 120 Å along the X, Y and Z axes and centred at 39.946, 40.191, 45.879 whereas the targeted docking grid box size was set to 70 × 70 × 60 Å dimension and centered at 43.946, 40.191, 33.879 of X, Y and Z coordinate, respectively. The docking simulations were performed for 100 runs using Lamarckian Genetic Algorithm (LGA).The potential energy arising from the interaction between each atoms present in the flexible molecules being docked and rigid macro molecules was stored in pre calculated grid box [15]. The docking of ligand was done around the identified

conserved region within the functional domain of the selected protein molecule [16] and the favorable interaction was selected on the basis of lowest binding energy (BE) calculated by Auto Dock which indicates for most favorable binding conformation between the macromolecule and ligand. The visualization and analysis of interaction between docked complexes was made using chimera 1.3 graphics.

Result and Discussion

Babesia bovis dihydroorotate dehydrogenase (BbDHODH) is a vital mitochondrial enzyme of pyrimidine biosynthesis which is targeted for various inhibitors to treat autoimmune diseases such as rheumatoid arthritis [17]. So this enzyme can be targeted for novel drugs for treatment of bovine babesiosis. Although the drugs like diminazene diaceturate, imidocarb, amicarbalide, quinuronium, acridine derivatives and imidocarb diproprionate have been tried against this protozoa, but all these induce drug resistance. So it prompts for search of novel chemotherapeutic compound i.e. atovaquone which has tremendous antimicrobial and antipneumocystis activity [18]. So detail information regarding physiochemical and structural characteristics of BbDHODH and its binding interaction study with the atovaquone is needed for development of suitable therapeutic regimen against babesiosis.

Primary structural characterization BBDHODH protein sequence

The retrieved BBDHODH protein sequence with Uniport accession number S6B413 has no experimental 3D structure although the biological and molecular process of it has well defined. This protein was found unreviewed in Uniport database which is a universally acceptable database to provide the scientific community with a comprehensive, high-quality and freely accessible resource of protein sequence and functional information [19]. It is shown that the retrieved protein sequence has 424 number of amino acid with the conserved domain from 72 – 386, which is provided detail in Table 1.

Table 1: The protein sequence retrieved from the Uni Prot.

Gene name	Protein name	Uni. Id	Sequence length	Domain	Organism Name	Compositional bias
BBOV_II007190	Dihydroorotate dehydrogenase	S6B413	424	72 – 386	*Babesia bovis*	410 – 424

Physiochemical characteristics

Different physiochemical parameters were computed on using ExPasy ProtParam tool and was shown in Table 2. The result suggested that the average molecular weight of BBDHODH protein is 45942.52 Da. The theoretical PI was found 9.35 which can be inferred
that it is a basic protein. It may be due large percentage of basic amino acids such as arginine and lysine (53%) in this protein [20]. The instability index of this protein was found 32.97 which is less than 40, indicating that the protein is very stable in nature and it may be due to occurance of less number of dipeptides in the composition [21]. The aliphatic index that defines the thermal stability of the protein was found 99.58 which is greater than 80, suggesting that this protein is more stable for a wide range of temperature. It might be due to the relative volume of this protein has occupied by large number of aliphatic side chain amino acids such as alanine, valine, isoleucine, and leucine [22]. However the Grand Average hydropathy (GRAVY) values was found --0.060 which is less than zero that implies that this protein is more hydrophilic in nature [23] which suggests large number of polar amino acid on the surface of this protein. This study also showed that the protein is intra mitochondria soluble in nature with hydrophobicity value of -0.060377 indicating that this protein makes cytochrome complex of electron transport chain. The ProtScale server analysis showed that there is no major peak in the hydropathy plot which is shown in Figure 1.

