Original Article

Cloning, sequencing, and *in silico* characterization of Omp 28 of *Salmonella* Typhi (strain MTCC 733) to develop r-DNA vaccine for typhoid fever

Anjani Saxena, ShantanuTamuly, M.K. Saxena

Animal Biotechnology Center, Department of Veterinary Physiology and Biochemistry, G.B.P.U.A and T, Pantnagar, Uttarakhand, India

Address for correspondence:
Dr. M. K. Saxena, Animal Biotechnology Center, Department of Veterinary Physiology and Biochemistry, G.B. Pant University of Agriculture and Technology, Pantnagar 263145, Uttarakhand, India. E-mail: mumteshsaxena@gmail.com

Abstract

Background: Typhoid is one of the most important diseases of human beings caused by *Salmonella* Typhi. There are many vaccine reported against *Salmonella* Typhi, but search for new candidate vaccine antigens is still going on because presently available vaccines have several limitations such as short-term immunity, high cost, and allergic reaction. Several approaches such as subunit vaccines, Vi polysaccharide, mutant vaccines, and r-DNA vaccines have been tested. r- DNA vaccines have shown some promising potential (targeted Omp). Omp 28 had shown very promising results and suggests that it should be used in further studies of animal protection against the disease. **Objective:** Cloning, Sequencing and *In silico* analysis of Omp 28 gene to develop r-DNA vaccine of *S.*Typhi. **Materials and Methods:** Omp 28 is made up of three identical subunits of 9.6 kDa showing PCR amplicon of 330 bp which has been cloned in the pJET vector. Recombinant clones has been sequenced, and data submitted to NCBI. Secondary structure was deduced by the Chou Fasman and Garnier method. The sequence of Omp 28 was studied for antigenic indexing, epitope mapping, and MHC mapping using various bioinformatics tool. **Results and Conclusion:** The sequence of Omp 28 has been assigned accession no GQ 907044.1 by NCBI. Secondary structure has shown it has more alpha region. Hydrophobic plot and surface probability plot shows most amino acids are surface exposed which is a requirement to develop a r-DNA vaccine. Antigenic sites are located within surface exposed regions and eight antigenic determinants are present in Omp 28. On Prosite analysis of Protein shown two motifs i.e. anaphylatoxin domain signature motif at position 219-252 and other one was iron sulphur binding region signature motif at position 36-44. On epitope analysis total six major B cell epitopes were observed which can provoke humoral immunity .On T cell epitope mapping several major epitopes has been found in case of MHC class I and MHC class II. It indicates that Omp 28 can provoke cell mediated as well as humoral immunity and can be proven a promising candidates of *Salmonella* Typhi.

Key words: Outer membrane protein, r-DNA vaccine, *Salmonella* Typhi

INTRODUCTION

Typhoid is one of the most important diseases caused by *Salmonellae* worldwide and it causes 60,0000 deaths per annum.[1] As per the WHO report the total cost associated with *Salmonella* is estimated at US$ 3 billion annually in the United States.[2] At present detail about the epidemiological or incidence rate of typhoid for any state is not available in India though there are numerous reports available which indicate that the typhoid is of great concern to India.[3,4] The emergence of multidrug-resistant *Salmonella* Typhi with increased virulence and high physiological adaptability has further complicated the disease management.[5,6] Vaccination plays an important role in preventing the disease. Presently, three approaches has been used for development of vaccine against typhoid these are i.e. whole cell typhoid vaccine,[7] Vi polysaccharide vaccine[8] and oral typhoid vaccine.[9] Whole cell typhoid vaccine prepared by inactivating virulent microorganism is safe and cheap, but causes local pain, inflammation and fever and it is not commonly used. Vi capsular polysaccharide vaccine of *S.*

