Fishing for vaccines against *Vibrio cholerae* using *in silico* pan-proteomic reverse vaccinology approach

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**Background:** Cholera, an acute enteric infection, is a serious health challenge in underdeveloped and developing world. It is caused by *Vibrio cholerae*, after ingestion of fecal contaminated food or water. Cholera outbreaks have recently been observed in regions facing natural calamities (i.e. earthquake in Haiti 2010) or war (i.e. ongoing civil war in Yemen 2016) where healthcare and sanitary setups have been disrupted as a consequence. Whole-cell Oral Cholera Vaccines (OCVs) have been in market but its regimen efficacy has been questioned. Reverse vaccinology (RV) approach has been applied as a successful anti-microbial measure for many infectious diseases.

**Methodology:** With the aim of finding new protective antigens for vaccine development, *V. cholerae* O1 (biovar eltr str. N16961) proteome was computationally screened in sequential prioritization approach that focused on determining antigenicity of potential vaccine candidates. Essential, accessible, virulent and immunogenic proteins were selected as potential candidates. The predicted epitopes were filtered for effective binding with MHC alleles and epitopes binding with greater MHC alleles were selected.

**Results:** In this study, we report lipoprotein *NlpD*, Outer membrane protein *OmpU*, Accessory colonization factor *AcfA*, Porin, putative and Outer membrane protein *OmpW* as potential candidates qualifying all the set criteria. These predicted epitopes can offer a potential for development of a reliable peptide or subunit vaccine for *V. cholerae*. 
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Abstract

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Introduction:

*Vibrio cholerae* is a prominent waterborne facultative pathogen which causes cholera disease which causes extreme dehydration and loss of electrolytes in patients [1]. Strains of *V. cholerae* O1 and O139 can be choleragenic. Further on O1 serogroup is divided into classical and El TOR biotypes [2]. Cholera is a notifiable endemic disease in developing and underdeveloped countries [3-6]. *V. cholerae* infections are a major factor with estimated annual global mortality around >100000 [7, 8]. The current seventh cholera pandemic is reported to be caused by Eltor Biotype strains while some regional epidemics have also been observed to have *V. cholerae* Eltor biotype strains as causative agents [9-11]. The toxigenic strains are capable of causing explosive outbreaks and epidemics in regions with devastated or poor sanitary infrastructure as observed in Haiti in 2010 [12-14]. Climate change and other factors have been noted to gain increased significance in outbreaks [4, 15]. Recent outbreaks of cholera are a result of poor sanitation, environmental pollution, natural and manmade disasters and unavailability of clean drinking water in affected areas [16-19]. Emergence and wide scale spread of antibiotic resistance in last six decades is a huge challenge [20-22]. Antimicrobial resistance has generally been a hindrance to the effective therapy of infectious diseases for as long as antibiotics have been used [23]. Despite the fact that during cholera treatment the antibiotics are limited as an adjunct to re-hydration, yet antibiotic usage has been observed to shorten the disease duration by 50% [24]. *V. cholerae* can also serve as reservoir for resistance mechanisms for horizontal transmission, as it is capable enough to procure and spread the resistance determinants via all forms of genetic transfer strategies [25-28]. A potent cholera vaccine could be effective in natural disasters or other humanitarian situations as it can provide immunity when given preventively.

Concurrent strategies have been aimed at development of oral formulations capable of imparting mucosal immunity. Few anti-cholera oral formulations were tested in humans. An early study developed formulations comprising of cholera toxin B-subunit and inactivated bacterial cells was tested from 1985 to 1989 in Bangladesh [29]. A recent clinical trial administered O-specific polysaccharide (OSP) to human subjects and demonstrated anti-OSP and vibriocidal antibody responses [30]. In another study long-term efficacy and protection was assessed for killed bivalent, whole-cell oral cholera vaccine in Haiti [31]. This study reported decrease in effectiveness of single dose oral vaccine in comparison to two doses over the period of 4 years. Currently various strategies have been employed to develop live attenuated cholera vaccines. A recently published
study reported development of a genetically engineered *V. cholerae* O1 strain CVD 103-HgR as a live attenuated vaccine [32]. Recently published Phase 3 clinical trial (Clinicaltrials.gov NCT02094586) of live oral cholera vaccine reported 94% vibriocidal antibody seroconversion rate 6 months post-vaccination [33]. This single dose cholera vaccine was developed using attenuated recombinant *Vibrio cholerae* O1 vaccine strain CVD 103-HgR. This clinical trial recruited over 3000 adult volunteers with 90% more efficacy in comparison to placebo group. One serious concern is regarding the safety of the vaccines. Yet similar formulations had faced efficacy and performance issues [3, 34, 35]. Possibility of horizontal gene transfer and reversion of live attenuated vaccine forms back to wild types with virulence spectrum and antibiotic resistance could aggravate the situation [36]. Under special conditions viral live attenuated vaccines have been reported to result in adverse effects [37-39].

