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Cloning, Sequencing and *In Silico* Characterization of OmpF Protein of *Salmonella typhimurium* for its Immune-Potential

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A B S T R A C T

Salmonella is a major foodborne bacterial pathogen responsible for millions of cases of infection and thousands of death in humans across the globe annually. Poultry and poultry products are considered to be the major source of *Salmonella* infection in humans. The economic loss caused by salmonellosis is very huge in case of poultry industry. Vaccination of the poultry is found to efficiently reduce the zoonotic risks associated with *Salmonella* infection. Virulence nature of outer membrane proteins of Gram-negative bacteria *Salmonella* is found to efficiently generate immunity in host system. Hence, several efforts have been made to develop an outer membrane protein based vaccine and were found to be successful. So, in the present study outer membrane protein F (OmpF) of *Salmonella typhimurium* was cloned, sequenced and *in silico* analysis was carried out to study its structural and immunological characteristics. Antigenic indexing, Epitope mapping and MHC mapping were performed using various prediction servers and online tools. Presence of eight major B cell epitopes indicated ability of OmpF to elicit humoral immunity. VaxiJen score of 0.85 further confirmed the antigenic nature of the epitope. T-cell epitope mapping identified several epitopes with high MHC binding affinity representing ability to provoke cell mediated immunity. The results from the study indicated that OmpF protein of *Salmonella typhimurium* is a strong candidate for developing recombinant vaccine against salmonellosis in poultry.

Introduction

Salmonellosis is a most commonly reported foodborne zoonoses in humans (Schlundt, 2004) capable of causing prolonged disease, possible treatment failure and increased severity of disease, mortality and societal costs. The major bacterial etiological agent of salmonellosis includes various pathogenic serovars of *Salmonella enterica*. Although more than 2500 serovars of *Salmonella enterica* have been identified, among these *S. typhimurium* was found to be second most common zoonotically important serovar isolated from humans globally (Hendriksen *et al.*, 2011; CDC, 2013). Many countries are facing this growing public health problem because of its antimicrobial resistance (Davies

and Davies, 2010; Mir *et al.*, 2015) and easy transmission of *Salmonella* bacteria through food and water. This organisms seems to be associated with gut of poultry, hence consumption of contaminated poultry meat, egg and its products consumption and contact with infected birds are the major transmission route to humans (Adak *et al.*, 2005).

In developing countries like India, poultry rearing is becoming a profitable sector in agrarian economy. Reports of *Salmonella* incidence from poultry stock of India indicate the possibility of transferring *Salmonella* from poultry to human populations where it can cause serious health issues (Kaushik *et al.*, 2014; Sudhanthirakodi *et al.*, 2016). Many strategic control programmes have been investigated to reduce the *Salmonella* burden from poultry flock. Vaccination along with other control measures is currently being used in poultry industry to reduce the zoonotic risk (Desin *et al.*, 2013). In last few years, vaccine control strategic programmes were in search of a suitable strong immunogenic vaccine candidate, this lead to an extensive study on outer membrane proteins of *Salmonella* (Hamid and Jain, 2008; Ghosh *et al.*, 2011). Outer membrane proteins are good surface immunogens (Nandakumar *et al.*, 1993), and are used in several studies to investigate the capability as a vaccine candidate in poultry (Meenakshi *et al.*, 1999; Khan *et al.*, 2003; Okamura *et al.*, 2012; Prejit *et al.*, 2013). Studies have also targeted in developments of diagnostic antigen with OMPs (Manoj *et al.*, 2015). OmpC, OmpF, OmpD are the major outer membrane proteins of *Salmonella* (Singh *et al.*, 2017). However, only limited studies were conducted on OmpF protein of *Salmonella Typhimurium*. Considering these factors, the present work was undertaken for detailed analysis of *omp*F gene, using various online immunological softwares and databases for gene analysis, to interpret information about the proteome to identify and characterize its antigenic properties.

