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Subtractive genomic approach toward introduction of novel immunogenic targets against *Clostridioides difficile*: Thinking out of the box

Narjes Noori Goodarzi [a], Sepideh Fereshteh [b], Omid Azizi [c], Hamzeh Rahimi [d], Negin Bolourchi [b], Farzad Badmasti [b,e,*]

[a] Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[b] Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran
[c] Health Sciences Research Center, Torbat Heydariyeh University of Medical Sciences, Torbat Heydariyeh, Iran
[d] Department of Molecular Medicine, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran
[e] Microbiology Research Center (MRC), Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT

Clostridioides difficile is one of the major causatives of nosocomial infections worldwide. Antibiotic-associated diarrhea, pseudomembranous colitis, and toxic megacolon are the most common forms of *C. difficile* infection (CDI). Considering the high antibiotic resistance of *C. difficile* isolates and the low efficacy of immunization with toxin-related vaccines, we suggested that surface-exposed and secreted proteins could be considered as potential immunogenic targets against CDI. Various immuninformatics databases were used to predict antigenicity, allergenicity, B-cell epitopes, MHC-II binding sites, conserved domains, prevalence and conservation of proteins among the most common sequence types, molecular docking, and immunosimulation of immunogenic targets. Finally, 16 proteins belonging to three functional groups were identified, including proteins involved in the cell wall and peptidoglycan layer (nine proteins), flagellar assembly (five proteins), spore germination (one protein), and a protein with unknown function. Molecular docking results showed that among all the mentioned proteins, WP_009892971.1 (Acd) and WP_009890599.1 (a C40 family peptidase) had the strongest interactions with human Toll-like receptor 2 (TLR-2) and TLR-4. This study proposes a combination of *C. difficile* toxoid (Tcd) and surface-exposed proteins such as Acd as a promising vaccine formulation for protection against circulating clinical strains of *C. difficile*.

1. Introduction

Clostridioides difficile is the most common causative agent of hospital-related infectious diarrhea leading to increased length of hospital stay, and costs. This microorganism's mortality is reported to be 6% on average and up to 13% in people over 80 years old [1]. *C. difficile* infection (CDI) mainly occurs following the administration of broad-spectrum antibiotics, and consequently dysbiosis in the gut microbiota [2,3]. *C. difficile* leads to a wide range of diseases from mild antibiotic-associated diarrhea to pseudomembranous colitis (PMC) and even more severe complications such as toxic megacolon and septic shock [4]. It was demonstrated that hospitalized individuals > 65 years old with recent antibiotic exposure are at the highest risk for *C. difficile* infections. Moreover, patients with inflammatory bowel disease (IBD) are also at increased risk of developing CDI. Although *C. difficile* as an anaerobe bacterium cannot tolerate on non-living surfaces for more than 15 minutes, the long survival of its spores on hospital inanimate surfaces and thier high resistance to disinfectants, leads to cross-contamination of patients with *C. difficile* spores, especially in the elderly [5]. The first way to manage CDI is to prescribe alternative antibiotics such as vancomycin and metronidazole. Even though vancomycin is the first-line therapy for severe CDI [6], there have been cases of reduced sensitivity of *C. difficile* isolates to this antibiotic recently [7,8]. More importantly, recurrent infections occur after antibiotic treatment in 20–30% of cases. In addition, half of patients with the recurrent infection, experience a second recurrence after the first recurrence [9].

Vaccination can be a reasonable alternative to combat antimicrobial resistance. In recent years, multiple toxin-based vaccines have been introduced and undergone clinical trials. Sanofi Pasteur toxoid, CDIFFENSE, a formalin-inactivated formulation of toxin A, and toxin B from

* Corresponding author. Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran.
E-mail address: fbadmasti2008@gmail.com (F. Badmasti).

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VPI 10463 strain were able to prevent CDI recurrences in the clinical trial phase II [10,11]. VLE 84, a recombinant fusion protein of toxin A, and B showed satisfying clinical trial results in phases I and II [12]. Pfizer, another recombinant toxin-based vaccine, has completed the trials of phases I and II, and phase III [13,14]. Even though vaccines and passive immunotherapies targeting toxins have shown promising results in reducing CDI recurrences, little is known about the toxoids' efficacy [15]. On the other hand, *C. difficile* toxoid vaccines only prevent toxin-mediated manifestations due to the neutralization of toxins, and thus are unable to prevent intestinal colonization of *C. difficile* [4]. Even, they may lead to an asymptomatic carriage and increased environmental dissemination of the spores [16]. Passive immunization against *C. difficile* toxins is an alternative and its effects are not a long-lasting journey. Thus, the development of vaccines targeting bacterial surface structures, involved in binding and colonization, can prevent CDIs and recurrences. To date, several recombinant proteins have been developed for active immunization against CDI including CdeC, CdeM [17], LMW- and HMW-SLPs [18], Cwp84 [19], FliC [20], GroEL [21], and Pilin [22]. However, some of these vaccine candidates did not develop a satisfactory immunity [19–22].

With the beginning of the genomics era, a great revolution in immunization has taken place. Genomic information has dramatically facilitated investigation of immunogenic targets to develop new protective vaccines against pathogenic microorganisms [23,24]. In comparison to traditional vaccine development methods, computational immunoinformatics using genomic data leads to more accurate investigations of vaccine candidates with lower processing time and costs [25]. Moreover, reverse vaccinology prompts the identification of novel unknown antigens, as well as the previously characterized antigens. This approach helps us to select proteins with B-cell and T-cell response promoting epitopes [26]. Hence, in this project, we explored new putative immunogenic candidates using a practical reverse vaccinology approach based on three different strategies, including known virulence factors in VFDB and literature, automated and manual reverse vaccinology.

