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Structural and functional studies of a 50 kDa antigenic protein from *Salmonella enterica* serovar Typhi

Yee Siew Choong[a,*], Theam Soon Lim[a], Ai Lan Chew[a], Ismail Aziah[b], Asma Ismail[b]

[a] Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800 Minden, Malaysia
[b] Institute for Research in Molecular Medicine, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

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ABSTRACT

The high typhoid incidence rate in developing and under-developed countries emphasizes the need for a rapid, affordable and accessible diagnostic test for effective therapy and disease management. TYPHIDOT®, a rapid dot enzyme immunoassay test for typhoid, was developed from the discovery of a ~50 kDa protein specific for *Salmonella enterica* serovar Typhi. However, the structure of this antigen remains unknown till today. Studies on the structure of this antigen are important to elucidate its function, which will in turn increase the efficiency of the development and improvement of the typhoid detection test. This paper described the predictive structure and function of the antigenically specific protein. The homology modeling approach was employed to construct the three-dimensional structure of the antigen. The built structure possesses the features of TolC-like outer membrane protein. Molecular docking simulation was also performed to further probe the functionality of the antigen. Docking results showed that hexamminecobalt, $Co(NH_3)_6^{3+}$, as an inhibitor of TolC protein, formed favorable hydrogen bonds with D368 and D371 of the antigen. The single point (D368A, D371A) and double point (D368A and D371A) mutations of the antigen showed a decrease (single point mutation) and loss (double point mutations) of binding affinity towards hexamminecobalt. The architecture features of the built model and the docking simulation reinforced and supported that this antigen is indeed the variant of outer membrane protein, TolC. As channel proteins are important for the virulence and survival of bacteria, therefore this ~50 kDa channel protein is a good specific target for typhoid detection test.

1. Introduction

Salmonella enterica serovar Typhi is the causative agent for typhoid fever, a multi-systemic enteric infection in humans [1,2]. The common antibiotics for typhoid treatment are fluoroquinolones but multi drug-resistance strains have evolved rapidly [3]. Vaccines for typhoid fever are available but cheaper and safer vaccines with higher efficacy via a single dosage is yet to be developed [4]. A total of 21.6 million new typhoid cases and 216,500 deaths per year [5,6] have prompted the research on new typhoid detection methods to replace conventional culture methods and biochemical test which are time consuming and lacked sensitivity and specificity [7–10]. The staggering typhoid incidence rate of 1100 cases per population of 100,000 in developing and under-developed countries [1,6] also emphasizes the need to develop a more affordable and accessible typhoid diagnostic test with high sensitivity and specificity. However, today's diagnostic methods which emphasized on the RNA-/DNA-based are complex, costly and unavailable where needed most. Thus, a rapid typhoid diagnostic test is necessary for early detection to prevent delay in treatment and management of possible outbreak.

Ismail et al. [11] reported a ~50 kDa antigenic protein specific for *S. typhi* which was later developed into TYPHIDOT®, a rapid dot enzyme immunoassay (EIA) diagnostic test with 87–90% sensitivity and specificity up to 95% [12]. However, the structure of the antigen has yet to be elucidated. In order to understand the function of the protein at molecular level, it is necessary to determine the three-dimensional structure of the protein since the biological function of a protein is dependent upon the structure that it adopts. The protein structures solved experimentally mainly by X-ray crystallography or NMR [13–19], are able to provide the detailed information about a protein structure but these methods are time-consuming and expensive. Unfortunately, the rate of solving the structure of new proteins by experimental means is still far behind the rate of new protein sequences being generated. Thus, there is considerable interest in using theoretical methods to predict the three-dimensional structure of a protein from its amino acid sequences.

Elucidating the structure of the 50 kDa antigen would be valuable in the quest to understand the biochemical functions and

* Corresponding author. Tel.: +60 46534837; fax: +60 46534803.
E-mail address: yeesiew@usm.my (Y.S. Choong).

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Table 1
Top 10 hits of different Enterobacteriaceae organisms from BLAST search against non-redundant protein database with the antigen from *Salmonella enterica* serovar Typhi.

Organism	Accession no.	Title	Sequence identity (%)/similarity (%)	*E*-Value
Citrobacter koseri ATCC BAA-895	YP_001455919.1	Outer membrane channel protein	91/96	<1.0 *e*−180
Escherichia fergusonii ATCC 35469	YP_002384075.1	Transport channel	89/94	<1.0 *e*−180
Escherichia albertii TWO7627	ZP_02900740.1	Outer membrane protein TolC	89/94	<1.0 *e*−180
Escherichia coli E22	ZP_03043238.1	Outer membrane protein TolC	89/94	<1.0 *e*−180
Citrobacter youngae ATCC 29200	ZP_03838598.1	Outer membrane channel protein	90/96	<1.0 *e*−180
Enterobacter cloacae	CAL18627.1	TolC protein	86/93	<1.0 *e*−180
Shigella boydii Sb227	ABB67411.1	TolC	89/94	<1.0 *e*−180
Shigella dysentariae Sd197	ABB63214.1	Outer membrane channel	89/94	<1.0 *e*−180
Shigella flexneri 2a str. 2457T	NP_838556.1	Outer membrane channel protein	89/94	<1.0 *e*−180
Klebsiella pneumonia 342	YP 002236547.1	Outer membrane protein TolC	85/92	<1.0 *e*−180

mechanisms of this antigen at the cellular level. The built model for this antigen could aid in optimizing typhoid diagnostic tests, therapeutic agents or vaccine development in the future via epitope(s) determination. Understanding the epitopes would help to increase the sensitivity and specificity for a recombinant antigen-based diagnostic test. Besides that, the built model can also aid in the design of specific complementary determining regions of the antibody in order to increase the antibody–antigen binding affinity for possible antibody-based diagnostic test. The structural and functional elucidation the antigen would also allow for the search for new inhibitor(s).