Materials and Methods

Bacterial strain

Reference strain *Salmonella typhimurium* E-2375 used in this study was procured from National Salmonella Center, Indian Veterinary Research Institute (IVRI), Izatnagar. Purity and identification of the culture was determined by primary and secondary biochemical test specific to *Salmonella*. Molecular confirmation was done with *Salmonella typhimurium* specific primers targeting the *stm* gene (Liu *et al.*, 2012). Culture was stored and maintained in Hektoen enteric agar at Department of Veterinary Public Health, College of Veterinary and Animal Sciences Pookode, Wayanad, India for further use. One Shot TOP 10 Chemically Competent *E. coli* cells (Invitrogen, USA) were used as host cell in the cloning reaction.

Cloning of *omp*F gene

Genomic DNA of *Salmonella Typhimurium* was isolated by using mini prep DNA isolation kit, Invitrogen (USA), from 2ml of overnight grown cultures in LB broth. Concentration and purity of the isolated DNA was checked and stored at -20°C until use. Sequence information of *Salmonella typhimurium* available on NCBI used for designing of *omp*F primers with the help of gene tool software (For- 5'-ATG ATG AAG CGC AAA ATC CTG GC-3' and Rev- 5' TCA GAA CTG GTA AGT AAT ACC GAC AG-3'), and synthesized at IDT, USA.

Amplification of *omp*F gene was carried out with 25 µl of PCR reaction mixture containing 50ng template DNA, 200 µM dNTPs, 0.2 µM each primers, 1X Taq buffer, and 0.1U of high fidelity Taq polymerase Invitrogen (USA) under following cycling condition. Denaturation at 95°C for 5 min followed by 34 cycles of denaturation (94°C for 40 sec),

annealing (56°C for 40 sec) and extension (72°C for 1min). Final extension was carried out at 72°C for 5 min. Amplified products were analyzed and then eluted from the gel using GeneJET Gel Extraction kit (Thermo Scientific, USA).

Eluted *omp*F PCR amplicon was ligated in to pCR 2.1 vector (Invitrogen, USA) and it was transformed to One Shot TOP 10 Chemically Competent *E. coli* cells by heat shock method. Randomly picked white colonies were screened for insert of desired size by colony PCR. Positive clones were grown in ampicillin containing media and plasmid DNA was isolated from the cloned cells by using Pure Link Quick Plasmid Mini Prep Kit (Thermo Scientific, USA). Plasmid was sequenced at DNA sequencing facility Agrigenome, Kochi. Deduced sequences were submitted to NCBI.

Sequence Similarity and Antigenic characterization of *omp*F

Sequencing result of *omp*F gene was analyzed for homology using Basic local alignment search tool (Altschul *et al.,* 1997). Multiple sequence alignment was performed by using CLUSTAL-W (Thompson *et al.,* 1994).

In silico translation and analysis of primary structure of outer membrane protein were performed using online bioinformatics tool (ExPASy translate tool). Based on primary structure, physiochemical properties of OmpF protein was also deduced using ExPASy ProtParam (Gasteiger *et al.,* 2005). The B cell epitopes of OmpF protein was identified and characterized using BepiPred prediction tool of IEDB analysis (Potocnakova *et al.,* 2016). The sequence was loaded into the tool window and searched for most potential linear epitopes. Antigenic peptides presented by MHC class I and II molecules was recognized by IEDB analysis of T cell epitope prediction tool.

The secondary structure of OmpF protein was predicted using SOPMA (Self- Optimized Prediction Method with Alignment) tool (Combet *et al.,* 2000). This tool evaluates the percentage of alpha helices, extended strand, beta turn and random coils. Protein Homology/analogy Recognition Engine V 2.0 (Phyre2) web portal for protein modeling was used to predict the 3D structure (Kelly *et al.,* 2015). SWISS-MODEL protein structure homology-modelling server was used to find structural homology of the protein (Schwede *et al.,* 2003).