2. Materials and methods

2.1. Identification of putative immunogenic candidates using three different strategies

2.1.1. Virulence factors and literature-based data mining

Known virulence factors of *C. difficile* were extracted from the Virulence Factor Database (http://www.mgc.ac.cn/VFs/) [27]. Other newly identified virulence factors were also extracted from the literature. Finally, the protein sequences were saved in FASTA format for further analyses.

2.1.2. Manual reverse vaccinology

The whole proteome of *C. difficile* strain R20291 was downloaded from the BacMap database (http://bacmap.wishartlab.com/) as the reference strain [28]. The proteome was saved in FASTA format and submitted in subcellular localization web tools such as LocTree3 (https://rostlab.org/services/loctree3/) [29], pLoc_bal-mgpos (https://www.ploc_bal-mgpos.com/) [30], and PSORTb (www.psort.org/psortb/) [31]. Only secreted and surface-exposed proteins were selected.

2.1.3. Automated reverse vaccinology

Automated reverse vaccinology was performed using the Vaxign database (http://www.violinet.org/vaxign/). In this analysis, the genome sequences of 13 *C. difficile* strains (all available strains in the Vaxign database) selected. *C. difficile* strain R20291 (belongs to ST1) was considered as the reference strain. The criteria were considered as follows: the number of transmembrane helices ≤ 1, adhesion probability > 0.51, and no similarity to human and mouse proteins [32–34]. Subcellular localization was assessed for gene-coding proteins, and only extracellular and secreted proteins were considered. All proteins of the three mentioned rounds were collected and the redundant proteins were removed and prepared for further analysis.

2.2. Consecutive protein analysis

2.2.1. Antigenicity and allergenicity determination of proteins

The antigenicity of secreted and surface-exposed proteins was determined using the VaxiJen tool (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with the cut-off ≥ 0.4 [35]. Allergenicity of the antigenic proteins was assessed using the Algpred 2.0 tool (https://webs.iiitd.edu.in/raghava/algpred2/batch.html) with cut-off ≥ 0.5 [36].

2.2.2. Sequence similarity of putative immunogenic targets against hosts proteomes

The sequence similarity of the above proteins against the host (*Homo sapiens*, taxid:9606 and Mus, taxid:10088) was investigated using the PSI-BLAST tool in the NCBI-BLASTP database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) [37]. Proteins that had similarity with the hosts were excluded from the study.

2.2.3. Determination of linear and conformational B-cell epitopes

Linear epitopes were identified using BepiPred v2.0 (http://www.cbs.dtu.dk/services/BepiPred/) with a threshold of ≥ 0.61 [38]. For each protein, the B-cell epitopes' ratio to the total number of amino acids was calculated (the number of amino acids in the B-cell epitope divided into total amino acids of each protein). Finally, proteins that were above the average ratio were selected. Tertiary structures (3D) of putative immunogenic candidates were characterized by the Robetta tool (https://robetta.bakerlab.org/) [39]. In the next step, ElliPro (http://tools.iedb.org/ellipro/) was used to identify the conformational B-cell epitopes with threshold of ≥ 0.8 [40].

2.2.4. Determination of MHC- II binding sites

T-cell epitopes were identified using the Tepitool (http://tools.iedb.org/tepitool/) in immune epitope database (IEDB) [41]. Human MHC-II binding sites were identified with a cut-off of the top 5% of peptides. For each protein, the MHC-II binding sites ratio to the total number of amino acids was calculated (the number of binding sites divided into total amino acids of protein). Finally, proteins that were above the average ratio were considered and the rest of the proteins were excluded.

2.3. Prevalence and conservation of immunogenic candidates

2.3.1. Prevalence of selected proteins

There were 54 completed genomes of *C. difficile* in the Genbank database. The genomes with completed annotation were downloaded using the CLC Genomics Workbench software (Qiagen, Hilden, Germany). The sequence types (STs) of selected strains were determined using the MLST 2.0 database (https://cge.cbs.dtu.dk/services/MLST-2.0/) [42]. Forty-four genomes which had common STs of *C. difficile* (including ST11, ST8, ST42, ST3, ST39, ST1, ST2, ST48, ST37, ST5, and ST54), were considered. See Supplementary Table 1. They were selected to assess the prevalence and conservation of selected proteins. NCBI protein-protein BLAST tool was used to detect the putative immunogenic candidates. The sequence of each protein in selected STs were saved in FASTA format and aligned using MegaX software for further investigations [43].

2.3.2. Conservancy analysis of selected epitopes

The conservation of continuous and discontinuous B-cell epitopes among selected genomes was investigated using the ConSurf web tool (https://consurf.tau.ac.il/) [44].

2.4. Protein domain search and physicochemical characteristics detection of putative immunogenic candidates

The conserved domain database, CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), was used to find the major protein domains. CDD is a part of NCBI's Entrez query and provides the annotation of protein sequences with the conserved domain's locations [45].

Further analyses of immunogenic candidates were performed via different databases. The number of amino acids, molecular weight, theoretical pI, estimated half-life, aliphatic index, and instability index were determined using the Expasy ProtParam server (https://web.expasy.org/protparam/) [46]. Alpha helix, beta-strand, disordered, and transmembrane (TM) helices were evaluated using the Phyre2 database (www.sbg.bio.ic.ac.uk/~phyre2/) [47]. The number of disulfide bonds was determined using the Prosite-Expasy database (https://prosite.expasy.org/). Adhesion probability was calculated using the Vaxign database (http://www.violinet.org/vaxign2) [33]. The functional class of proteins was predicted using the VICMpred database (http://www.imtech.res.in/raghava/vicmpred/).