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Typhi was developed from the wild type *S*.Typhi strain.[8] It is not economical. Attenuated live bacterial vaccine (LBV) is an attenuated mutant strain of *S*. Typhi. One of its drawbacks is the requirement for multiple doses for optimal immunogenicity. Therefore, there is a need of a better vaccine. Outer membrane protein (Omps) has been tested extensively and has shown good potential as vaccine candidate. Omps interface the cell with the environment, has important virulent factors and played a significant role in the pathobiology of gram-negative bacteria and bacterial adaptation.[10] Over the past years, several *Salmonella* Omps have been investigated as potential vaccine candidates and diagnostic antigens.[11] The molecular structure and function of Omps and their respective genes[12,13] have been studied. However, only a small number of Omps have so far been characterized.[14] Study of other gram-negative bacteria demonstrated that porins represent the most abundant class of Omps that are protective and show some degree of antigenic heterogeneity among different strains.[15] Porins play a key role in the uptake and disposal of small hydrophilic compounds and a potential role as immunogens in diagnostic assays and vaccination.[16] Outer membrane proteins of *Salmonella* Typhi are immunogenic and among them Omp28 had shown very promising results. Antibodies against Omp28 were detected by ELISA in 43% of sera from typhoid fever convalescent patients or antisera from mice immunized with Omp 28 gave a positive bactericidal test killing 50% of *S*. Typhi cells in serum dilution of 1/80 and 1/320.[17] Complete amino acid sequence and location of Omp 28 of *S*.Typhi has also been analysed.[18] As it is difficult to isolate Omp(s) in bulk for vaccine purpose, this study was undertaken to clone and sequence and *in silico* analysis of Omp 28 gene of *S*.Typhi to further explore the possibility of r-DNA vaccine of *S*.Typhi.

MATERIALS AND METHODS

Bacterial strains

Culture of *Salmonella* Typhi (MTCC 733) was obtained from Institute of Microbial Technology, Chandigarh and was maintained in Growth Medium 3. *Escherichia coli* DH5α used in the cloning experiments was purchased from Bangalore Genei and grown in Luria broth (LB). The culture of *Salmonella* was revived on Luria Bertani broth (Hi Media, India) and Luria Bertani agar and the purity of the culture was tested using colony character on Brilliant Green Agar (Hi media, India). Single colony was inoculated in LB broth and tested by *Salmonella* specific PCR[19] and biochemical tests.

Cloning of Omp 28 gene

Genomic DNA of *Salmonella* Typhi was isolated by the C-TAB method.[20] For amplification of Omp28 gene following primers were used which were designed using the complete amino acid sequence reported earlier.[18]

Primer 1: ATG AAT AAA TTC TCC CTT GC
Primer 2: TTA TTT TGA GAG TTC TTT CTT GA

Twenty-five microliters of the PCR reaction mixture containing 40 ng of genomic DNA, 20 pmole of each primer, 200 μM of each dNTPs, 1.5 mM $MgCl_2$, 2U of jumpstart polymerase (Sigma, USA) were amplified by PCR and were under standard conditions in a thermal cycle with the following programme, i.e. initial denaturation at 94° C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min annealing at 46°C for 1 min, 68°C for 1 min and the final elongation was carried out at 68°C for 5 min. The amplified product was loaded on 1.5% agarose gel and eluted from gel using a QIA quick gel extraction kit (Qiagen, USA). After blunting the amplified product, it was cloned into the pJET cloning vector (Qiagen, Germany). Clones were inoculated in LB ampicillin tubes and the plasmid was isolated by the alkaline lysis method and an insert from plasmid was released by digestion with *Xba* I and *Not* I restriction enzymes. The recombinant clones were screened to obtain insert of desired size verified by colony PCR amplification. The cloned product was sequenced by Ocimum Biosolutions Ltd., Hyderabad. The sequence was submitted to NCBI and assigned the accession no. GQ 907044.

Sequence similarity and phylogenetic analysis

The sequence was subjected to homology search using BLASTn.[21,22] The sequence showing maximum similarity with Omp28 was subjected to multiple sequence alignment by CLUSTALW[23] and a phylogenetic tree was constructed based on the protein sequence using the UPGMA method (DNASTAR, USA).

Protein structural and functional analysis

The Omp28 gene sequence was translated to the protein sequence using the translation tool (http://ca.expasy.org/tool). Translated Omp protein was subjected to protein domain functional analysis using Pfam version 23.0,[24] Prosite version 20.37,[25] Psort tools, and SWISS-PROT model analysis.[26] *In silico* translation and analysis of primary structure of outer membrane protein were performed using online bioinformatics tools.[27] On the basis of primary structure, physiochemical properties of Omp28 protein were also deduced using the same software. Protein secondary structure and antigenic analysis were also performed by PROTEAN[28,29] Program (DNASTAR, USA).Protein 3D structure has been made by submitting translated sequence of Omp 28 gene sequence in Bhagirath online server.