In this study, we aimed to construct the three dimension predictive model of the antigen. We also elucidated its functionality via molecular docking simulation and validated the docking simulation by performing mutations on the built structure. Results obtained show that the architecture of the built model resembles the variant of outer membrane protein TolC.

2. Methodology

The sequence of the antigen was obtained from GenBank (id: CAD07712.1). Signal peptide prediction was performed by SignalP 3.0 [20], PrediSi [21], SIG-Pred [22], SOSUIsignal [23] and SPEPLip [24] servers. The secondary structure prediction was made by Jpred [25], Phyre [26], and PSIpred [27] and SSpro [28] servers. The sequence was used to search against Basic Local Alignment Search Tool (BLAST) [29] for non-redundant (nr) protein sequences database. The sequence was further searched against BLAST for suitable templates in RCSB Protein Data Bank (PDB). Multiple sequence alignment with members of Enterobacteriaceae family was performed by ClusterW2 [30]. From the results obtained from BLAST and ClusterW2, outer membrane protein, TolC of *Escherichia coli* (PDB id: 1EK9 [31], 1TQQ [32] and 2VDD [33]) were selected as the templates for the construction of residue 25 to 448 of the antigen. Proteasome from *Saccharomyces cerevisiae* was selected as the template for the C-terminal 43 residues (residue 449–491) of the antigen.

MODELLER 9v7 [34] was employed to build the three dimensional model for the antigen. A total of 100 models were randomly generated. A model with the best discrete optimized potential energy (DOPE) [35] was chosen. The built model was subsequently energy minimized with 45 cycles of the steepest descent followed by 5 cycles of conjugate gradient from the basic minimize- Sander module of Amber version 8 [36] to reduce unfavorable contacts and stesric clashes. Ramachandran plot [37] from PROCHECK [38] was employed to verify the constructed model. Secondary structure calculation of the constructed model was done using STRIDE [39].

Docking of hexamminecobalt, $Co(NH_3)_6^{3+}$, to the binding site of the antigen was performed by AutoDock version 3.0.5 [40]. PM6 charges for $Co(NH_3)_6^{3+}$ were calculated by MOPAC2009 [41] as recommended by AutoDock 3.0.5 [42]. The antigen was added with polar hydrogen atoms using the program *protonate* and the partial charges were loaded using *kollua.amber* option of AutoDock. The grid maps with $60 \times 60 \times 60$ points and a spacing of 0.375 Å were generated under AutoGrid of AutoDock. The molecular docking was performed employing Lamarckian genetic algorithm (LGA) with pseudo-Solis and Wets local search and with the following parameters: population size of 50; energy evaluations of 1,500,000; maximum generations of 27,000; translational step of 0.2 Å; orientational and torsional step of 5.0°; crossover rate of 0.80; mutation rate of 0.02; elitism of 1; local search rate of 0.06; 300 iterations per local search with termination value of 0.01; consecutive successes or failures before doubling or reducing local search step size of 4 and a total of 100 docking runs. Same docking simulation approach was performed towards a single point mutation on the antigen of D392A, D395A and, double mutations of both D392A and D395A. Mutant type antigen was mutated from wild type antigen using HyperChem 7.0 (Hypercube Inc., FL).

3. Results

Data from the GenBank showed that the antigen consists of 491 residues (amino acids). Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.gov/) against non-redundant protein database showed that this antigen has at least 85% and 92% for sequence identity and similarity, respectively, with the outer membrane channel protein of Enterobacteriaceae family, namely *Citrobacter*, *Escherichia*, *Enterobacter*, *Shigella* and *Klebsiella* (with

Table 2
Templates obtained from BLAST search against RCSB protein data bank (PDB) with the antigen from *Salmonella enterica* serovar Typhi.

Organism	PDB id.	Title	Sequence identity (%)/similarity (%)	*E*-Value
Escherichia coli	1TQQ	Chain A, structure Of TolC in complex with hexamminobalt	89/94	<1.0 *e*−180
Escherichia coli	2VDD	Chain A, crystal structure of the open state of TolC outer membrane component Of multidrug efflux pumps	91/96	<1.0 *e*−180
Escherichia coli	1EK9	Chain A, 2.1a X-ray structure Of TolC: an integral outer membrane protein and efflux pump compnent from *Escherichia coli*	91/95	<1.0 *e*−180
Saccharomyces cerevisiae	1G0U	Chain I, a gated channel into the proteasome core particle	33/43	7.5
Saccharomyces cerevisiae	1RYP	Chain J, crystal structure of the 20S proteasome from yeast at 2.4 Å resolution	33/43	7.5