2.5. Comparative analysis and scoring of proteins based on quartile method

The conserved proteins were analyzed based on the quartile method scoring. This method is based on assessing nine physio-chemical properties, including functional class (virulence, cellular process, information and storage and metabolism molecule), hydropathy index, instability index, antigenicity, B-cell epitopes ratio, T-cell binding sites ratio, surface-exposed linear B-cell epitopes, surface-exposed conformational B-cell epitopes, and adhesion probability [48]. The sum of all scores for each protein was considered as the final score. Proteins with scores > 20, were considered as suitable immunogenic targets.

2.6. Molecular docking and immune simulation

The shortlisted proteins, selected based on physio-chemical features and quartile method, were investigated for their binding affinity to human TLR-2 (PDB:2Z7X). The protein-protein docking was done using pyDockWEB (https://life.bsc.es/pid/pydockweb/default/index) [49]. In addition, immune simulations of the proteins with HLAs including

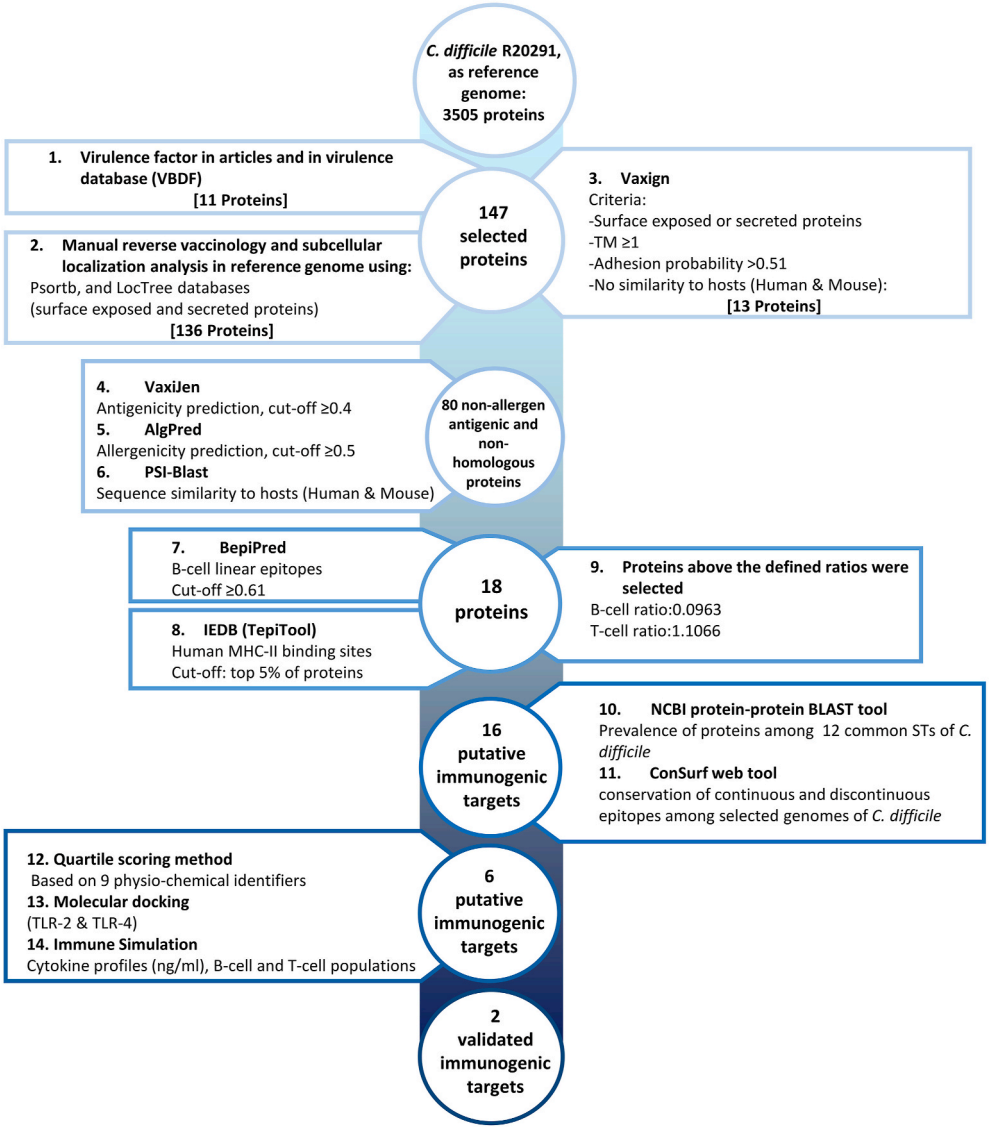

Fig. 1. The workflow used for identification of new putative immunogenic candidates against *C. difficile* via reverse vaccinology method. This project introduced the immunogenic targets were investigated through three different routes, VFDB, automated and manual reverse vaccinology. Finally, two proteins met the criteria of putative immunogenic candidates. VBDF: Virulence Factor Database, TM: Transmembrane Helices, ST: Sequence Type.

MHC-I (A0101 and B0702) and MHC-II (DRB1_0101) was checked using C-ImmSim tool (https://kraken.iac.rm.cnr.it/C-IMMSIM/index.php) [50]. The number of injections was considered 1 time without LPS for each immunogenic target.

3. Results

3.1. Collection of protein targets

Using the VFDB database and literature reviews, automated and manual reverse vaccinology methods, 11, 13, and 136 extracellular proteins were identified, respectively. After excluding redundant proteins, 147 putative candidates remained for further analyses. See Fig. 1.

3.2. Antigenicity and allergenicity of proteins

One-hundred and forty-seven proteins were submitted in VaxiJen, and 117 proteins were considered as antigen. Out of 117 antigenic proteins, only 88 proteins met the criteria and were considered as non-allergens.