Table 2: Physiochemical characteristics of BbDHODH protein

Parameters	Value
Number of amino acids	424
Molecular weight	45942.52
Theoretical pI	9.35
Total number of negatively charged residues (Asp + Glu)	40
Total number of positively charged residues (Arg + Lys	53
Instability index	32.97
Aliphatic index	99.58
Grand average of hydropathicity (GRAVY)	-0.060
Solubility	Soluble
Hydrobhobicity	-0.060377
Sub cellular localization	Intramitochondrial

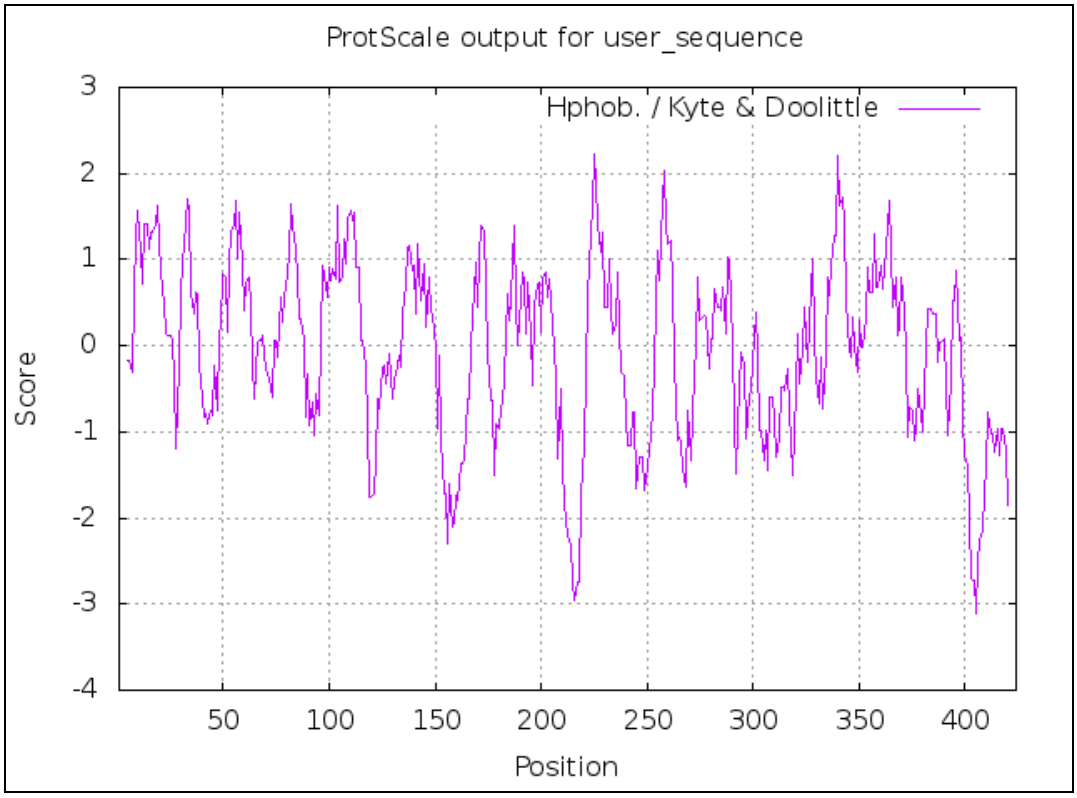

Fig 1: Showing the Hydropathy plot of protein under ProtoScale platform

Prediction and characterization of secondary structures of BBDHODH

This study revealed that three principal secondary structures are present in BBDHODH protein shown in Table No 3. In our designed secondary structure of the protein, it has been shown there is more abundance of random coil (53.30%), followed by alpha helix (27.12% and then extended strand (19.58%) which inferred that the protein is very flexible and stable in nature. More abundance of random coils indicates more flexibility in conformation which revealed that this protein is an important enzyme in pyrimidine synthesis [24]. It is also found that this protein has three domains between 7 2 – 386 amino acid residues i.e. an amino (N)-terminal domain of unknown function, a central catalytic domain, and a carboxy (C)-terminal DNA-binding domain.