the *E*-value of above the threshold; Table 1). Table 2 shows that the template search against PDB revealed that the antigen has 89–91% sequence identity with TolC outer membrane protein of *Escherichia coli* (PDB id: 1EK9 [31], 1TQQ [32] and 2VDD [33]; Table 1). Based on the BLAST result against both non-redundant protein database and PDB, 3 templates (PDB id: 1EK9, 1TQQ and 2VDD) were chosen to model the three-dimensional structure of the antigen. However, all templates are without the N-terminal 24 residues (residue 1–24) and C-terminal 43 residues (residue 449–491). Thus, an effort was made to verify the possibility of the N-terminal 24 residues of the antigen as a signal peptide. Data from all 5 unrelated signal peptide prediction servers showed consistent results where the N-terminal 24 residues from the antigen is indeed the signal peptide, which also correlates with the experimental data [33]. Experimental data also showed that the C-terminal 43 residues were removed by protease digestion in order to acquire crystal formation [31,32]. BLAST against PDB for the C-terminal 43 residues obtained 2 templates from proteasome of *Saccharomyces cerevisiae* (PDB id: 1G0U and 1RYP), which has 33% sequence identity with the 43 residues (Table 2). Therefore, the three dimensional structure for the total of 467 residues (residue number 25–491) of the antigen was modeled. Secondary structure prediction on the 467 residues on 4 servers showed similar result (Table 3). The predicted number of helixes and sheets is 10–13 and 4–5, respectively, from 4 different consensus prediction servers.

Comparison of templates TolC of *E. coli* (1EK9, 1TQQ and 2VDD) with the built model showed low differences with a root mean square deviation (RMSD) of 4.2, 4.6 and 4.3 Å, respectively. Ramachandran plot from PROCHECK was employed to examine the stereochemical quality of the built model. Result indicates that more than 95% of the residues have phi and psi angles in the most favored regions. An overall G-factor of 0.16 indicates a good quality built model. The residues in the disallowed region were located outside of the binding pocket, thus no further action was taken to further improve the backbone folding of these residues. The validation of the built model was again confirmed by secondary structure calculation on the built model. Results from STRIDE showed that the packing of the helices and sheets was similar to that of the templates (Fig. 1) as well as and consistent with the results from the consensus prediction (Table 3).

Fig. 2a shows the built structure of the antigen. The built structure is an assembly of a trimeric single pore channel (~140 Å in long axis). Each monomer consists of a total of eight β-strands and ten α-helices (Fig. 2b). The upper end of the structure is a β-barrel formed by 12 antiparallel β-sheets domain (~40 Å in long axis, ~40 Å in external diameter and ~25 Å in internal diameter), each monomer has 4 strands and each strand is connected by loops. The side chains of phenylalanine and tyrosine at this domain were found to be projected out from barrel (Fig. 3). The lower end of the built structure consists of both the α-helices barrel and the equatorial domain (mixed of β-sheets/α-helices). The built structure shows a partially close conformation with coiled coils at the proximal end (periplasmic entrance) where the side chains of a helix fits nicely in between the space created by the side chains of the adjacent helix (Fig. 4a and b). The periplasmic entrance of the channel is ~10 Å in diameter.

Docking simulation showed that binding site of hexamminecobalt, $Co(NH_3)_6^{3+}$, is located at the periplasmic entrance of the protein (Fig. 5a and b). The nitrogen atoms of hexamminecobalt were 2.9–3.2 Å from carbonyl oxygen atoms of D392 and D395 from the built structure, which is within the proposed distance for hydrogen bonding [43]. Results from multiple sequence alignment among other Enterobacteriaceae showed highly conserved regions for D392 and D395 of the antigen (Table 4). From Table 5, the molecular docking simulation of hexamminecobalt on the mutant type

Table 3
Consensus prediction on the residue 25-491 of the 50 kDa antigen by Jpred [25], Phyre [26], PSIPRED [27] and SSpro [28].

```
Sequence ENLMQVYQQARLSNPELRKSAADRDAAFEKINEARSPLLPQLGLGADYTYSNGYRDANGINSNETSASLQLTQTLFDMSKWRGLTLQEKAAGIQDVTYQTDQQTLILNTANAYFKVLNAI
Jpred    --HHHHHHHHHH--HHHHHHHHHHHHHHHHHHHHHH-----EEEEEEEEEE----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
Phyre    --HHHHHHHHHH--HHHHHHHHHHHHHHHHHHHHHH-----EEEEEEEEEE----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
PSIPRED  --HHHHHHHHHH--HHHHHHHHHHHHHHHHHHHHHH-----EEEEEEEEEE----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
SSpro    --HHHHHHHHHH--HHHHHHHHHHHHHHHHHHHHHH-----EEEEEEEEEE----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH

Sequence DVLSYTQAQKEAIYRQLDQTTQRFNVGLVAITDVQNARAQYDTVLANEVTARNNLDNAVEELRQVTGNYYPELASLNVEHFKTDKPKAVNALLKEAENNRNLSLLQARLSQDLAREQIRQA
Jpred    HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH---HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----------HHHHHHHHHH-HHHHHHHHHHHHHHHHHHHHHH
Phyre    HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH---HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----------HHHHHHHHHH-HHHHHHHHHHHHHHHHHHHHHH
PSIPRED  HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH---HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----------HHHHHHHHHH-HHHHHHHHHHHHHHHHHHHHHH
SSpro    HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH---HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----------HHHHHHHHHH--HHHHHHHHHHHHHHHHHHHHHH

Sequence QDGHLPTLNLITASTGISDTSYSGSKTNSAQYDDSNMGQNKIGLNFSLPLYQGGMVNSQVKQAQYNFVGASEQLESAHRSVVQTVRSSFNNINASISSINAYKQAVVSAQSSLDAMEAGYS
Jpred    H----  EEEEEEEEEEE----------EEEEEEEEEE-----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
Phyre    H-----EEEEEEE----------EEEEEEEEEE-----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
PSIPRED  HHH---EEEEEEE----------EEEEEEEEEE-----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
SSpro    H----EEEEEEEEE----------EEEEEEEEE-----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH

Sequence VGTRTIVDVLDATTTLYDAKQQLANARYTYLINQLNIKYALGTLNEQDLLALNSTLGKPIPTSPESVAPETPEQDAAADGYNAHSAAPAVQPTAARANSNNGNPFRH
Jpred    H----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----HHHHHHHHH----------HHHH----------HHHHHHH----------
Phyre    ----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----HHHHHHHHHH----------HHHHHHHH----------
PSIPRED  H----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----HHHHHHHHHH----------HH----------HHHHHH----------
SSpro    ----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----HHHHHHHHHH----------HHHHHHHH----------
```