Reverse vaccinology (RV) approach is the vaccine development strategy in the genomics era. This approach predicts vaccine candidates by screening genome and proteome, evaluates using algorithms and computational tools for proteins with best suitable properties as a potential vaccine agent [40]. In contrast to conventional vaccine development strategies, RV strategy provides rapid vaccine design and reduces the dependency on conventional animal testing based screening for getting a potentially suitable candidate. A number of vaccines have been developed for pathogens such as *Streptococcus pneumoniae* [41], serogroup B *Neisseria meningitides* (MenB) [42], *Cryptosporidium hominis* [43], *Mycobacterium tuberculosis* [44], and *Bacillus anthracis* [45].

This study is aimed at identification of extracellular and outer membrane proteins that can serve as better antigen targets for *V. cholerae*. We report lipoprotein NlpD, Outer membrane protein OmpU, Accessory colonization factor AcfA, Porin, putative and Outer membrane protein OmpW as potential candidates qualifying all the set criteria. These predicted epitopes can offer a potential for development of a reliable peptide or subunit vaccine for *V. cholerae* in calamities hit regions as preemptive preventive protection. This is the first study to report vaccine target prediction using reverse vaccinology and reductive screening approach against *V. cholerae* O1 biovar eltor.
Methodology

We adopted our previously devised computational framework (Fig. 1) that includes three comprehensive steps for prediction of prospective vaccine candidates for *V. cholera* as described in detail [46].

1. **Pre-Screening of primary data**

   The steps involved Pre-Screening of primary data include retrieval of *V. cholerae* O1 (biovar eltr str. N16961) proteome from uniprot [47]. Subcellular localization was predicted using the primary sequences of the *V. cholerae* proteome PSORTb V3.0 [48] and CELLO v2.5 [49]. Database of Essential Genes (DEG) (http://tubic.tju.edu.cn/deg/) version 10.4 provided the essentiality information of the proteins [50]. Virulence check was performed using Virulence Factor Database (VFdb) for identification of potential virulence proteins [51]. These steps were adopted to identify vital virulence proteins and respective epitopes to be subjected to peptide vaccine discovery.

2. **Screening of selected proteins**

   Screening of selected proteins was performed for their suitability of prospective immuno-protective potential. The criteria included appropriate molecular weight (<110 kDa estimated via ExPASy Compute pI / Mw Tool [52]), prediction of antigenic and virulence potentials, protein structural details and human homologue search. The crystalline structures for these proteins were obtained from structural database Protein Data Bank (PDB) [53] or developed using SWISS-MODEL server [54] and interactions within pathogen and with host proteins and cluster of orthologous groups COG were studied using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) [55].

3. **Epitope Selection**

   In third step epitopes were predicted using multiple approaches via different algorithms in order to obtain broad spectrum epitopes. The predicted epitopes were screened to obtain epitopes capable of efficient binding to higher numbers of MHC alleles [56]. *Continuous B-Cell Epitopes* were predicted using BcePred server [57]. BCPreds server was employed for prediction of 20-mer B-cell epitopes [58]. ABCpred, an artificial neural network based
B-cell epitope prediction server was also used for predicting B-cell epitopes [57]. Default threshold values were used for each server. Propred and PropredI servers were used to investigate epitope interactions with MHC I and II alleles [59]. While antigenicity and IC50 calculations were performed with the help of MHCPred [60]. Using proteins’ three dimensional epitopes were visualized using Discovery studio v4.1 [61].