Table 3: Secondary structure of BbDHODH protein

Types	Alpha helix	Extended strand	Random coil
Percentage	27.12%	19.58%)	53.30%
Length	115	83	226

Building of Model and refinement of proposed drug target

Our study revealed that the *Eimeria tenella* dihydrorate dehydrogenase (PDB ID: 6AJ5**)** and human dihydrorate dehydrogenase (PDB ID**:** 4OQV)) have been solved to a resolution of 3.5 Å and 1.23 Å, respectively. However, the *Eimeria tenella* dihydrorate dehydrogenase was chosen as the template based on its higher resolution. Further the alignment of the amino acid sequences of the DHODH protein from *B.bovis* (UniProtKB ID: S6B413) and *E. tennela* (PDB ID: 6AJ5A) resulted identity and similarity 42% and 70% respectively and two conserved amino acid residues have been identified shown in Figure 2A. The homology modeling resulted ten protein models with different discrete optimized protein energy (DOPE) shown in Table 4. The model structure such as target.B99990002.pdb with the lowest DOPE score (-41275.21484) was assessed and subsequently used for further analyses as protein attains the lowest free energy during the native state [25]. Further, the structural superimposition of BbDHODH model with template 6AJ5 A chain both before and after energy minimization (Figure 2B) revealed a Root Mean Square Deviation (RMSD) score of 0.112 Å indicating that the structures were closely related [26].

Table 4: Showing Ten BbDHODH models created by Homolog modeling (Modeller 9.21**)**

Sl. No.	Models	molpdf	DOPE score	GA341 score
1.	target.B99990001.pdb	3006.58813	-40389.25000	1.00000
2.	target.B99990002.pdb	3052.81763	-41275.21484	1.00000
3.	target.B99990003.pdb	3046.19580	-40735.70313	1.00000
4.	target.B99990004.pdb	3106.62988	-40874.49219	1.00000
5.	target.B99990005.pdb	3118.87329	-40921.98438	1.00000
6.	target.B99990006.pdb	3014.70874	-40868.46094	1.00000
7.	target.B99990007.pdb	3129.51782	-41017.08984	1.00000
8.	target.B99990008.pdb	3060.83960	-40796.19531	1.00000
9.	target.B99990009.pdb	3127.06982	-40473.57422	1.00000
10.	target.B99990010.pdb	3101.32593	-40804.48438	1.00000