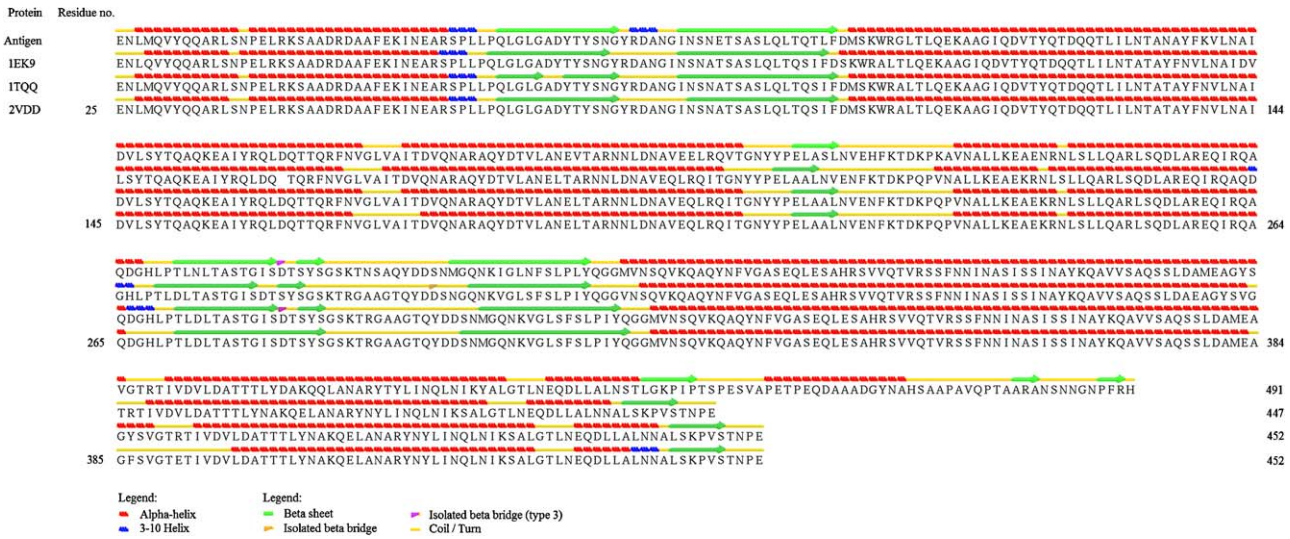

Fig. 1. Secondary structure comparison of the built model and the templates.

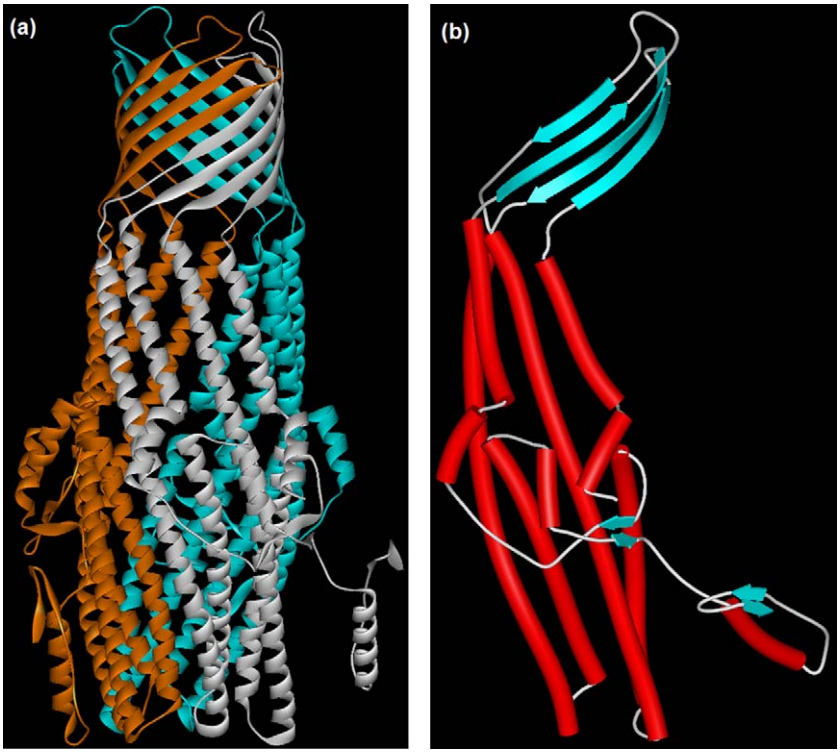

Fig. 2. (a) The side view of the built model for the antigen. Each monomer is colored individually. (b) The single monomer of the built model. The β-sheets are cyan, the α-helices are red while the coils and turns are white. All figures were generated with WebLab ViewerLite 3.20 (Molecular Simulation Inc., San Diego).