Finally sequences of selected proteins from other virulent strains were obtained from members of *V. cholerae* NCBI Taxonomic group (TAXID: 666). The predicted antigenic regions were analyzed via BioEdit Sequence Alignment Editor, for sequence divergence against *V. cholerae* representative strains and consensus sequences were obtained for respective vaccine candidate for inter-strain immune-protection against *V. cholerae*. 
Results:

Primary data retrieval

We selected *V. Cholerae* O1 biovar eltor str. N16961 as a reference strain for our vaccine prediction strategy. Unlike other prokaryotes, *V. cholerae* contains two circular chromosomes. It is a unique biotype due to hemolysin production. Using the virulence factor database (VFDB), proteomic data of virulent strain of *V. Cholerae* was obtained. Genomic visualization of curated virulence factors was performed using server ‘Island Viewer 4: An integrated interface for computational identification and visualization of genomic islands’ (http://www.pathogenomics.sfu.ca/islandviewer/), as shown in Figure 2 [62].

Subcellular Localization of Screened Targets

Subcellular localization is the most critical screening criterion. Antigens exposed at the surface are more accessible to immune system. We scrutinized proteins exposed at pathogen’s surface with potential role as antibiotic resistance determinants. Proteomic sequences were subjected to subcellular localization analysis which is a crucial step in screening out potent vaccine candidates' identification. The proteome was screened based on subcellular location, number of transmembrane helices and minimum adhesion probability. In total, 47 proteins (*Supplementary Table 1*) were predicted as potential vaccine candidates consisting of 21 outer membrane, 19 extracellular and 7 periplasmic proteins as shown in Figure 3. These proteins had less than 1 transmembrane helices and an adhesion probability greater than 0.51, the cut off value to assign a protein as an adhesin. Moreover, these proteins showed no similarity to human proteins [63]. The antigenicity scores were predicted using VaxiJen v2.0 server to further refine the selection. This software predicted antigenicity of proteins from FASTA-submitted amino acid sequences based on their physiochemical properties. This feature is characterized according to an antigenic score. Our Vaxijen analysis predicted 45 antigenic potential vaccine candidates out of 47 proteins with antigenicity scores greater than 0.41. An antigenicity score of over 0.40 indicates protein antigenicity [64]. To be more specific we selected proteins giving the antigenicity score equal to or greater than 0.7. As a result we obtained 5 prioritized proteins, details in *Table 1*.

3. PPI interactions and COG analysis:
The predicted proteins were studied for their potential biological roles and proteomic interactions. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) provides essential information regarding interactions of desired proteins [65].

Intra-specie protein-protein interactions were calculated for the selected proteins using STRING database online network analysis tools Figure 4. STRING database also provided the Cluster of Orthologous Groups (COG) analysis tools. COGs analysis was conducted on the basis of protein sequence similarity and conserved domains in comparison to reported proteins in the database.

For COG protein functional categorization, 4 out of the given 5 proteins fall into “outer membrane/membrane” group whereas 1 was identified as ATPase-coupled sulfate with transmembrane transporter activity.

3D Structures of Prioritized Vaccine Candidates:

Structural information is vital for proteinaceous targets before predicting immunogenic domains. Availability of crystalline structures for the selected protein was checked in experimental structural database Protein Data Bank (PDB) [66]. One crystalline structure available for protein NlpD (PDB id 2gu1) was retrieved. For other selected proteins suitable templates were searched within PDB. Protein structures were predicted using SwissModel server via homology modelling approach. 3D structures of the Prioritized protein targets are given in Figure 5.

Predicted Prioritized Vaccine Targets

Epitope mapping

Peptide vaccines are more convenient and safer than the contemporary vaccines. As it includes only the immunogenic epitopes rather than full three dimensional structures obtained from pathogens. Immunogenic potential is primarily dependent on Major Histocompatibility Complex (MHC) binding affinity. Thus predicting the epitopes with higher binding potential for MHCs is necessary to design peptide vaccines [56]. The prioritized proteins were subjected to primary sequence based antigenic and virulence epitopes prediction. Since there are multiple algorithms for prediction of antigenic epitopes, thus multiple servers were used for evaluation of selected vaccine candidates. Primary sequences of the proteins were subjected to alignment
independent antigenic prediction based on physicochemical properties of proteins. Proteins having score > 0.4 were considered antigenic. The resultant antigenic proteins were subjected to further studies.