Results and Discussion

Purity of the procured *Salmonella typhimurium* culture was confirmed using biochemical characterization and PCR analysis using *Salmonella typhimurium* specific gene primers. The culture was found to be MR+, VP-, Urease-. In PCR analysis 984 bp characteristic amplicon of *stm* gene was obtained. These results confirmed the identity of the strain.

PCR amplification of *omp*F gene

PCR Amplification of *omp*F gene was carried out using genomic DNA isolated from *S. typhimurium*, which resulted in a PCR amplicon of size 1092 bp (Fig. 1). The amplified product was purified using gel extraction kit, and cloned in to pCR 2.1vector using TA cloning kit and was transformed to One Shot TOP 10 Chemically Competent *E. coli* cells.

Screening of the transformed colonies on selective agar plates with ampicillin was done by colony PCR (Fig. 2). Plasmid with *omp*F gene insert was isolated using Pure Link Quick Plasmid MiniPrep Kit (Thermo Scientific, USA) and, sequenced at Agrigenome, Kochi Sequenced data with complete cds of *omp*F gene was submitted in

NCBI Gene Bank (Accession number MF593888).

Sequence analysis of *S. typhimurium ompF* gene

The complete *omp*F gene sequence of *Salmonella typhimurium* has shown 100 % identity to outer membrane sequences of many serovars of *Salmonella enterica* like *Typhimurium*, Heidelberg, Saintpaul etc. by NCBI BLAST search.

Bioinformatics analysis using ExPASy ProtParam analysis revealed that 363 amino acid containing OmpF protein is having 42.412 KDa size, of these 51 amino acids were strongly basic (+Ve) (K+ R), and 31 were strongly acidic (−Ve) (D+E) in nature with a theoretical pI value of 4.51. Instability index, aliphatic index and Grand average of hydropathicity (GRAVY) was found to be 13.22, 61.46, -0.607, respectively. Proteins with instability index less than 40 indicates that the stability of protein in test tube. Here the OmpF protein is having an instability index of 13.22, showing its stable nature.

It has been previously reported that, OmpF protein is resistant to high temperature and denaturing agents and preferably expressing at low osmolar conditions (Verma *et al.,* 2009). Aliphatic index of a protein is considered as positive factor for increasing the thermo stability of protein and GRAVY is indicative of hydrophobicity or hydrophilicity of protein. Both the parameters proved that this protein is thermo stable (61.46) and hydrophilic (-0.607) in nature. The estimated half-life of the protein is 30 hrs in mammalian reticulocytes (in vitro), >20 hours in yeast (*in vivo*) and >10 hrs in *E. coli* (*in vivo*).

Pfam analysis revealed that OmpF protein of *S. typhimurium* belongs to Gram negative porin family. Swiss model analysis showed, 92.8 per cent structural similarity with OmpF of *S. typhi*. Prosite analysis identified five motifs in OmpF protein of *S. typhimurium*. Casein kinase II phosphorylation site at five locations (47-50, 83-86, 127-130, 258-261, 263-266), single tyrosine kinase phosphorylation site (52-58), N-myristoylation site at nine locations (104-109, 141-146, 154-159, 166-171, 172-175, 194-199, 207-212, 288-293, 305-310), three protein kinase phosphorylation sites (150-152, 158-160, 215-217) and N- glycosylation sites at three locations (184-187, 156-259, 272-275). Antigenic characterization of translated protein sequence was done by VaxiJen v2.0 antigen prediction server (Doytchinova and Flower, 2007) and OmpF protein was found to be antigenic in nature with an overall antigen prediction score of 0.85. OmpF protein is found to be more antigenic on comparison with OmpC of *S. typhimurium* (score 0.73).

Protein structural and functional analysis

ExPASy translate tool translated the nucleotide sequences of *omp*F to deduce amino acid sequences. On comparison with deduced amino acid sequence of OmpF protein of *S. typhimurium* with other protein sequences available in Gene bank revealed that 100% homology with phosphorin (PhoE) protein of *Salmonella enterica*.