B-cell and T-cell epitope prediction

To find out the B-cell epitope regions of Omp 28 protein, the Bepripred prediction tool of IEDB analysis resources was used. A critical step in developing immune response against pathogens is the recognition of antigenic peptides presented by MHC Class I and II molecules.[30] Peptides are divided into binders and nonbinders and binding affinities of MHC Class I and II are calculated by epitope database analysis.

RESULTS AND DISCUSSION

Purity of culture was confirmed by biochemical characterization and *Salmonella*-specific PCR. In biochemical characterization culture was MR+, VP-, Urease– which was identical to standard biochemical tests of *Salmonella* Typhi. In *Salmonella*-specific PCR, an amplicon of 496 bp was obtained which corresponds to the expected amplicon of *Salmonella* by this method.[19]

Cloning and sequencing of the Omp 28 gene

Omp 28 gene is made up of three identical subunits and the primer was designed corresponding to one subunit of protein. The PCR product of the corresponding size (330 bp) was obtained when the genomic DNA of *Salmonella* Typhi was amplified with these primers [Figure1a]. The amplified products were separately cloned into the clone JET cloning vector (Fermentas, USA) and transformed into DH5α competent cells. Recombinant clones have been selected by colony PCR [Figure1b]. The restriction digestion of each of the recombinant clones with *Xba* I and *Not* I enzymes confirmed the presence of an approximately 330 bp sequence within the recombinant plasmid for Omp 28 gene [Figure1c].

Nucleotide sequence accession number

The sequence of the cloned insert has been submitted to NCBI, Genbank data and has assigned an accession no GQ 907044.1. The length of this gene is 330 bp and GC content of 40–45%.

Sequence analysis of Omp 28 gene

The nucleotide sequence of Omp 28 revealed a putative open reading frame beginning with an ATG initiation codon which remained open to the end of the sequence obtained. The 330 bp functional Omp was found to be 325 bp in length having the GC content of 40–45%. The Omp 28 gene of *Salmonella* Typhi shows maximum homology with *Salmonella* enterica serovars Typhimurium, Paratyphi, and Enteritidis by NCBI BLAST search. The Omp 28 gene of *Salmonella* Typhi shows maximum homology with *Salmonella* enterica serovars Typhimurium, Paratyphi, and Enteritidis by NCBI BLAST search. The Omp 28 gene of Indian isolates of *S.* Typhi serotypes was aligned for homology with the reported sequence of *S.* Typhi strains MDR 9, Omp C, Omp F, and AG3 different strains, and the percentage of identity and divergence among 15 strains of *S.* Typhi has been depicted in Figure 2. The result of phylogenetic tree analysis showed a close relationship between Indian isolate of *S.* Typhi Omp 28 gene to *S.* Typhi Omp C and Omp F gene of *S.* Typhi.

Analysis of amino acid sequence and secondary structure predictions

The deduced sequences of Omp 28 gene of *S.* Typhi contain 16 acidic charged amino acids and 11 are basic in nature. From them, 30 amino acids are charged and 31 are polar in nature. When the amino acids sequences were compared, it was observed that histidine was missing in the total amino acid sequence and there are two cysteine and three methionine amino acids are present. PI value of Omp 28 was found to be 4.53. The C-terminal of Omp 28 gene has characteristic amino acids as reported, but the terminal amino acid was lysine in place of phenylalanine which have been found more common in outer membrane protein. The presence of lysine in place of phenylalanine may be responsible for localization of Omp 28 in periplasm. The physiochemical analysis of Omp 28 using PROTEAN

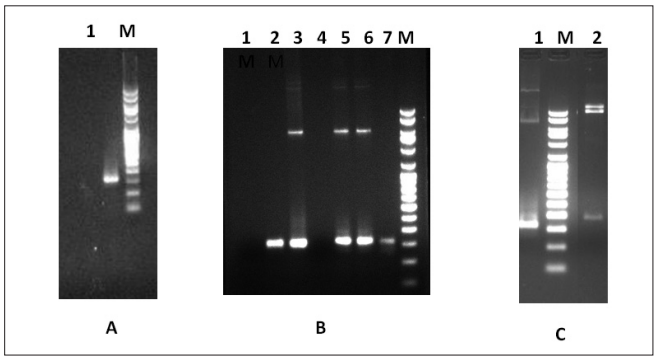

Figure 1: (a) Specific Omp 28 gene PCR of S.Typhi MTCC 733, Lane 1: Omp 28 gene PCR on S.Typhi MTCC 733, Lane M: Low range DNA ruler Plus(Genei), (B) Colony PCR for screening of recombinant clones, Lane 1: Negative Control, Lane 2: Positive Control, Lane 3-7: PCR amplicon of Clones, Lane M: Low range DNA ruler Plus(Genei), (C) Restriction Enzyme Digestion with Not I and Xba I to release insert, Lane 1: Positive amplicon of selected clone, Lane M: Low range DNA ruler plus(Gene), Lane 2: Digested Plasmid with Not I and Xba I to release insert