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B.bovis      28   SVLYNVLMPLFRNYLDPEVAHKLSITALKLGIAPVDYSVDPPVIQSRLKDVVFFNPIGMA   87
                  V+Y  L+P   N+ DPE+AH + +     G  P D   D P +   +K + F  P+G+A
E.tennela    28   EVVYG-LLP--ENFPDPELAHDMVMWLAAKGYLPYDLERDDPELSVNIKGLTFHTPVGLA   84
B.bovis      88   AGYDKQVEVPLQILRMGFGFVEVGTVLPLPQEGNPKPVMFRLHDSKALINCCGFNSVGLE   147
                  AG+DK  E PL   +MGFGFVEVGT+ P PQ GNPKP +FRL    A+IN CGFNS GL+
E.tennela    85   AGFDKNAEAPLNFCKMGFGFVEVGTITPKPQLGNPKPRIFRLAKDHAIINRCGFNSAGLD   144
B.bovis      148  VAKARLKRVRKKQASDPLTKDFMIGVSVGKNRTGDILADTVNA-------VKGVAPYADY   200
                  V + RL++V + +  D L +  ++GV++GKN+      DTVNA       VK V  +ADY
E.tennela    145  VVEPRLEKVSRDRWHDRLERHCVLGVNIGKNK------DTVNAEDDIREGVKRVGRFADY   198
B.bovis      201  IAINVSSPNTPNLRDNQKREPLIAVISAARSALEIVNTEIK-------------------   241
                  + IN+SSPNT  LR  Q+R+ L ++I+AA+S LE +    +
E.tennela    199  LVINLSSPNTKGLRTLQQRDHLRSIITAAQSELEKLEERSRAQRERDGHSNPSEEEEAAS   258
B.bovis      242  ----SKGAE--FNNTTKKVPLLLIKISPDVSRQELEDIADISLTHHVDGIIATNTTITRD   295
                     ++ AE  F   T K PLL +KI+PD++ +E  DIAD++L   +DG+I TNTTI R
E.tennela    259  SDSVTRKAEQFFPTQTGKRPLLFVKIAPDLTDEEKRDIADVALETGLDGLIVTNTTIQRP   318
B.bovis      296  NVVPSDLKTAGNPKGGLSGRPLKTMSKKIVYGLYELTEGKIPIIACGGISTAQDALEMIE   355
                    + S+ K   +  GGLSGRPLK MS K V  +Y++T G++ IIA GGI T  DA + I
E.tennela    319  ESLRSESK---HETGGLSGRPLKAMSTKCVSDMYKMTNGQVAIIASGGIETGLDAYKRIR   375
B.bovis      356  AGASLCQIYTALVYEGPGIPSRMKNGLADLLMKKGYLNVSEAIGAEH------RRRKPT   408
                  AGAS  ++YT+++Y GP +  R+K+ L ++L + G  NV +AIG +H      R RKPT
E.tennela    376  AGASAVEVYTSMIYRGPIVARRVKDELLNILNQAGIYNVQDAIGLDHRPPKKKRVRKPT   434
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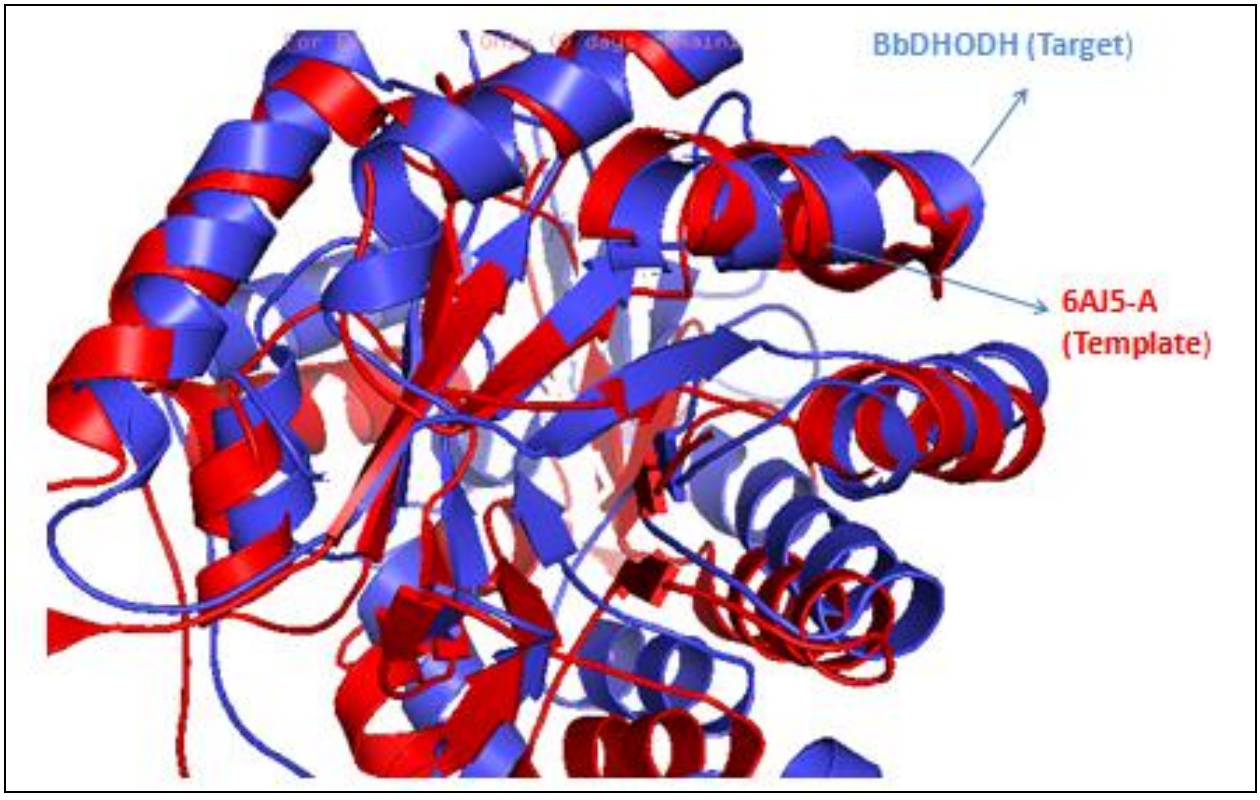
(A)