3.3. Homology analysis of putative immunogenic targets with host proteomes

Using the PSI-BLAST tool, eight homologous proteins with homo sapiens, taxid:9606 and Mus, taxid:10088 were excluded and 80 non-homologous proteins were remained.

3.4. Determination of linear B-cell epitopes and human MHC-II binding sites

Of the 80 proteins of the previous stage, 18 selected proteins rated higher than defined thresholds for T-cell and B-cell linear epitopes ratios. See Fig. 2.

3.5. Prevalence and conservation of immunogenic candidates

Immunogenic candidates were detected in 11 common STs of *C. difficile*. Two proteins including CdtB (WP_009890823.1) and hypothetical protein (WP_012816046.1) were excluded due to low prevalence among these STs. The remaining 16 putative immunogenic candidates were selected for further analysis. The putative immunogenic candidates were as follows: FlgK (WP_009892584.1), FlgE (WP_009888081.1), FlgG (WP_009888104.1), FlgD (WP_009888079.1), FliD (WP_009888060.1), DUF3794 domain-containing protein (WP_009890363.1), glucosaminidase domain-containing protein (WP_009890365.1), NlpC/P60 family protein (WP_009889145.1), C40 family peptidases (WP_009892230.1 and WP_009890599.1), cell wall binding protein Cwp21 (WP_009893883.1) and Cwp12 (WP_009893729.1), polysaccharide deacetylase (WP_003436667.1), N-acetylmuramoyl-L-alanine amidase (WP_009893723.1), Acd (WP_009892971.1) and S8 family peptidase (WP_009890437.1). See Supplementary Table 2.

3.5.1. B-cell epitope prediction and sequence conservation

The BepiPred analysis showed that all of 16 putative immunogenic candidates have B-cell linear epitopes with a score ≥ 0.65. The number of linear epitopes of each protein was as follows: Acd (6), WP_009892230.1 (5), Cwp12 (5), S8 family peptidase (3), WP_009890599.1 (3), FlgK (4), FliD (3), Cwp21 (3), FlgG (3), epitopes, N-acetylmuramoyl-L-alanine amidase (3), DUF3794 domain-containing protein (2), polysaccharide deacetylase (1), glucosaminidase domain-containing protein (1), FlgE (1), FlgD (1), and NlpC/P60 family protein (1).

Moreover, 3D modeling was successfully performed for all candidates, and all 16 proteins had conformational B-cell epitopes in ElliPro analysis. The number of conformational B-cell epitopes was as follows: S8 family peptidase (9), NlpC/P60 family protein (8), Cwp12 (8), WP_009890599.1 (7), Cwp21 (6), polysaccharide deacetylase (6), Acd (5), WP_009892230.1 (5), glucosaminidase domain-containing protein (5), FlgK (4), FlgE (4), FlgG (4), FliD (3), FlgD (3), WP_009890599.1 (3), and N-acetylmuramoyl-L-alanine amidase (2).

The conservation profile of all linear and conformational B-cell epitopes has been characterized in Supplementary Table 2. Conservation analysis of linear and conformational B-cell epitopes showed that the surface-exposed proteins including Acd, FlgG, WP_009888104.1, and WP_009890599.1 (a C40 family peptidase) were highly conserved among circulating strains. The conformational B-cell epitopes on the 3D model of proteins were shown in Fig. 3.

3.5.2. Protein conserved domain search

3.5.2.1. Proteins involved in the cell wall structure. In this study, four cell wall hydrolase (CWH) proteins were detected as putative immunogenic

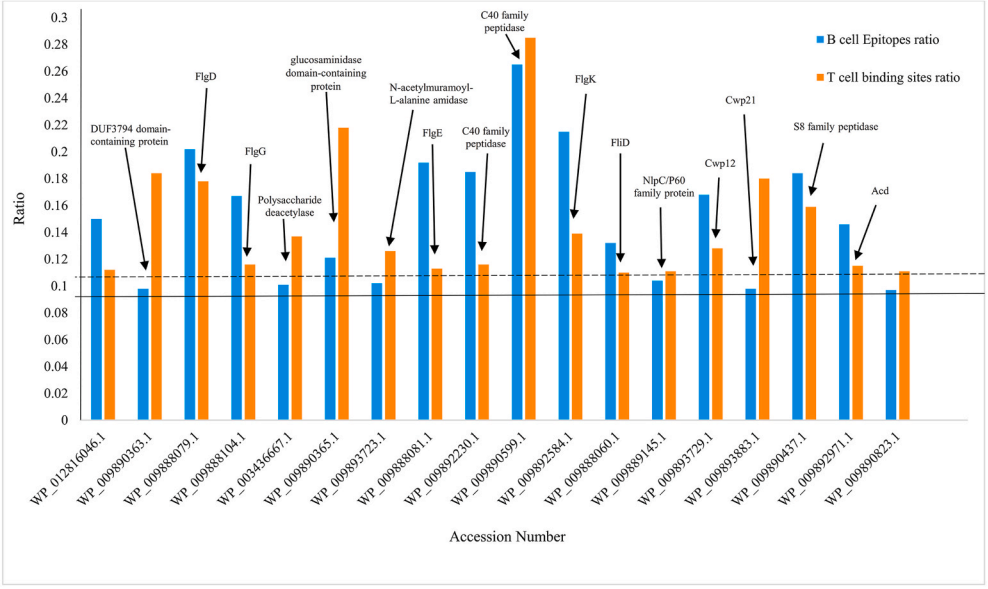

Fig. 2. The ratios of B-cell epitopes and MHC-II binding sites. Eighteen proteins had the highest score for both ratios, simultaneously. For each protein, the B-cell epitopes' ratio to the total number of amino acids was calculated (the number of amino acids in the B-cell epitope was divided into total amino acids of protein). The dotted line showed the mean of the B-cell epitope ratio (0.0963). The ratio of MHC-II binding sites to the total number of amino acids was calculated (the number of binding sites divided into total amino acids of protein). The continuous line shows the mean T-cell epitope ratio (0.1066).