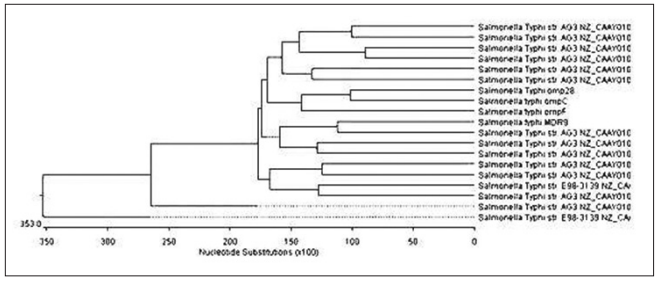

Figure 2: Phylogenetic Tree made of omp 28 gene with different serovars of *S.*Typhi

software showed a typical properties of containing a signal peptide [Figure 3]. Psorb analysis of Omp 28 gene also indicates its location in periplasm same as reported earlier.[18] In secondary structure analysis three membrane spanning α- helices were deduced [Figure 4].

Antigenic characterization and Motif analysis of Omp 28 gene

Antigenic characterization of the protein was done with PROTEAN.[28] Several hydrophilic peaks which represent surface exposed domains were present in Omp 28. These variable domains may represent unique epitopes. The surface probability plot indicates that most of the amino acids are surface exposed. The antigenic index creates a linear surface contour profile of the protein. Antigenic sites are located within a surface exposed region of a protein and the PROTEAN programme offers a mean for predicting potential antigenic determinants. Hydrophilic domains corresponding to the cell surface exposed domains were highly variable in their amino acid composition [Figure 3a]. There are so many domains present which are surface exposed and it is a requirement for development of vaccine.[Figure 3b]. Eight antigenic determinants or epitopes were found in Omp 28 protein with protean software[28] [Figure 3c].The overall structure of Omp 28 is conserved; however, there were small variable regions in the proteins of each serotypes which may work as strain-specific epitopes.

Prosite analysis showed the presence of two motifs in Omp 28, i.e. the anaphylatoxin domain signature motif at position 219–252 other one was the iron sulfur binding region signature motif at position 36–44 [Table 1]. On Pfam analysis no similar protein could be identified in the database. Omp 28 gene was submitted for Swiss-Prot model analysis, but this gene does not match with available models in the Web site. Translated Omp 28 gene sequence has been submitted to Bhagirath online server and 3 D Structure has been obtained [Figure 5].

B-cell and T-cell epitope mapping

To find out the B-cell epitope regions of Omp protein, the Bepripred prediction tool of IEDB analysis resources was used. The sequence was loaded into the tool window and searched for most potential linear epitopes [Figure 6]. A total of six B-cell epitopes were analyzed [Table 2]. Among these six epitopes, mainly three were found to be more immunogenic as predicted by the scores of epitope. A peptide length of 5 or more amino acids are supposed to be good B-cell epitope. A critical step in developing immune response against pathogens is the recognition of antigenic peptides presented by MHC Class I and II molecules. Peptides are divided into binders and nonbinders, and binding affinities of MHC Class I and II are calculated by epitope database analysis and ranked according to their percentile. We have analysed our sequence for MHC Class I and II binding peptide epitope. For MHC Class II, a percentile rank for each of the four methods (ARB, Combinatatorial library SMM-align and

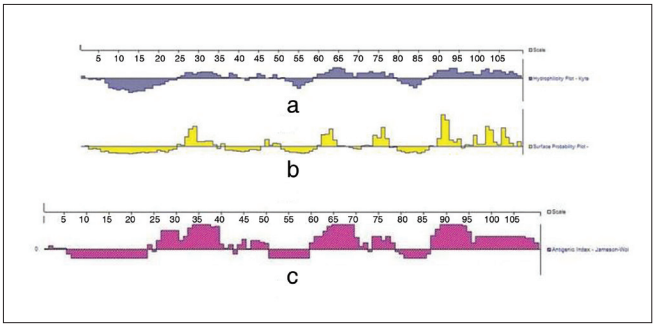

Figure 3: Hydropholicity, Surface Probability Plot and Antigenic Index of translated sequence of Omp 28 gene, (a) Hydrophobic Plot, (b) Surface probability plot, (c) Antigenic determination