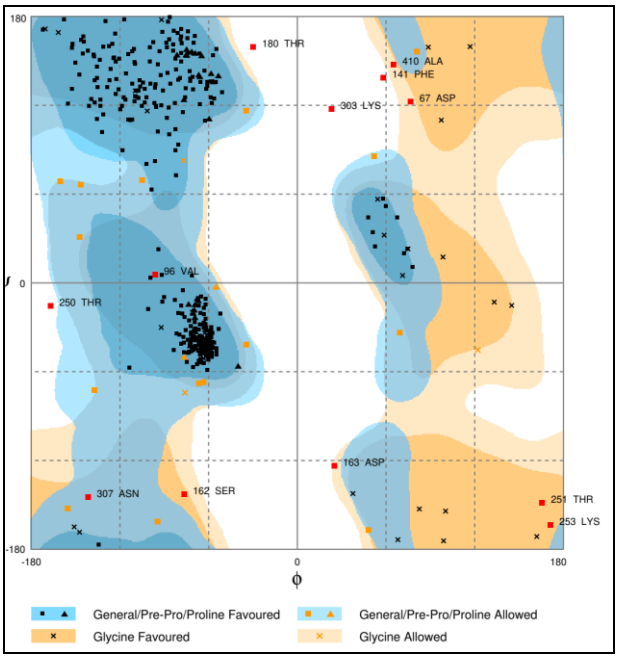
B)

Fig 2: The conserved amino acid residues DHODH between *B. bovis* and *E. tennela* (PDB ID: 6AJ5, Chain A) are identified and deciphered (A). The structural super imposition between identified drug target DHODH (blue color) of *B. bovis* and template (PDB ID: 6AJ5, Chain A) (red color) of *E. tennela* represented (B).

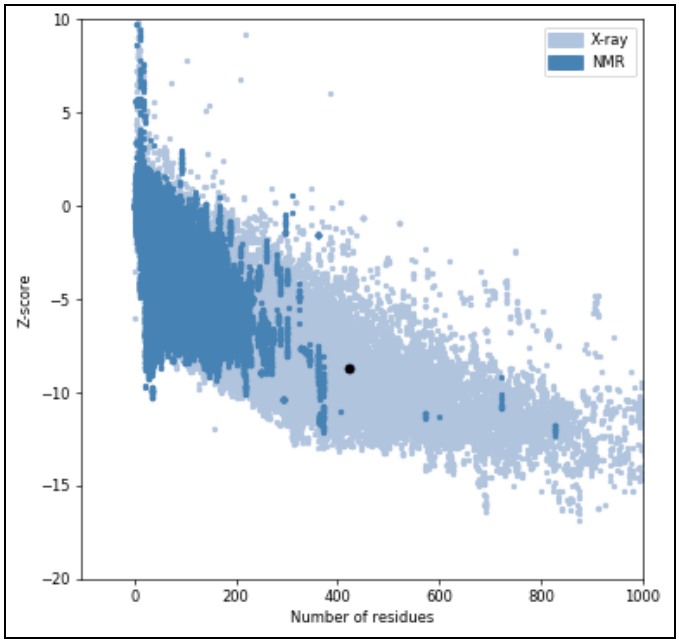
Quality verification of the predicted structure

The quality of the best BbDHODH model was verified through different Insilco platform before going to further studies. The RAMPAGE analysis displays the Ramachandran plot which explains the stereochemistry of the main chain torsion angles such as Phi, Psi angle pairs, describing the overall stability of the protein model [27] shown in Figure 3A indicate that 92.4%, 4.7%, 2.8% of residues fall under most favored, allowed and outlier region respectively, confirming that the predicted model is of good quality [28]. Further the ProSA-web analysis revealed a Z score of -8.74 for BbDHODH model (Figure 3B) indicating that the protein is remained well within the range of native conformation of experimental structures [29]. The ProQ analysis resulted Levitt-Gerstein (LG) and Max sub score of 4.690 and 0.271, indicating the predicted BbDHODH model is of extremely good model as the LGscore>4 is considered as extremely good for protein model [30]. Further the ERRAT programme