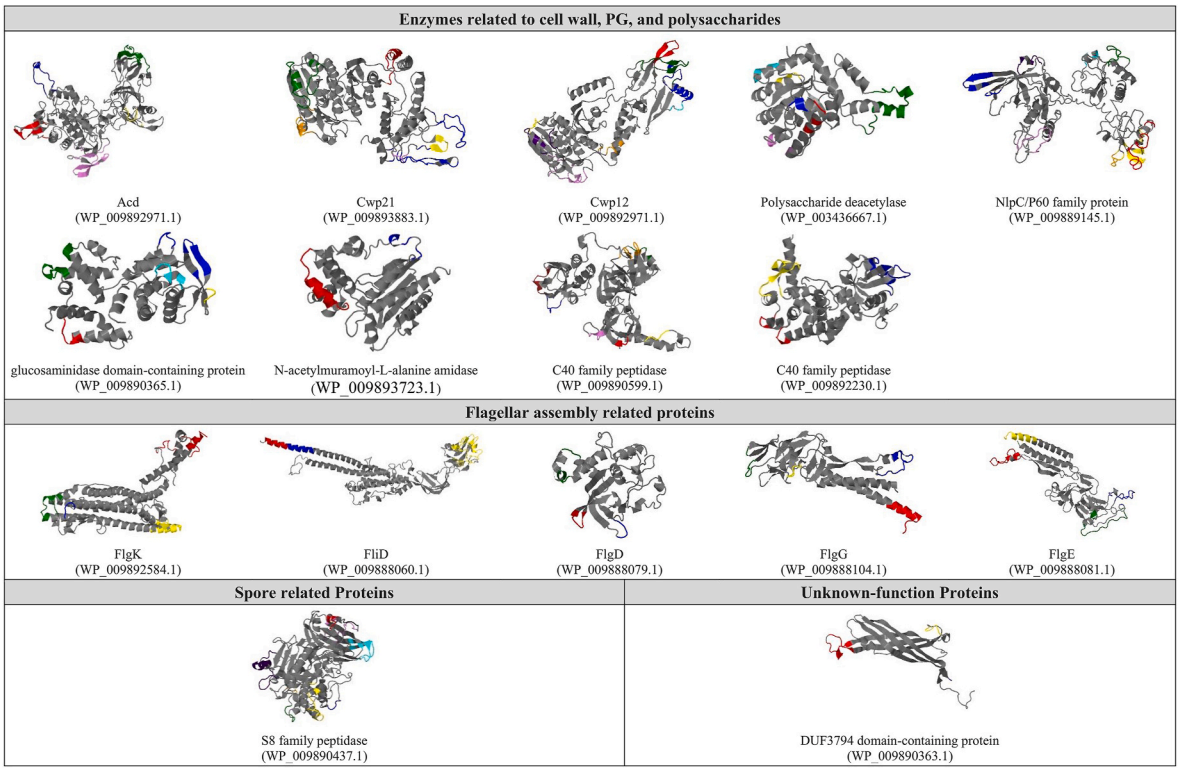

Fig. 3. The conformational B-cell epitopes of 16 putative immunogenic candidates were extracted using the ElliPro. The color and the score of each protein have shown in Supplementary Table 2. These proteins belonged to three functional groups including proteins involved in the cell wall and peptidoglycan (10 proteins), flagellar assembly (5 proteins) and spore germination (1 protein), and one DUF3794 domain-containing protein was identified as a vaccine target. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

candidates. WP_009890599.1 and WP_009892230.1 are C40 family peptidases. WP_009890599.1 contains two conserved domains, YgiM and Spr domains (a cell wall hydrolase from NLPC_P60). YgiM is an uncharacterized conserved domain, contains an N-terminal SH3 domain. WP_009892230.1 contains two domains including Lyz-like superfamily and NLPC_P60. The Liz-like superfamily members are involved in the hydrolysis of beta-1,4- linked polysaccharides.

WP_009890365.1, a phage-related CWH, has only one conserved domain from the glucosaminidase superfamily. This family includes mannosyl-glycoprotein endo-beta-N-acetyl glucosaminidase. WP_009889145.1 is another phage CWH consisting of two conserved domains, one from NLPC_P60, and the other from 3D_domain superfamily.

WP_009893723.1 contains one conserved domain, named N-acetylmuramoyl-L-alanine amidase (MurNAc-LAA). MurNAc-LAA is one of the peptidoglycan hydrolases (PGHs) found in bacteria and bacteriophages or prophages. These PGHs are involved in the degradation of the peptidoglycan.

WP_003436667.1 contains the conserved CE4_SmPgdA_like domain, which is a polysaccharide deacetylase.

Cwp12 consists of three conserved domains: S-layer_Clost superfamily, CAP domain, and Big_3 superfamily and is likely to have a role in adhesion and invasion.

Cwp21 has four conserved domains, one S-layer_Clost superfamily domain, and N-terminal cell wall binding domains followed by three PepSY domains.

Acd, a mannosyl-glycoprotein Endo-beta-N-acetyl glucosamidase, has four conserved domains; one LytD superfamily which is a beta-N-acetyl glucosaminidase and has an essential role in carbohydrate transport and metabolism. Two YgiM superfamily domains and one SH3_3 domain.

There is no available information about the conserved domains of DUF3794 domain-containing protein in the CDD database. The family is found to be association with LysM domain which provides an anchoring to extracellular polysaccharides such as peptidoglycan and chitin.