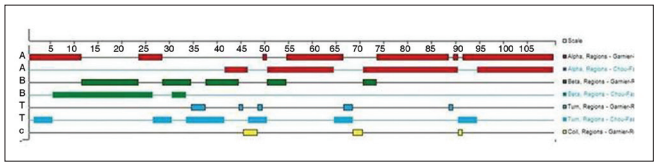

Figure 4: Determination of Secondary structure of translated sequence of Omp 28 gene

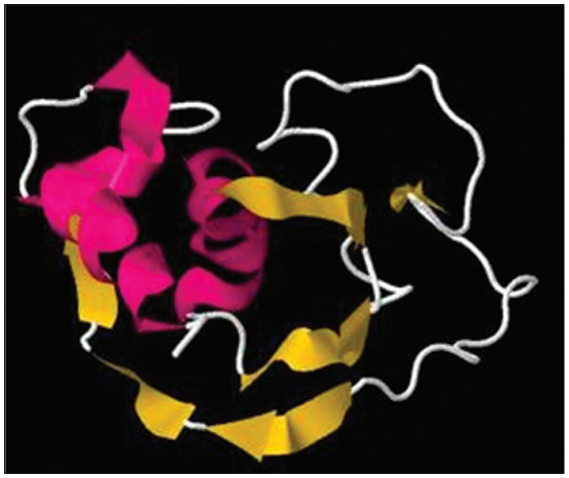

Figure 5: 3 D Structure of translated sequence of Omp 28 gene of *S.*Typhi

Table 1: Motif analysis of translated sequence of Omp 28 gene of *S.*Typhi

Motif Analysis of OMP 28 gene

Found Motif	Position	Prosite	Description	Related sequene
ANAPHYLATOXIN_1	219-252	PS01177	Anaphylatoxin domain signature	27
2FE2S_FER_1	36,44	PS00197	2Fe-2S ferredoxins, iron- sultfur binding region signature	211

Sturniolo) was generated by comparing the peptide score against the scores of five million 15 mers selected from SWISS-PROT database. A small percentile rank indicates high affinity. A graph was been plotted between percentile rank and amino acid position. There are different types of MHC class I alleles in the case of mouse, i.e. H_2kk, H_2kd, H_2Ld. IEDB recommend that low percentile peptides are good binders. Omp 28 gene is having 16 major epitopes for H_2kk, 14 epitopes for H_2kd, and 10 epitopes for H_2Ld for MHC Class I [Figure 7a]. There are three alleles for MHC Class II molecules for mouse, i.e., H_2IAb, H_2Ied, H_2Iad. IEDB recommend that top percentile peptides are good binders for MHC Class II molecules. Translated sequence of Omp 28 gene is having 27 for H_2Iab, 64 for H_2Ied, and 23 epitopes for H_2Iad which has been found in the case of MHC class II molecules [Figure 7b]. Recognition of a peptide bound to MHC molecules by a T-cell receptor is a critical stage, and it is done by H_2Iab binding prediction. It has been analyzed through multiple sequence alignment tools that *S*. Typhi Omp 28 consists of eight variable regions on comparison with other porins with well known crystal structures. These variable regions have been found to be on the outer side of the membrane, and therefore, they have high probability to be presented for B-cell recognition and elicit immune response. These findings clearly depict that Omp 28 has recognized B-cell epitopes and as it shares maximum similarity with OmpC of *S*. Typhimurium (98%), these variable regions can be strongly predicted to act as possible B-cell epitopes capable of eliciting immune response. OmpC of *Salmonella* Typhi has also been purified using salt extraction procedures and its epitopes have been mapped. It is found to be a trimer made of 16 stranded β-barrel monomers and is a major cell surface antigen from the human pathogen *Salmonella* typhi.[31,32] Sequencing revealed that the C-terminus of Omp 28 has typical characteristic of Omps. The last residue at the C-terminus, phenylalanine has been reported to be highly conserved among outer membrane proteins and is essential