Secondary structure analysis using SOPMA tool, it was observed that in OmpF, four types of structures were present, of these 19.01 % were alpha helix, 28.37 % extended strands, 6.06 % beta turns and 46.56 % random coils (Fig. 3). On 3D structure using Phyre2 web portal for protein modeling, prediction and analysis software, output data had a confidence in the model: 333 residues (92%) modelled at >90% accuracy (Fig. 4).

Table.1 Major B-cell epitopes

No.	Start	End	Peptide	Peptide length
1	19	35	TANAAEIYNKDGNKLDL	17
2	38	38	K	1
3	40	40	V	1
4	44	95	WTTTGDSKNADQTYAQIGFKGETQINTDLTGFGQWEYRTKA DRAEGEQQNS	52
5	108	108	A	1
6	110	110	VGSIDYGRN	9
7	120	120	G	1
8	126	152	ESYTDMAPYFSGETWGGAYTDNYMTSR	27
9	154	154	G	1
10	176	198	QYQGKNQDNHSINSQNGDGVGYT	23
11	205	243	GFGVTAAYSNSKRTNDQQDRDGNGDRAESWAVGAKYDAN	39
12	252	254	AET	3
13	257	257	M	1
14	262	276	NTVTDTVEMANKTQN	15
15	294	313	SYVQSKGKQLNGAGGSADLA	20
16	317	318	QA	2
17	321	323	TYY	3
18	341	358	ENDYSSSYVGTDDQAAVG	18

Table.2 Prediction of T-cell epitopes of OMP of *S. typhimurium*

Allele	Start	End	Peptide	Percentile rank	IC50 value
H2-1Ab	140	154	AYEFDGFGVTAAYSN	2.86	530
	141	155	YEFDGFGVTAAYSNS	2.9	531
	139	153	MAYEFDGFGVTAAYS	3.35	623
	138	152	TMAYEFDGFGVTAAY	3.4	630
	137	151	YTMAYEFDGFGVTAA	3.49	637
	142	156	EFDGFGVTAAYSNSK	4.33	801
	143	157	FDGFGVTAAYSNSKR	4.38	791
	251	265	DLAKYIQAGATYYFN	6.44	784
	250	264	ADLAKYIQAGATYYF	6.46	778
	249	263	SADLAKYIQAGATYY	6.67	775
H2-1Ad	250	264	ADLAKYIQAGATYYF	8.66	4770
	180	194	YDANNVYLAAVYAET	9.51	1575
	247	261	GGSADLAKYIQAGAT	9.58	4292

Fig.1 PCR amplification of *omp*F gene of *Salmonella typhimurium*; Lane M: 1kb ladder, Lane 1-2: PCR amplicons of *omp*F gene (1092bp)

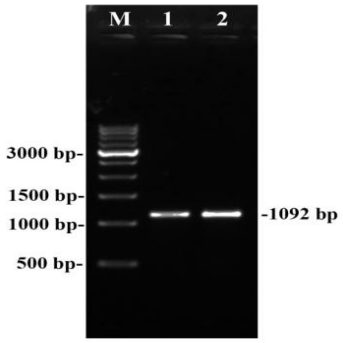


Fig.2 Colony PCR confirming *omp*F gene in pCR 2. 1vector; Lane M: 1kb ladder Lane 1-5: Positive transformed colonies, Lane 6: Positive control

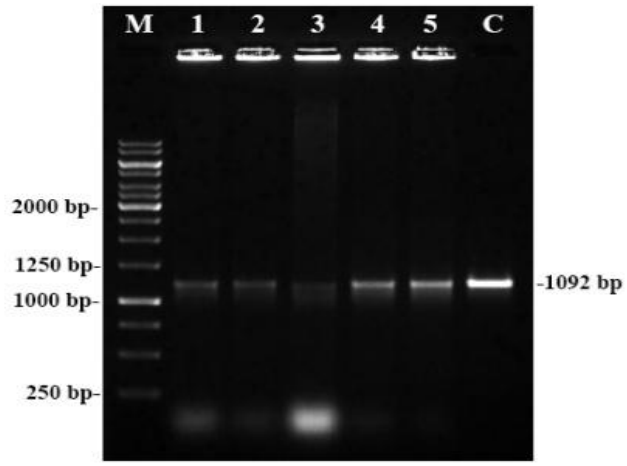


Fig.3 Secondary structure of OmpF of *Salmonella typhimurium*, with 4 types of secondary structures; Alpha helix (19.01 %), Extended strands (28.37 %), Beta turns (6.06%) and Random coils (46.56 %)