3.5.2.2. Proteins involved in flagella assembly. Several proteins in our study were involved in the flagellar assembly. FlgG is the flagellar basal body rod protein, and FlgE is the flagellar hook protein. Both of these proteins have a conserve domain from the FlgG superfamily, named flagellar basal body rod protein FlgG. FlgK has a conserved domain called flagellar hook-associated protein FlgK. FlgD has also one conserved domain called flagellar hook assembly protein. FliD contains one conserved domain called flagellar hook-associated protein-2.

3.5.2.3. Protein involved in spore germination. WP_009890437.1, a germination-specific protease (GSP), has a conserved domain named peptidase S8 family domain. This protein converts the spore peptidoglycan hydrolase (SleC) precursor to an active enzyme during spore germination. GSP is produced by a gene cluster containing *cspA*, *cspB*, and *cspC*, positioned upstream of the 5′ end of *sleC*.

3.5.2.4. Unknown-function protein. There is no available information about conserved domains of WP_009890363.1 in the CDD database. This protein is a DUF3794 domain-containing protein. VirulentPred analysis demonstrated that this protein is probably highly virulent (Score = 0.99).

3.5.3. Physio-chemical characteristics

The ProtParam analysis demonstrated that the estimated half-life of all proteins was (mammalian reticulocytes, *in vitro*), > 20 h (yeast, *in vivo*), and > 10 h (*E. coli*, *in vivo*). VICMpred analysis showed that among the 16 shortlisted proteins, five were virulence factors, four were metabolism molecules, six were involved in cellular process, and one

was involved in information and storage. Other physio-chemical features of proteins are presented in Supplementary Table 3.

3.5.4. Shortlist proteins

Sixteen proteins were evaluated by quartile method. The scores for the proteins were as follows: WP_009890599.1 (33), Acd (29), FlgG (25), WP_009892230.1 (23), FlgK (21), N-acetylmuramoyl-L-alanine amidase (21), FlgD (20), S8 family peptidase (20), NlpC/P60 family protein (19), DUF3794 domain-containing protein (18), polysaccharide deacetylase (18), FlgE (18), FliD (18), glucosaminidase domain-containing protein (17), Cwp21 (16) and Cwp12 (15). Finally, six proteins with a score > 20 were confirmed as shortlist proteins, including Acd, FlgK, FlgG, N-acetylmuramoyl-L-alanine amidase and C40 family peptidases (WP_009892230.1 and WP_009890599.1). See Fig. 4.

3.5.5. TLR-2 and TLR-4 docking and immune simulation

Molecular docking of selected proteins with TLR-2 and TLR-4 were as follows: Acd (−42.177 and −57.810), FlgK (−27.704 and −28.823), N-acetylmuramoyl-L-alanine amidase (−26.787 and −50.178), FlgG (−26.925 and −37.866), WP_009892230.1 (−28.223 and −37.217) and WP_009890599.1 (−29.945 and −37.217). See Table 1. Molecular docking results revealed that Acd and WP_009890599.1 had the strongest interaction with TLRs. Thus, the immune simulation of these two proteins was evaluated. The C-ImmSim results demonstrated that cytokine profiles (ng/ml), B-cell and T-cell populations (cells per mm^3) were dramatically increased after 1-week post-injection of Acd and WP_009890599.1. See Fig. 5.

4. Discussion

C. difficile is responsible for antibiotic-associated diarrhea in hospitalized patients by an imbalance of the intestinal micro-flora [51]. Due to the prolonged survival of the spores on inanimate objects and high resistance to bactericidal agents, disinfectants, and antibiotics [6], alternative solutions are necessary to prevent the CDI. Immunization by vaccines can reduce antibiotic usage and resistance through the reduction of disease incidence [52]. Toxin-based vaccines, although are effective in preventing infection-associated symptoms, they are likely unable to prevent colonization. It seems that toxoid A and B (TcdA and B) are the most popular candidates of vaccine formulations against CDI [53]. These vaccines have shown effectiveness against toxigenic strains in hamster or mouse models. However, the changing epidemiology of *C. difficile*, diverse phylogenic isolates and non-toxigenic strains present new challenges for CDI treatment. Furthermore, several studies have demonstrated that colonization with non-toxigenic strains is associated with a reduced disease incidence, which suggests that vaccination along with components other than toxins could be a better way to prevent the disease manifestations [4].

In terms of vaccinology, an ideal or promising immunogenic candidate should be exposed on the bacterial surface (outer membrane or extracellular proteins), have a high antigenicity value, be conserved among circulating strains, and be expressed during bacterial infection. In addition, the ideal immunogenic candidates should play an important role in bacterial pathogenesis [54,55]. In this study, we considered all above-mentioned criteria and introduced 16 non-toxic putative immunogenic candidates against *C. difficile* using the reverse vaccinology method, bioinformatics analysis and literature-based data mining. To the best of our knowledge, this is the first comprehensive study investigating reverse vaccinology of *C. difficile*.

Appropriate initial screening of protein targets plays an essential role in reverse vaccinology, reducing researcher's time and increasing the precision of the study. Vaxign database considers the most important criteria (subcellular localization, TM helix and adhesion probability) for proposing protein candidates at the same time. It was approved that proteins with TM helix ≤1 are more desirable because they can be cloned and expressed more efficiently [56], and adhesion proteins are considered critical because they induce host cell responses and mediate bacterial invasion [57]. However, the highest number of suitable immunogenic candidates (136 proteins) were found using the manual method of reverse vaccinology, suggesting the powerfulness of this approach compared to other methods. This approach can be considered the best route proposing the top candidates.