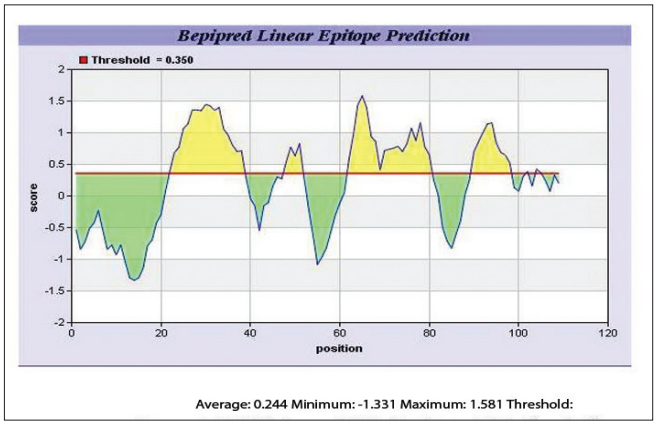

Figure 6: Major B cell epitopes of translated sequence of Omp 28 gene of *S*.Typhi

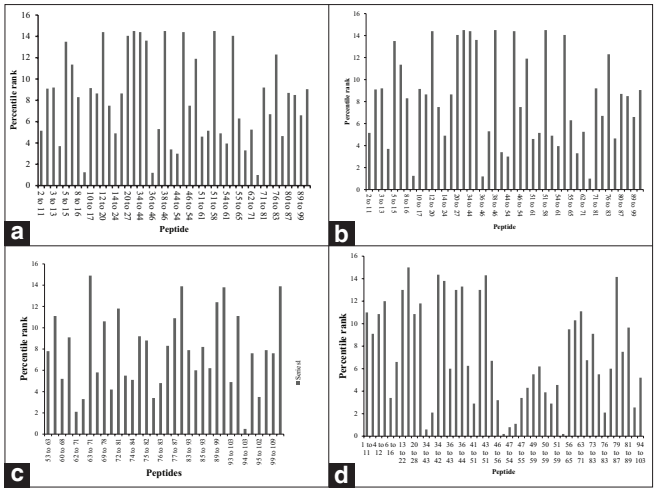

Figure 7a: MHC I epitopes identified through IEDB epitope prediction tool (a) MHC H-2-Kd allele, (b) MHC I H-2-kk allele first graph, (c) MHC I H-2-kk allele second graph, (d) MHC I, H-2-Ld graph

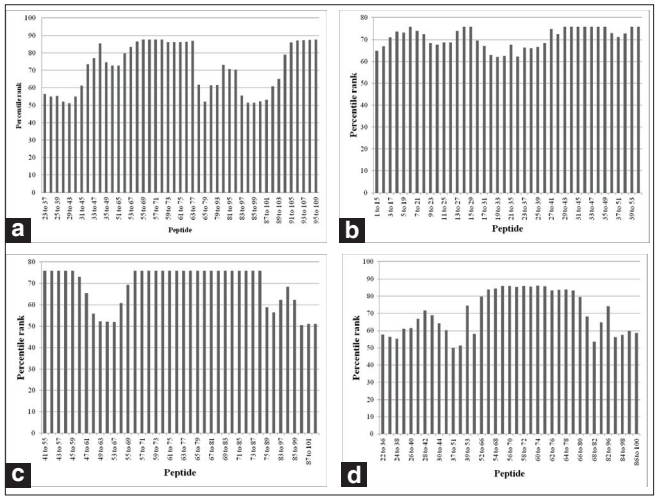

Figure 7b: MHC II epitopes identified through IEDB epitope prediction tool, (a) MHC II H-2-Iab, (b) MHC II H-2-Ied allele first graph, (c) MHC II H-2-Ied allele Second graph, (d) H2-IAd Graph

Table 2: Predicted B cell epitopes of translated sequence of Omp 28 gene

Start position	End position	Peptide	Peptide length
22	38	VNAATDTTKTNVTPKGM	17
48	51	PQTM	4
62	80	DEDFKGGDYVDFQETETTA	19
90	98	KNPQSELSK	9
102	102	E	1
104	105	KK	2

for stability and correct assembly of protein into the outer membrane.[32] The 15 C-terminal amino acid residues of Omp 28 including the terminal phenylalanine were found to be hydrophobic in nature which is important for incorporation of the protein in the membrane. These results indicate the immunogenic importance of Omp 28 isolated from *S*. Typhi outer membrane and strongly suggest that it should be used in further studies of animal protection against the disease caused by this pathogenic bacterium.

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