Out of the 47 predicted *V. cholerae* vaccine candidates, proteins with the antigenicity score greater than 0.7 were filtered through VaxiJen 2.0. Only the epitopes with P value greater than 0.9 were selected for each protein and antigenicity scores were further analyzed specific for all epitope sequences. MHCPred was used for antigenicity and IC\(_{50}\) calculation for the selected epitopes [67]. MHCPred covers a range of different human MHC allele peptide specificity models. These include Class I (HLA-A\(^*\)0101, HLA-A\(^*\)0201, HLA-A\(^*\)0202, HLA-A\(^*\)0203, HLA-A\(^*\)0206, HLA-A\(^*\)0301, HLA-A\(^*\)1101, HLA-A\(^*\)3301, HLA-A\(^*\)6801, HLA-A\(^*\)6802 and HLA-B\(^*\)3501) and Class II (HLA-DRB1\(^*\)0101, HLA-DRB1\(^*\)0401 and HLA-DRB1\(^*\)0701) alleles [67]. Moreover, MHC II epitopes were studied in detail using EpiDOCK that predicts binding to the 23 most frequent human MHC class II proteins: 12 HLA-DR, 6 HLA-DQ and 5 HLA-DP proteins. These alleles cover more than 95% of the human population. EpiDOCK is freely accessible at: [http://epidock.ddg-pharmfac.net/](http://epidock.ddg-pharmfac.net/). The epitopes were prioritized based on the number of binding alleles to the given epitope sequences. Consequently, 10 epitopes were prioritized Table 2.

The prioritized epitopes were aligned with the available strains of *V. cholerae*. Sequences of the potential targets were obtained from 100 members of *V. cholerae* NCBI Taxonomic group (TAXID: 666). The predicted antigenic regions were analyzed via BioEdit Sequence Alignment Editor, for sequence divergence against *V. cholerae* representative strains and consensus sequences were obtained for respective vaccine candidate. The multiple sequence alignment for the selected epitopes showed that these peptide sequences are conserved in *V. cholerae*, as shown in Figure 6.
Discussion

In this study we adopted reverse vaccinology based reductive screening and fished out 5 immunogenic proteins harboring 10 peptide epitopes as potential vaccine candidates in *V. cholerae* proteome. Reverse vaccinology is a genome/proteome based approach for vaccine development that has been proved effective [68]. Reductive screening is performed based on parameters i.e. protein essentiality, subcellular localization, host homology and effective immunogenicity for predicting an effective vaccine candidate. Computer aided screening process is more convenient, accurate and fast in comparison to the contemporary vaccine development which depends on hit and trial approach. This strategy studies key aspects of the pathogen i.e. genome, essential metabolism, virulence and protein-protein interactions and incorporates this information for determining the prospective vaccine candidates prior to any wet lab experimentations [56]. One of the key limitation is that this strategy is primarily focused on prediction of peptide epitopes based on amino acid sequences of the proteins. Hence the long known immunogenic potential of nonprotein antigens (i.e. Lipopolysaccharides) couldn’t be accounted in this strategy [69-71]. But addition of such known epitopes as adjuvants is a good overcoming approach for this limitation [72, 73]. Another prominent limitation could be the high mutation rate of the viral surface proteins [74, 75]. The prospects of reverse vaccinology approach have been discussed in detail in our previous study [46].

Peptide vaccines theoretically have several advantages over conventional and recently developed DNA vaccines [76]. Lesser cost and convenient synthesis with improved safety and stability are the key features which have been demonstrated in various studies [77, 78]. Conventional vaccines are overburdened with unnecessary antigens which divert immune response resources thus might result in a chaos which lacks the required dedicated for eliminating the threat thus impedes the vaccine efficacy [79]. As in case of cholera, whole cell vaccines were only able to impart varying protective efficiency (39-60%) in studies conducted in Bangladesh and Vietnam [80, 81]. While live attenuated vaccine was unsuccessful in generating long term protective response [82]. One interesting inconsistency is the comparative efficacy of cholera vaccines in developed and developing countries [83]. While a notable recent exception was observed in South Sudan [84].
Considering these factors, a novel strategy was vital to achieve protection against this pathogen.