Table 4
Multiple sequence alignment of the antigen with different Enterobacteriaceae organisms. D392 and D395 of the antigen are highly conserved among the other Enterobacteriaceae organisms. These aspartic acids were located closely with the inhibitor, $Co(NH_3)_6^{3+}$. The conserved regions are indicated as *.

50kDa	**VRSSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT**	**397**
Escherichia coli	VRSSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT	398
Shigella flexneri	VRSSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT	398
Shigella boydii	VRSSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT	400
Shigella dysenteriae	VRSSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT	400
Escherichia fergusonii	VRSSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT	400
Escherichia albertii	VRSSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT	398
Citrobacter youngae	VRSSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT	396
Citrobacte koseri	VRSSFNNINASISSINAYKQAVVSAQSSLDANEAGYSVGTRTIVDVLDAT	398
Enterobacter cloacae	VRSSFNNVNASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT	397
Klebsiella pneumoniae	VRSSFNNVNASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDAT	398
	*******:********************** **********	

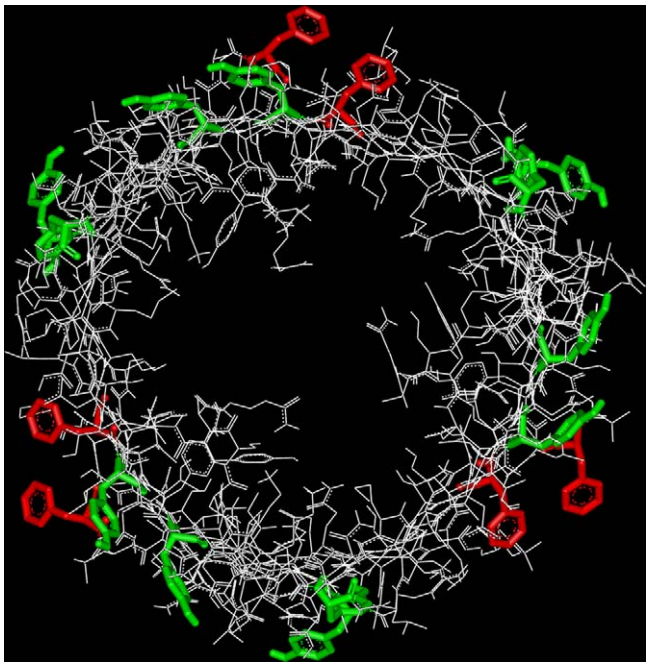

Fig. 3. The residues at the β-barrel domain (white line representation). Aromatic side chain of phenylalanine and tyrosine that are projected out from the β-barrel are in red and green stick representation, respectively. Figure was generated with WebLab ViewerLite 3.20 (Molecular Simulation Inc., San Diego).

(single point mutation: D392A and D395A and double mutations of both D392A and D395A) antigen showed that the mutation has significantly decreased the binding affinity (compared to wild type) towards the inhibitor. However, the binding site of the inhibitor in the single point mutation antigen is the same as in the wild type. Table 5 also shows that the double mutations resulted in the lost of binding ability with the inhibitor (with the inhibition constant of 0.00). Docking result showed that the inhibitor is no longer located in the periplasmic entrance of the antigen (Fig. 6) but has moved to the side of the entrance.

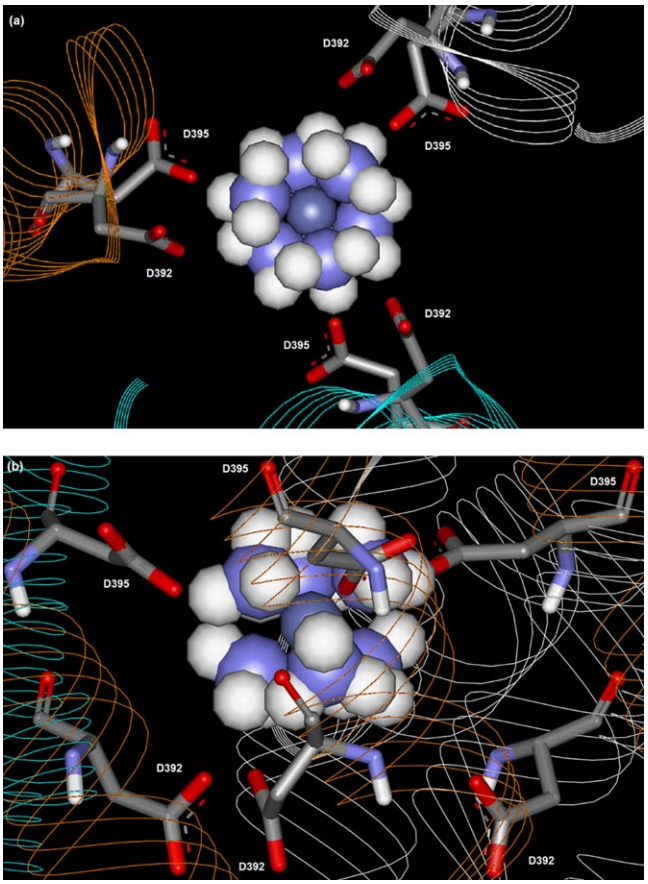

Fig. 5. The docked conformation of hexamminecobalt, $Co(NH_3)_6^{3+}$ (CPK representation) in the binding site (D392 and D395 in stick representation) of the built model (line ribbon representation). (a) Viewed from the periplasm entrance. (b) Viewed from the side. All figures were generated with WebLab ViewerLite 3.20 (Molecular Simulation Inc., San Diego).