In the present study, we only included highly prevalent and conserved proteins. Prevalence and conservancy of immunogenic candidates are two important criteria to obtain a broadly protective vaccine. The term prevalence means that the proteins are widely distributed among the circulating strains and thus are extensively response mediating. From the other side, high conservation of proteins implies their lower alterations in time passage providing their long-term efficacy [58,59]. Keeping in mind, some proteins were excluded from this study. For example, CdtB protein was found to have an acceptable value according to the study criteria, but; was excluded due to low prevalence among the

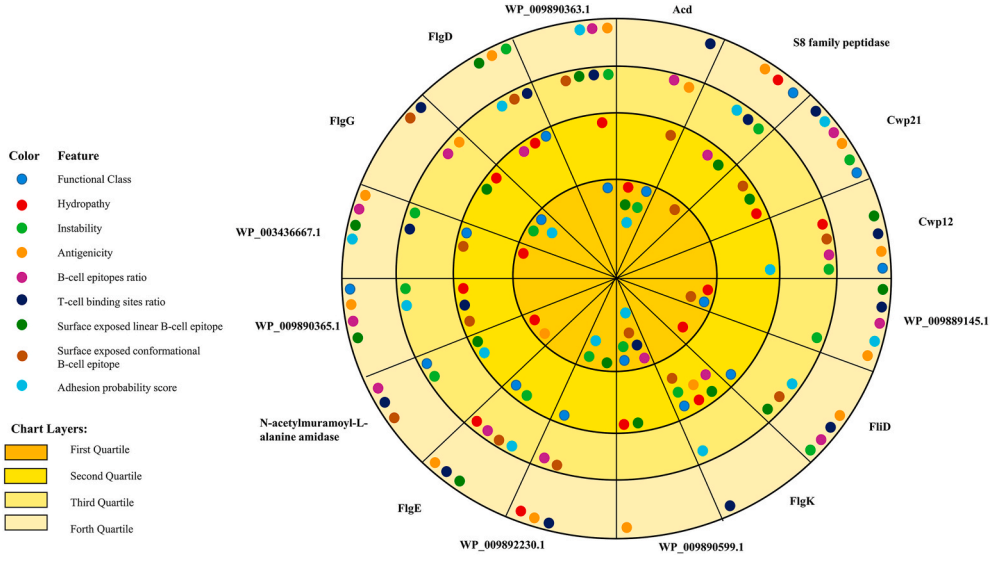

Fig. 4. The assessment of 16 immunogenic targets based on quartile method. The Table (left hand) shows nine protein features considered in the quartile scoring. Each color spot shows a feature. Acd, FlgK, FlgG, N-acetylmuramoyl-L-alanine amidase, WP_009890599.1 and WP_009892230.1 had score >20 and considered as the shortlisted proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Data on molecular static dockings of seven selected proteins in *C. difficile* with human TLR-2 and TLR-4.

Docking Results	Proteins											
	TLR-2						TLR-4					
	Acd	FlgK	NALA[a]	FlgG	WP_009890599.1	WP_009892230.1	Acd	FlgK	NALA[a]	FlgG	WP_009890599.1	WP_009892230.1
Electrostatics	−24.843	−11.175	−26.292	−11.199	−15.473	−13.372	−37.884	−10.910	−62.621	−44.886	−27.960	−6.458
De-solvation	−21.010	−20.499	−4.370	−19.598	−17.385	−19.930	−19.936	−22.012	12.639	3.425	−19.964	−36.178
Van-der Waals forces	36.758	39.699	38.755	38.715	48.134	40.784	0.103	40.994	−1.961	35.951	19.455	54.191
Total	−42.177	−27.704	−26.787	−26.925	−29.945	−28.223	−57.810	−28.823	−50.178	−37.866	−45.978	−37.217

[a] NALA, N-acetylmuramoyl-L-alanine amidase

clinical strains with widespread STs.

The conserved domains analysis and physicochemical features showed that our putative immunogenic candidates belonged to three functional groups including proteins involved in the cell wall synthesis, flagellar assembly and spore germination. In addition, an unknown-functioned protein (WP_009890363.1) met the criteria of a vaccine candidate. According to the VirulentPred results, this protein is highly virulent, but; its exact role in the pathogenesis of *C. difficile* is still unknown. Thus, further *in vitro* and *in vivo* studies are necessary to determine the role of this protein in the pathogenesis of *C. difficile*.

The physicochemical properties are essential factors for the optimal selection of immunogenic proteins. These properties directly determine the biological behavior of the peptide and influence other vaccine-related processes. The acceptable stability and half-life of all 16 selected proteins indicates that cloning and expression of these proteins in a suitable host seems worthy for further investigation.

The quartile measures the spread of values above and below the mean by dividing the distribution into three points-lower quartile, median, and upper quartile, to form four intervals based on the values of each variable. The quartile plot combines multiple criteria including physicochemical properties, B-cell and T-cell epitopes ratio and number of conformational epitopes and helps the researcher to compare all proteins from different aspects at a glance.

In this study, five CWHs were identified as promising vaccine candidates. The central catalytic domains in CWHs of *C. difficile* are NLPC_P60 and glucosaminidase. The NLPC_P60 domain has a role in the cleavage of N-acetylmuramate-L-alanine linkages and the 4–3 linkage between D-Glu and m-DAP residues. The glucosaminidases hydrolyze the glycosidic bond in the cell wall sugar backbone [60]. In a study by Kaus GM et al., deletion of polysaccharide deacetylase (WP_003436667.1) resulted in lowered number of muropeptides containing glucosamine residues and increased number of muropeptides containing GlcNAc residues. This suggests that PgdA is a major peptidoglycan deacetylase in *C. difficile* [61]. Moreover, the Acd (WP_009892971.1) protein is another CWH with lytic activity on the peptidoglycan layer of some Gram-positive bacteria, including *C. difficile*. It hydrolyses bonds between N-acetylglucosamine and N-acetylmuramic acid in peptidoglycan [62]. The physiological functions of Acd have not yet been investigated [63].