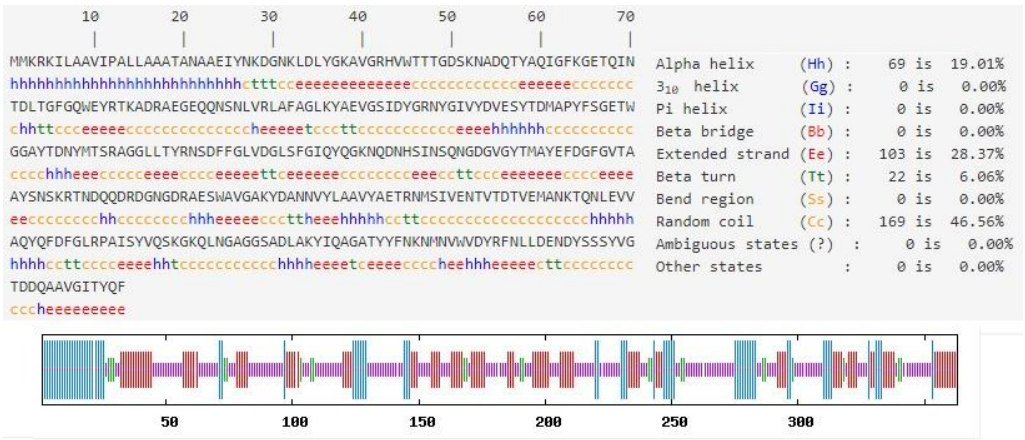


Fig.4 Predicted tertiary structure of OmpF protein of *Salmonella typhimurium* in three different angles generated using Phyre2 protein modeling tool

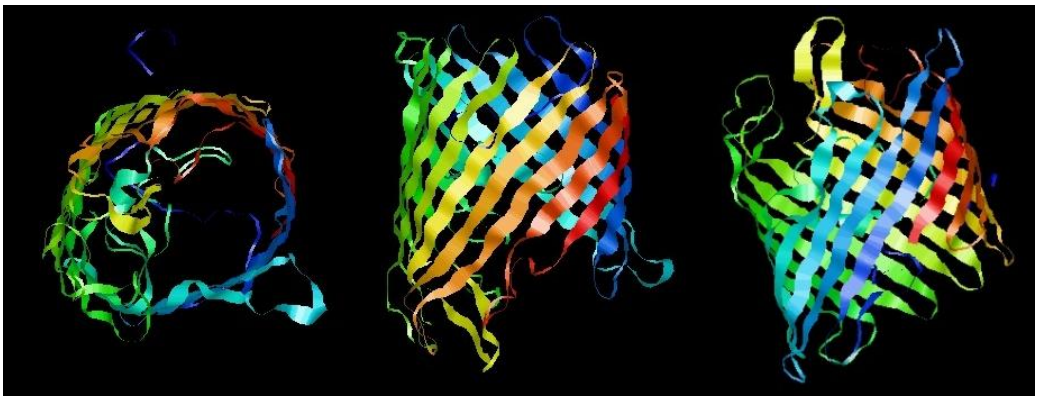


Fig.5 SWISS-MODEL analysis showing OmpF of *Salmonella typhimurium* having 99.4% sequence similarity with OmpF of *S. typhi* and has a similar stable homo-trimer 3D structure