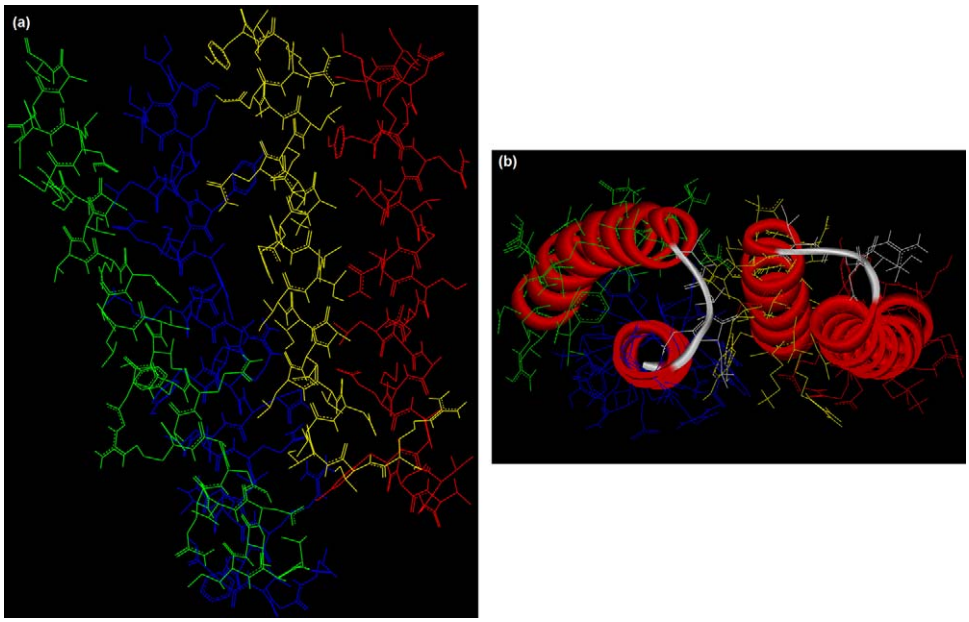

Fig. 4. (a) Side view of two pairs of coiled coils helices (stick representation) in the periplasmic entrance of one monomer in the built model. Each helix is colored individually. (b) The coiled coils helices in the periplasmic entrance of one monomer in the built model. Side chains (stick representation) of each helix are colored individually. Backbone of the helices is in ribbon representation. All figures were generated with WebLab ViewerLite 3.20 (Molecular Simulation Inc., San Diego).

Table 5
Docking results of hexamminecobalt, $Co(NH_3)_6^{3+}$, in the built model (wild type-WT and mutant type-MT).

	Antigen			
	Wild type (WT)	Mutant type (MT)		
		D392A	D395A	D392A and D395A
Free energy of binding (kcal/mol)	−7.05	−4.86	−4.94	−3.63
Inhibition constant (K_i)	6.58 e−06	2.72 e−04	2.39 e−4	0.00

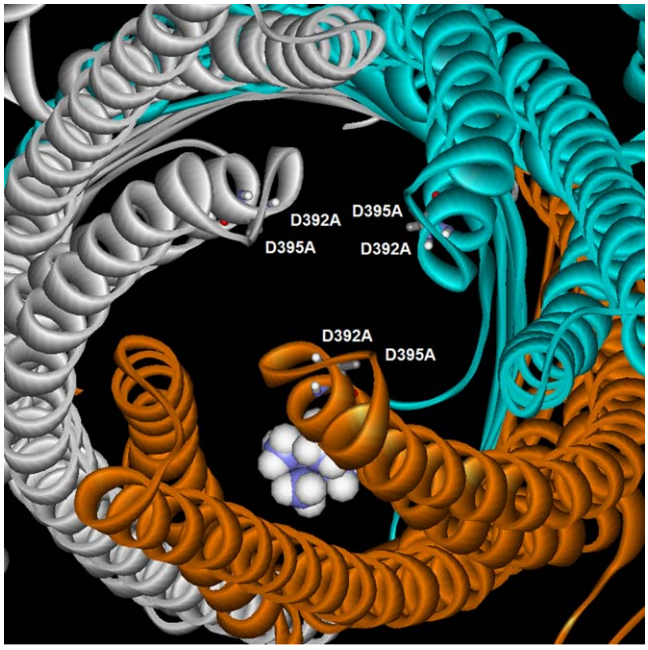

Fig. 6. The docked conformation of hexamminecobalt, $Co(NH_3)_6^{3+}$ (CPK representation) in the binding site of the double point mutation (D392A and D395A: stick representation) on the antigen (ribbon representation) viewed from the periplasm entrance. Figure was generated with WebLab ViewerLite 3.20 (Molecular Simulation Inc., San Diego).

4. Discussion

BLAST, a web-based program, has been widely used to search the regions of local similarity between sequences. This program is able to compare an unknown protein sequence to a sequence database and calculate the statistical significance amongst the matches. The function of an unknown protein can be inferred from other known homologous proteins based on their sequence and structural similarity. Thus, it is postulated that this antigen might be an outer membrane channel protein as BLAST result showed that this 50 kDa antigen has high sequence identity and similarity with that of 50 kDa, an outer membrane channel protein for the Enterobacteriaceae family.