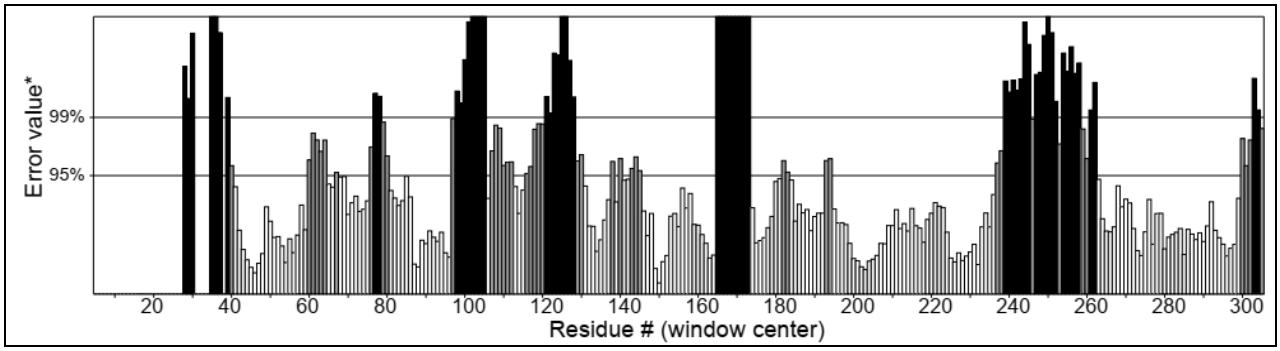
showed the overall quality value for DHODH model of *B. bovis* was 68.7339% (Figure 3C), which may be inferred that, this model is more reliable for the further studies [31]. In addition, Verify3D plot of the modelled protein (Figure 3D) showed PASS and the 3D environment profile resulted 83.47% of the residues have averaged 3D-1D score⩾0.2, which suggests the modelled protein is structurally valid one [32].



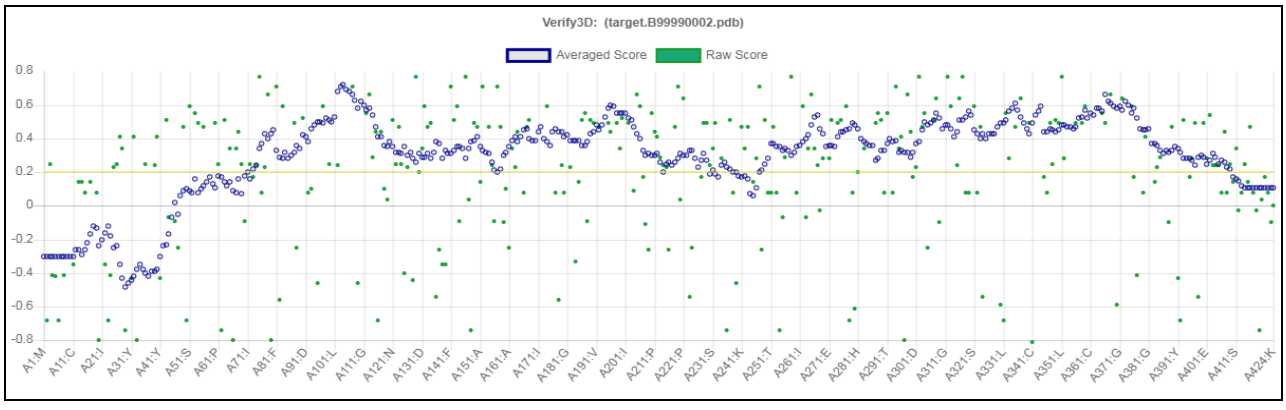
A)



B)



C)



D)

Fig 3: Ramahandran plot by RAMPAGE presented in (A), z plot which describes the overall quality of model evaluated and deciphered (B), Quality verification plot of the energy minimized model of the BbDHODH performed using ERRAT shown in (C), Verify 3D plot (D)

Selection of Ligand

Previous studies suggested that atovaquone has tremendous antimilarial and antipneumocystis activity; hence the chemical structure of it was downloaded from Pub Chem database of NCBI web server (Table 5), and structure was shown in Figure 4. This therapeutic property might be due to its blockade effect on electron transport that results in inhibition of various metabolic enzymes linked to nucleic acid and ATP synthesis [33].