Reported prioritized targets included lipoprotein \textit{NlpD}, outer membrane protein \textit{OmpU}, accessory colonization factor \textit{AcfA}, putative porin, and outer membrane protein \textit{OmpW}. These predicted proteins are involved in important virulence mechanisms of \textit{V. cholerae}. Role of lipoprotein \textit{NlpD}, has been studied in reference to cell division and intestinal colonization by the pathogen. Septal peptidoglycan (PG) amidase, \textit{AmiB} is involved in separation of daughter cells at the end of cell division process [85]. \textit{AmiB} is regulated by \textit{NlpD} in \textit{V. cholerae} [86]. Both of these processes are important for pathogen’s survival in host intestine. Another predicted potential target accessory colonization factor \textit{AcfA} is of peculiar interest as it has been subjected to edible vaccine [87]. Targeting \textit{NlpD} and \textit{AcfA} could provide passive therapeutic potential as immune inactivation would impede the pathogen’s ability to colonize and multiply in the small intestine.

Among these vaccine candidates, we obtained 2 outer membrane proteins (OMPs), \textit{OmpU} and \textit{OmpW} that also serve as antibiotic resistance determinants. In vibrio species OMPs are studied to play vital roles as porins in iron, phosphate and sugar acquisition as well as in bacterial attachment to solid surfaces [88]. While \textit{OmpU} has been reported to be involved in conferring polymyxin B sulfate resistance [89]. We consider \textit{OmpU} as an important vaccine candidate selected via our computational framework as it is not only involved in host cell invasion but also confers antibiotic resistance [90]. Moreover, it has also been used as an effective vaccine candidate in other vibrio species such as \textit{V. alginolyticus} and \textit{V. harveyi} in \textit{Lutjanus erythropterus} and \textit{Scophthalmus maximus}, respectively [91, 92]. Such studies provide good examples of how a reverse vaccinology strategy can be used for systematic vaccine design against drug resistant microbial pathogens.

Another important predicted potential vaccine candidate is \textit{OmpW}. It’s a characteristic outer membrane protein expressed by \textit{V. cholerae} and has been used to identify infectious agent via different PCR based detection techniques. Studies have reported this protein to be conserved and harbors immunogenic properties [93, 94]. Considering its abilities, \textit{OmpW} could be a good candidate for developing a broad spectrum and effective vaccine.

Interestingly when we analyzed our screened results with a recent antibody profiling study of the \textit{V. cholerae} O1 protein immunome, 9 overlapping antigens were observed [3]. These antigens were: Organic solvent tolerance protein (VC0446), outer membrane protein \textit{OmpU} (VC0633), toxin co-regulated pilin (VC0828), outer membrane protein \textit{OmpV} (VC1318), neuraminidase...
(VC1784), hemolysin-related protein (VC1888), and flagellar proteins/components (VC2142, VC2143, VC2187). Among these 9, outer membrane protein OmpU (VC0633) was common in the most effective antigens reported in the final selection of the both studies. While toxin co-regulated pilin (VC0828) was among our initial screening list but it is reported as one of the most effective by RC Charles et. al. One possible reason of the screening results could be the difference in the adopted screening strategies. Our strategy was purely computational which derived all the calculations using only peptide sequences of the proteins which has short coming as it could only be applied for peptide antigens while on the other hand antigens other than proteins do have their immuno-protective potential i.e. the O polysaccharide, LPS, etc. These overlapping proteins in the two investigations provide confidence to our prediction.
Conclusion

With the aim of finding new protective antigens for vaccine development, in this study, we report lipoprotein NlpD, Outer membrane protein OmpU, Accessory colonization factor AcfA, Porin, putative and Outer membrane protein OmpW as potential candidates qualifying all the set criteria. These predicted epitopes can offer a potential for development of a reliable peptide or subunit vaccine for *V. cholerae*. 
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Figure 1

Reverse vaccinology screening process overview.

Reverse vaccinology approach based computational framework for prediction of vaccine candidates for Vibrio cholerae O1 (biovar eltr str. N16961) [34].
Figure 2

*V. Cholerae* O1 El Tor Genomic Analysis for virulence and antibiotic resistance genes.