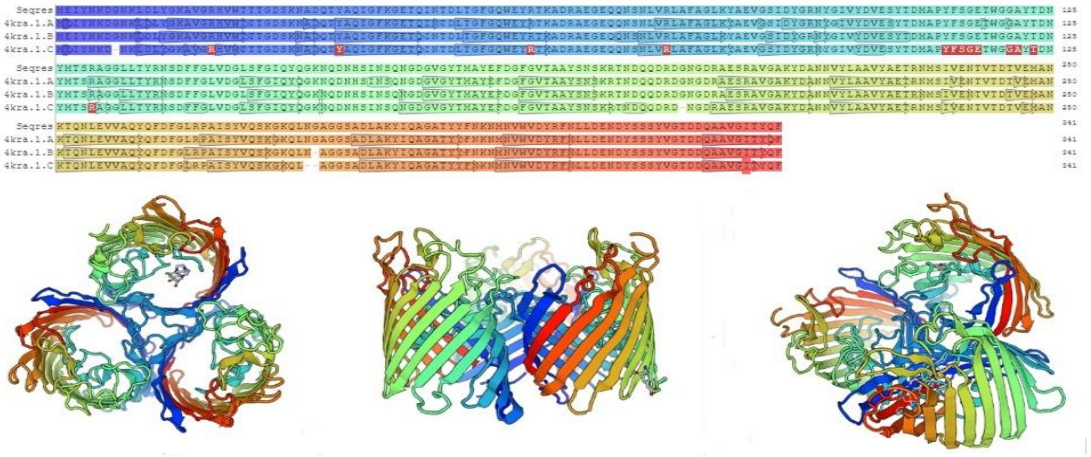


Fig.6 B-cell epitope prediction through IEDB online epitope prediction tool

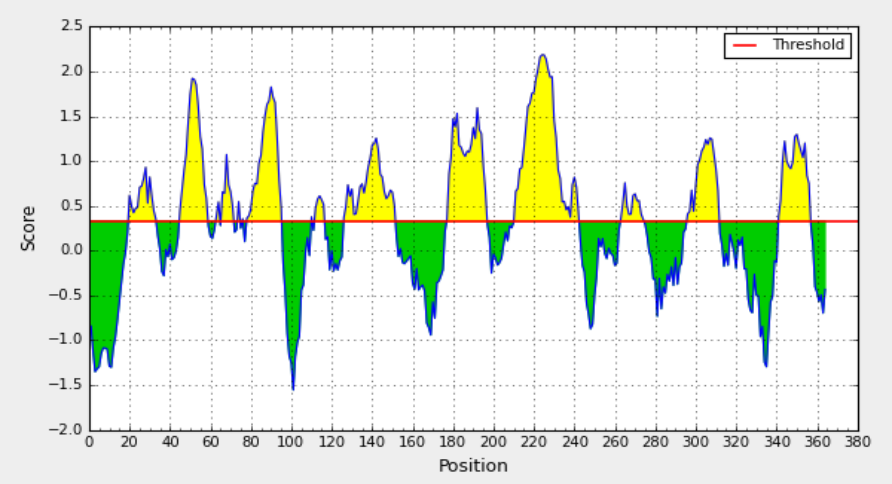
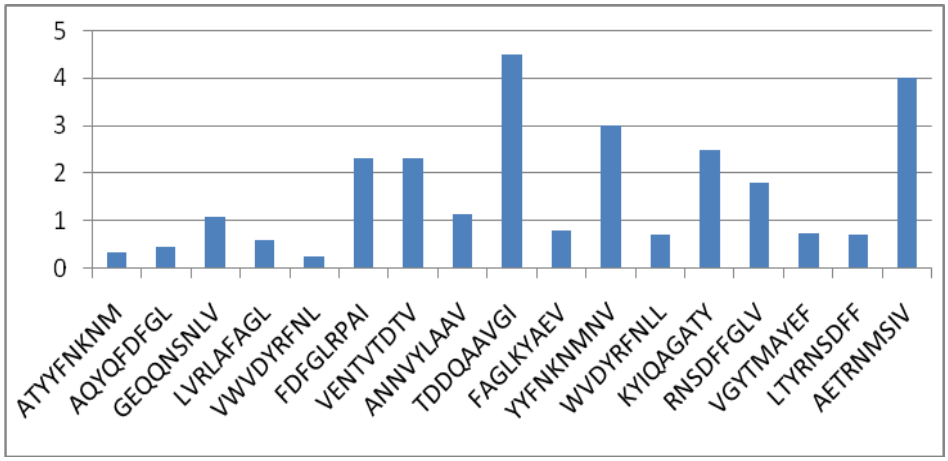