Table 5: Pharmacological information about atovaquone

Drug Name	Group (Approved)	Molecular Weight	Accession Number	CAS Number	Molecular Formula
Atovaquone	Small molecule	366.8	DB01117	95233-18-4	$C_{22}H_{19}ClO_3$

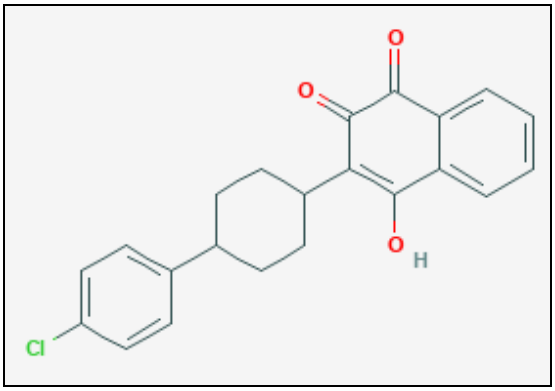


Fig 4: Chemical structure of drug Atovaquone

Molecular docking studies

Molecular docking experiment between BbDHODH and atovaquone resulted eight different energetically favorable binding conformations within the active pocket of proposed drug target. Among these, the conformation with lowest binding energy (-9.73 kcal/mol) was selected for further analysis as lowest binding energy is generally preferred for favorable binding mode during the docking of small compounds with target protein molecule which may be due to the stability of docked structure [34]. The binding inhibition constant was found 73.57 µM which was the lowest among all conformations suggesting as strong inhibitor of BbDHODH protein. It was found that there was existence of various interactions including hydrogen, hydrophobic and electrostatic between protein and ligand docked complex Figure 5A, which may be due to presence of hydrophilic, hydrophobic as well some positively and negatively charged amino acid residues within the binding pocket [35] shown in Table 6. Our study revealed that the hydrogen bond was formed with the amino acid residue Asp 264 where as strong hydrophobic interactions were existed due to presence of aminoacid Leu 270, Iso 324 and Gly 327 within 4 Å distance from the ligand shown in Table 8 and Figure 5B and more over, presence of residue Arg 267, Asp 264 and Glu 293 contributes the electrostatic interaction which may conferred that atovaquone can be used a strong inhibitor against DHODH of *B. bovis*. The crystal structure of the binding interaction shown in Figure 6

Table 6: Polar contact information obtained from docking calculation

Ligand	Binding energy (kcal/mol)	Inhibition constant	Residues	Atoms	Distance (Å)
Atovaquone	-9.73	73.57mM	ASP 264	O8--H	1.887
			ARG 267	O1--NH1	2.336
			LEU 270	H43--O	1.640
			GLU 293	H41--OE1	1.743
			ISO324	H31--O	1.987
			GLY 327	H51--O	1.654