Dark and light purple dots represent the curated virulence factors and antibiotic resistance genes in the both *V. cholerae* chromosomes. The orange and blue bars represent the algorithms used by server for prediction of genomic islands i.e. SIGI-HMM, IslandPath-DIMOB respectively. The red bar represents the integrated results for all the methods used. The inner most circle indicates the GC skew for both chromosomes. The Figure was generated using IslandViewer 3 (http://www.pathogenomics.sfu.ca/islandviewer/).
Figure 3

Subcellular Localization of Screened Targets:

Screening process yielded total of 47 proteins as potential vaccine candidates. Subcellular localization yielded after CELLO analysis (EC; extracellular, OM; outer membrane & P; periplasmic).
Figure 4

Protein – Protein Interaction Analysis:

Prioritized protein targets were subjected for PPI assessment using STRING database. (A) Interaction suggests involvement of OmpC and OmpW in flagellar development and adhesion Inflammation (B).
Figure 5

3D structures of prioritized proteins

Predicted and crystal structures of prioritized proteins. NlpD had predetermined crystal structure in PDB which was retrieved (PDB id 2gu1). For the rest of the proteins homology models were predicted using Swiss Model server.

*Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.
Figure 6

Multiple protein sequence alignment of protein targets was performed among 100 members of V. cholerae NCBI Taxonomic group (TAXID: 666).

Colors indicate the following: red: residue ED; yellow: residue G; blue: residue KR; turquoise : residue YFA; magenta: residue H; green: residue LIMV; grey: others. The sign “—”(dash) means no amino acid aligned.
**Table 1** (on next page)

List of predicted prioritized *V. cholerae* vaccine candidates.

Details of predicted *V. cholerae* vaccine candidates based on genome sequence analysis.
Table 1. Details of predicted *V. cholerae* vaccine candidates based on genome sequence analysis.

| Protein Accession  | Protein name                         | Localization     | Adhesin Probability | Transmembrane helices | Antigenicity | Pfam domains | Functional description                         |
|--------------------|--------------------------------------|------------------|---------------------|------------------------|--------------|--------------|-----------------------------------------------|
| NP_230184.1        | lipoprotein NlpD                      | Outer Membrane   | 0.654               | 0                      | 0.7878       | PF01476      | Membrane protein                              |
| NP_230282.1        | outer membrane protein OmpU          | Outer Membrane   | 0.563               | 0                      | 0.74         | PF00267      | Outer membrane protein (porin)                |
| NP_230492.1        | accessory colonization factor AcfA    | Outer Membrane   | 0.550               | 0                      | 0.7709       | PF13505      | ATPase-coupled sulfate transmembrane transporter activity |
| NP_231488.1        | porin, putative                      | Outer Membrane   | 0.518               | 0                      | 0.7463       | PF13609      | Outer membrane protein (porin)                |
| NP_233253.1        | outer membrane protein W             | Outer Membrane   | 0.640               | 0                      | 0.7774       | PF03922      | Outer membrane protein                        |
Table 2 (on next page)

List of Prioritized Epitopes.

Prioritized *V. cholerae* vaccine candidates based on epitope mapping
Table 2. Prioritized *V. cholerae* vaccine candidates based on epitope mapping.

| #  | MHC class | Index | Epitope          | Antigenicity |
|----|-----------|-------|------------------|--------------|
| 1  | MHCI      | 6     | LYSFRLGLLL       | 1.4424       |
|    | MHCII     | 3     | GLLLFCSLL        | 1.5179       |
| 2  | MHCI      | 3     | YSDNGEDGY        | 1.6821       |
|    | MHCI      | 6     | SYISYQFNL        | 1.8449       |
|    | MHCII     | 3     | YISYQFNLL        | 1.5362       |
| 3  | MHCI      | 6     | ALFSLGLDY        | 1.6604       |
|    | MHCII     | 6     | FSFEINYSS        | 1.5186       |
| 4  | MHCI      | 2     | YGDGTTLGY        | 1.8772       |
|    | MHCI      | 3     | RTRNSHIKK        | 1.9183       |
| 6  | MHCII     | 1     | TFMVQYYFG        | 1.4084       |