Due to the difficulties in expression [44] and crystallization [45], till date, there are only 663 membrane proteins with known three dimensional structures, of which less than half are unique proteins (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Unfortunately, the structure of this 50 kDa outer membrane protein of *S. typhi* has yet to be determined. Together with the known general topology of the outer membrane channel protein in Enterobacteriaceae family, therefore, this research employed comparative modeling approach to investigate the structure and function of the antigen. Comparative modeling (or usually is termed as homology modeling), one of the protein structure prediction tools, utilizes experimentally solved 3-dimentional structure to construct an atomic resolution model of the target protein from its given amino acid sequence. Previous studies have shown that this approach has been proven to be successful for those targets with at least 30% of sequence identity with the template [46–51]. Solving the structure of this antigen may also lead to the possible structural optimization for higher specificity and sensitivity in typhoid diagnostic test. This is due to the epitope(s) location determination from the structure that will enable the improvement of the binding affinity towards specific antibody. The improvement in binding affinity for antigen–antibody interaction will therefore facilitate its application in diagnostics, as well as in the design of therapeutic agents and the development of vaccine candidates.

The overall architecture of the built model was the assembly of a homotrimeric long cylinder protein with 467 residues. The characteristic of the built model also resembles the structure of known transmembrane proteins in Gram-negative bacteria [31–33,52–55]. The built model shows similar structure as in TolC transmembrane channel protein with the combination of β-barrel domain projecting from the membrane, across the periplasmic space with α-helices domain and the equatorial domain (mixed of β-sheets/α-helices). The upper part of the structure is open and could provide solvent access, while the lower part is narrowed to a near close conformation as resemble in the templates. The structure shows that this antigen might be able to serve as an ion channel but the conductance could depend on the open or close conformation at the lower end, which is similar with the characteristic of TolC and its analogues [31,56–59].

The loops which is connected to the strands in the β-barrel domain have a relatively high atomic displacement with conformational mobility, thus might act as a "lid" to access into the top end of the β-sheets domain [31]. However, it is believed that the built structure with an open conformation at the upper part of the structure might not be as important in the transport of drugs or peptide as the opening of this "lid" is spontaneous and is not provoked by, e.g., voltage or ions [31,55,56]. The β-barrel domain of the built model consists of 16 strands, which is within the number of strands that has been characterized on all the bacterial outer membrane proteins [60–65]. The 40 Å long axis of the β-barrel domain should also ideally fit into a lipid bilayer membrane which is typically 30 Å [66,67]. The built structure is a transmembrane protein as it consists of a β-barrel domain and one of the permissible transmembrane structural is with the β-barrel motif [66,67]. The β-barrel domain of the built model also consists of aromatic residues, specifically phenylalanine and tyrosine, which form a ring around the base of the β-barrel. These residues are usually found in a typical β-barrel membrane protein to define the inner edge of a lipid bilayer of membrane [68]. Furthermore, the assembly of β-barrel proteins is also a common structural feature for the outer membrane protein for Gram negative bacteria [63,69,70]. Therefore, the β-barrel domain in the built model suggests that this part of the protein is anchored in the outer membrane of *S. typhi*.

The lower part of the built structure is the left twisted antiparallel α-helices barrel. The end of this α-helices domain consists of the conventional 6 pairs of two-stranded coiled coils. The structure of the coiled coils in the built model is consistent with the findings where these coiled coils will control the opening or closing of the access into the α-helices domain [31–33,52–55]. In these coiled coils, the side chains of a helix are a perfect fit and pack into the space between the knobs formed by its neighboring helix, which correlates with the commonly found structure of coiled coils [71–75]. The un-twisting of the helices that results in the breaking of intra-monomer and inter-monomer hydrogen bond and electrostatic interaction at the coiled coils are proposed

to be the mechanism of the channel opening to the periplasm [55,56].

The equatorial domain in the built model is the mixture of β-sheets/α-helices which bundled against the helical barrel. The model without 43 residues on the C-terminal from the templates was found extend from the equatorial domain and bundled against the periplasmic entrance of the α-helices barrel. We believe that this part of the protein might contribute to the open or close state of the periplasmic entrance to the α-helices barrel. This is due to the fact that the uncoiling movement to open the tunnel entrance of the TolC protein is triggered by protein–protein interaction as proposed by Koronakis et al. [31], Koronakis [53], Andersen et al. [55] and Bavro et al. [33]. Same observation was detected from molecular dynamics simulation of TolC and its analogues [56–59]. The hypothesis from Koronakis et al. [31], the equatorial domain of a TolC protein is one possible recognition site on the recruitment of TolC by the inner membrane translocase.

The outer membrane protein TolC is a 140 Å long channel homotrimer with three domains (β-barrel domain, α-helices domain and mixed α/β domain). The β-barrel domain is 40 Å in height while the α-helices domain is 100 Å in height. The coiled coils at periplasmic end of TolC control the open or close state to the channel. Each monomer consists of four β-sheets, two long α-helices and four short α-helices [76,77]. The overall architecture of the built model resembles the TolC protein and its analogues, thus it is believed that the antigen would be a variant of TolC protein.