Fig.7 MHC I epitopes identified through IEDB epitope prediction tool



SWISS-MODEL analysis the *S. typhimurium* OmpF protein with known protein structures homology analysis showed 99.4 % similarity to *Salmonella typhi* OmpF, which indicate that the protein has a similar stable homo-trimer structure. The structure homology and 3D homo-trimer structure of OmpF protein is shown in Figure 5.

B-Cell epitope prediction

Major B cell epitopes of OmpF protein were evaluated for potential linear epitope using Bepipred prediction tool (Fig. 6). A total of 18 B cell epitopes were predicted by IEDB epitope prediction tool (Table 1). On analyzing the epitope prediction scores, 8 of the epitopes were found to be highly immunogenic in nature. An epitope having 10 or more amino acid are generally considered to be a good B cell epitope to elicit humoral immune response, in case Omp F protein we found very long epitopes making it a high potential vaccine candidate. In a similar study conducted in OmpC by Jha *et al.,* (2012) there were only 13 B cell epitopes, and the epitope scores were less in comparison to OmpF in this study.

T-cell epitope prediction

On MHC class I analysis using IEDB resource based on IC-50 value, 17 MHC-I significant epitopes were identified of these 4 were found to be high affinity epitopes (IC50 value <50 nM) and 13 epitopes with intermediate affinity (IC50 value <500 nM) (Fig. 7).

In case of MHC class II there are three different types H2-1Ab, H2-Ad and H2-Ed alleles in mice. A high affinity MHC binding epitope should have a percentile rank ≤ 10 and IC50 value at ≤1000nM. IEDB also recommended that peptide with low percentile rank have good binding capacity. In case of H2-1Ab, 10 high affinity binding sites were found and H2-1Ad has only three (Table 2). The interaction between MHC molecules in antigen presenting cells and predicted epitope binding site may have the ability to elicit T cell mediated immunity (Doytchinova and Flower, 2007; Schuler *et al.,* 2007). Reports of some studies stated that epitope having ability to trigger T-cell and B-cell mediated immunity could be beneficial to develop peptide based vaccine (Barh *et al.,* 2009; Barh *et al.,* 2010). Therefore presence of these epitopes in OmpF protein can make use of it as an epitope based sub-unit vaccine for broad range of *Salmonella* serovars. The presence of phenylalanine as the last residue (hydrophobic residue) at the C-terminus is highly conserved among outer membrane proteins. Phenylalanine at C terminus provide stability and proper assembly of protein into the outer membrane (Ruffolo *et al.,* 1996)

Surface proteins of *Salmonella* have been considered as a potential vaccine candidate. Some of the outer membrane protein had been studied and described its immunogenic potential (Jha *et al.*, 2012; Saxena *et al.*, 2012; Jha *et al.*, 2015).To date, there are only limited studies were conducted on OmpF protein of *Salmonella typhimurium*. The functional unit of OmpF porin is a homo-trimer and is formed by 16-stranded beta-barrel with three beta barrel monomers in its outer membrane (Balasubramaniam *et al.*, 2012). Trimer assembly and beta barrel are essential for epitope presentation.

This trimeric porin will be expressed in usual culture condition (Nikiado, 1994). Compared to *E coli, Salmonella* specific conformational epitope is more stable in nature (Arockiasamy *et al.*, 2004). The results from *in silico* analysis indicate that OmpF is having better properties on comparison with OmpC protein, with high immunogenic potential. Further investigations need to be carried out to understand the complete vaccine potential of OmpF protein, and this will reveal the full potential of the protein in future.

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