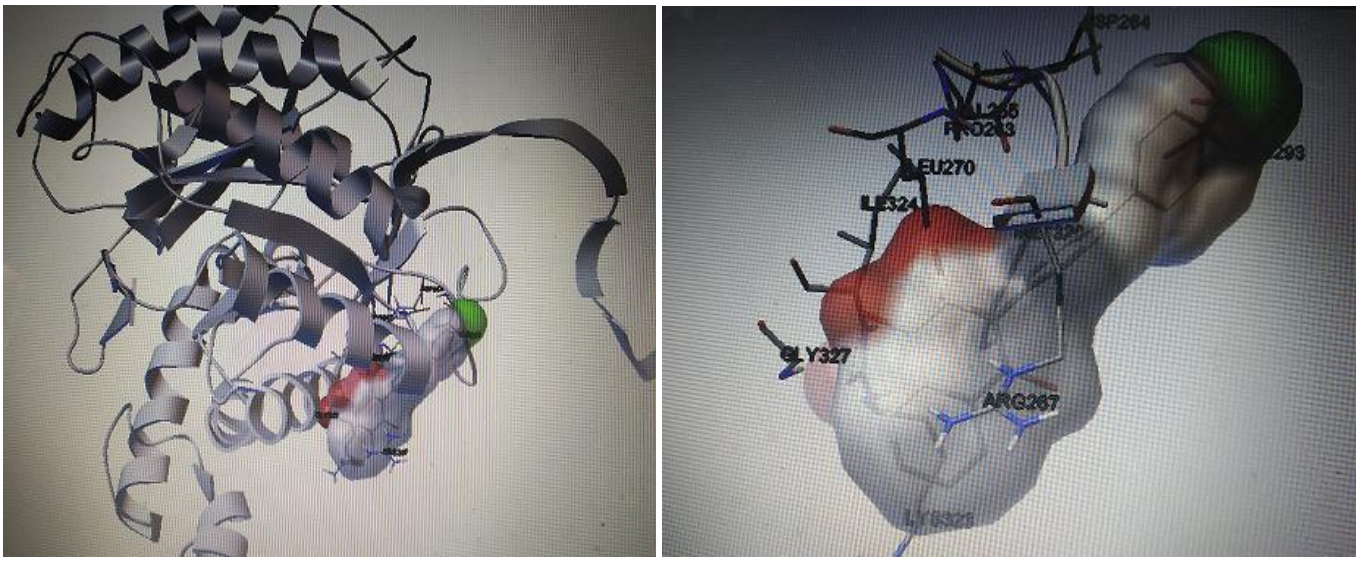


Fig 5: Interaction profile of Atovaquone with proposed drug target DHODH protein of *B. bovis* presented in (A); Amino acid residues participated in hydrogen bonding and interacting within a distance of 4Å in the active pocket of BbDHODH protein with Atovaquone was deciphered in (B)

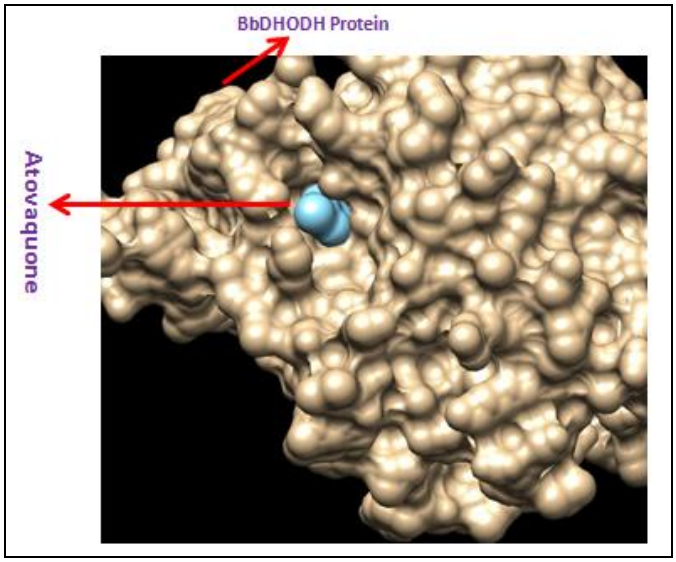


Fig 6: Figure showing the crystal structure of binding interaction between BbDHODH and Atovaquone

Conclusion

This study was concluded BbDHODH, an important enzyme of pyrimidine biosynthesis can be considered as potential drug target specific antibabesial drug to combat this babesiosis menace. The primary and secondary structure analysis revealed that this protein is very stable, mitochondrial, and hydrophilic. Moreover, the lowest binding energy (-9.73 kcal/mol) and least inhibition constant (73.57 µM) of Atovaquone with the target during docking study explains its strong inhibitory effect on *babesiosis*. This presumption was also confirmed through the observation of strong hydrophobic, electrostatic and hydrogen bonding interactions between protein-ligand complex. Hence, atovaquone would be suggested as most potent therapeutic compound against the proposed drug target (DHODH) of *B. bovis.* So this study would provide a good platform towards designing a novel drug against *B. bovis* and which would be further validated through *in vitro* experiments

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