The results of this study may open a new insight into the pathogenesis of *C. difficile*. For example, two cell wall binding proteins named Cwp12 and Cwp21 were discovered as important virulence factors and immunogenic candidates. The Cwp21 contains three PepSY domains. It has been speculated that secreted proteins containing PepSY domains are involved in microbial environmental control and pathogenesis. Cwp12 contains a bacterial immunoglobulin-like (Big) domain that plays a key role in host cell adhesion and invasion [64]. We identified five proteins associated with the flagella apparatus. *C. difficile* flagella play a role in attachment facilitating intestinal colonization. According to the previous studies, the *flgE* deleted mutants showed less toxicity and adherence ability than the wild-type strain [65]. Péchiné et al. reported that rectal vaccination of mice by combinations of FliD, flagellar preparation, Cwp84, and cell wall extract, significantly reduced the colonization level [66]. Some studies indicated that the FliC and the cap protein FliD bind to murine mucus *in vitro* [67]. It was reported that recombinant FliC and FliD proteins could bind specifically to the mucus of mice, proposing the presence of a receptor for flagella in murine mucus. Thus, *C. difficile* flagella play a role in attachment, which facilitates the infection in the gut. In addition, according to the previous studies, the *flgE* mutant showed less toxicity and adherence ability than the wild-type strain [65].

We considered TLR affinity of our candidates as a criterion as they are a class of membrane-spanning single receptors and play vital roles in the recognition of invading organisms by innate immune cells. TLR-2 and TLR-4, two important TLRs, play important roles in the immunity against many Gram-positive or Gram-negative bacteria. Our molecular

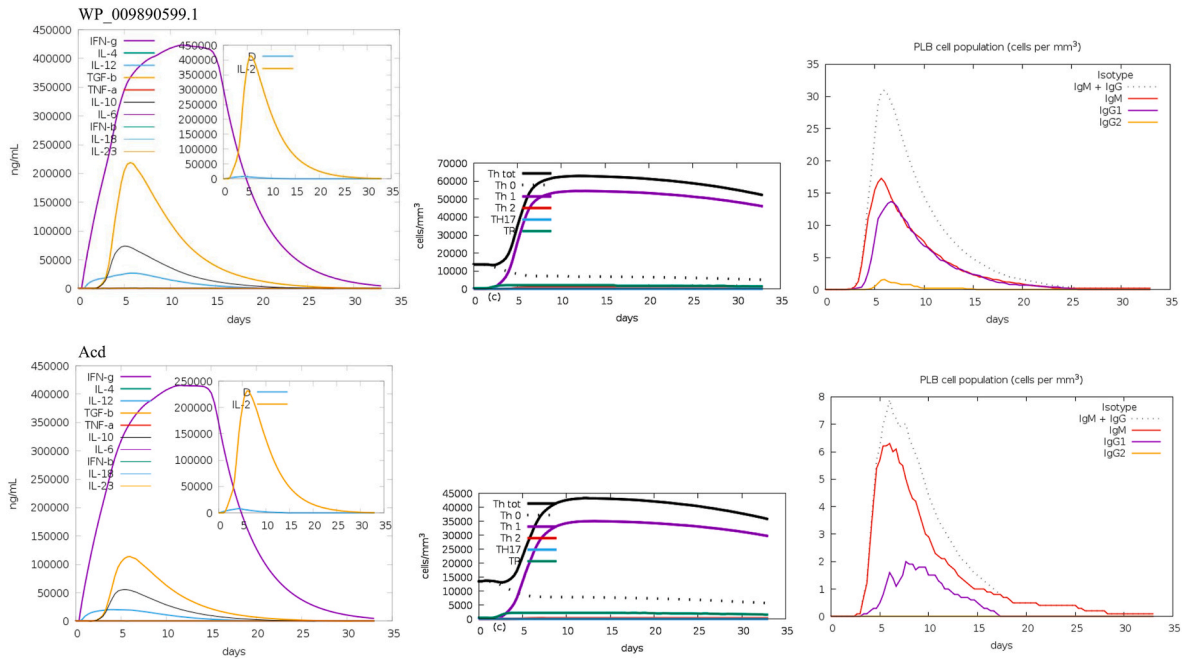

Fig. 5. The results of immune simulations revealed that WP_009890599.1 and Acd exhibit potent immune reactivity. Data show the cytokine profiles (ng/ml), B-cell and T-cell populations (cells per mm^3). One-week post-injection the immunological reactions represented the increases in cytokine concentrations and immune cell populations.

docking analysis showed that the Acd protein with the highest affinity (−42,177 and −57,810) binds to TLR-2 and TLR-4 and can elicit a strong immune response.

5. Conclusion

Considering all criteria for an excellent immunogenic target as a putative vaccine, Acd (WP_009892971.1) and WP_009890599.1 (a C40 family peptidase) are excellent immunogenic targets. Although CdtB was an immunogenic target, it had low prevalence among clinical isolates. Based on our results, we suggested a combination of toxoid (Tcd) of *C. difficile* and surface-exposed proteins (*e.g.* Acd) as a novel vaccine formulation to protect against circulating clinical strains of *C. difficile*.

Ethics approval and consent to participate

This article does not contain any studies with human or animals.

Consent for publication

This article does not contain any individual person's data in any form.

Availability of data and materials

The datasets or analyzed during the current study are available in the NCBI repository, (https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/535/).

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CRediT authorship contribution statement

Narjes Noori Goodarzi: Formal analysis, Investigation, Writing - original draft. **Sepideh Fereshteh:** Formal analysis, Writing - original draft. **Omid Azizi:** Writing - editing. **Hamzeh Rahimi:** Visualization, Writing- editing. **Negin Bolourchi:** Data curation, Writing- editing. **Farzad Badmasti:** Conceptualization, Data curation, Methodology, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

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