Outer membrane channel, together with inner membrane protein and a bridging protein is an example assembly of an efflux pump that transports drugs and proteins [32,78–81] across the membrane in Gram negative bacteria, which includes *Salmonella* spp. Thus, these channel proteins are important for the survival of the bacteria during host infection and they mediate multidrug resistance and contribute to virulence of the bacteria [82–88]. A well studied efflux pump is the tripartite assembly of TolC (outer membrane protein) [78,89], AcrA (periplasmic protein), and AcrB (inner membrane protein) of *E. coli*. This efflux pump uses proton motive force for the translocation of acridine and other drug-like compounds [33]. Another one well studied protein export systems in *E. coli* involved TolC, HlyD (periplasmic protein) and HlyB (inner membrane protein) to translocate hemolysin toxin (HlyA) [53,54]. Literature also shows that the lack of outer channel membrane protein or one of its homologue reduces bacterial survival and attenuates virulence, thus suggesting that these proteins may be the potential targets for chemotherapeutics [31,82]. This 50 kDa antigen which resembles the outer membrane TolC protein could have the ability to use the similar tripartite mechanism for protein (such as toxins and enzymes) export and drug efflux. The periplasm entrance of this antigen could possible remain at close conformation until it is recruited by inner membrane translocases/transporters such as AcrA/AcrB or HlyD/HlyB and their variants [31]. The entrance will be opened when it interacts with these inner-membrane tranlocases. This will then form a complete and functional export or efflux pump which connects the extracelluar space to the inner membrane.

As previously mentioned, TolC protein is central in the expulsion of diverse molecules from the cell. Thus, the TolC inhibitor, hexamminecobalt, $Co(NH_3)_6^{3+}$, was chosen for functional verification on the built model. Literature also showed that aspartic acid at residue 392 and 395 are important as the periplasmic entrance for TolC channel permeability and contribute to high affinity ligand binding site in which ligand will form hydrogen bonds [32]. When these aspartic acids were replaced with alanine (non-polar and hydrophobic residue), the high ligand binding affinity was eradicated [32]. Results from molecular docking simulation showed that the built model was able to form favorable hydrogen bonds with hexamminecobalt. The binding free energy of hexamminecobalt thus suggests that this inhibitor is able to block the efflux function of the built model. We also performed point mutation(s) and constructed the mutant type antigen from the built wild type model to further verify its efflux function. The docking of hexamminecobalt into the mutant types (single point mutation: D392A and D395A and double mutations of both D392A and D395A) showed the correlation of the observed experimental data [32] with our calculation. Results indicated that the mutant type protein (either single point mutation or double point mutation) has hindered or lost the binding affinity with the inhibitor, thus losing its function as an efflux pump. Therefore, as previously indicated, 50 kDa antigen posses similar the efflux pump function as in TolC protein.

BLAST result shows that the 50 kDa antigen has a high sequence identity with the TolC of other members of the Enterobacteriaceae family. Even with such high sequence identity, this antigen is still unique for *S. typhi*. This is evident from the immunoblot test using the sera of patients with fever that has demonstrated that the 50 kDa protein is only recognized by typhoid sera. No cross reaction was observed from the typhoid patients sera with *E. coli* [11]. Several proteins have been identified to be essential for host invasion such as SicA [90], SipB [91], Syc [92] and heat shock proteins [93–95]. This could be attributed to these similar chaperone proteins which is essential in the pathogenesis and host invasion of *Salmonella*. These chaperone proteins might be forming complexes with the 50 kDa protein which could lead to the specific binding and interaction. Several reports also showed various virulence and proliferation factors in *Salmonella* in relations to transmembrane protein in the outer membrane region such as InvG [86], OmpC and OmpF [87], OmpR [85], PrgH and PrgK [88]. The cross-link of the chaperon proteins with more than one transmembrane protein could be another contributor to the specificity of the 50 kDa antigen. As the virulence of *S. typhi* is different from other members of its family, therefore the specificity of the 50 kDa protein could be attributed from the pathogenesis of *Salmonella*. The conformations formed by the 50 kDa antigen could be another possibility how this antigen could be distinguished from other members of the Enterobacteriaceae family. The good shape complementarily is important between the interacting surfaces as it is the key to specific antibody–antigen recognition mechanism [96–98]. Therefore, the loops which are exposed to the extracellular region in the 50 kDa protein might have distinct conformation or shape compared with other members of its family. This specific conformation can only be recognized by the typhoid antibodies, thus the key factor to the specificity of this antigen. However, the differences between the 50 kDa with other the member in Enterobacteriaceae can only be solved with further study.

5. Conclusions

We combined structural modeling and molecular docking simulation to provide the understanding on the structure and function of the 50 kDa protein of *S. typhi*. In this work, it was found that the antigen possesses similar characters and functions with TolC protein of *E. coli*, which is important as a multidrug efflux channel protein. The structural feature of the β-barrel confirms that this domain is embedded in the outer membrane of *S. typhi* while the α-helices domain is at the periplasmic region. Therefore, the built model is a transmembrane protein. The characteristic of the built model also resembles the features of known outer membrane protein TolC. Molecular docking simulation results showed that hexamminecobalt, $Co(NH_3)_6^{3+}$, able to form hydrogen bonds with the built model, namely aspartic acid of residue 392 and 395. This indicates that the hexamminecobalt is able to inhibit the built model as well as in the TolC protein. Docking results also showed that the functionality of the built model can be hindered by single

point (D392A, D395A) and double point (both D392A and D395A) mutations. The findings from docking simulation correlated with experimental data for TolC protein. Together with the structure and functionality of the built model, we have yielded conclusive evidence that this ~50 kDa antigen is indeed a variant of TolC protein. The pathogenesis, virulence and survival of bacteria depend on channel proteins. Therefore, this 50 kDa channel protein is a good candidate for detection test. This is the first report on the structural and functional prediction of the 50 kDa antigen of *S. typhi*. It is hoped that this work derives some basis on the further improvement on the binding affinity of the antigen with the specific antibody for the purpose of typhoid diagnostics, therapeutic agents or vaccine candidate development.

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