In silico prediction and expression analysis of vaccine candidate genes of *Campylobacter jejuni*

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ABSTRACT *Campylobacter jejuni* (***C. jejuni***) is the most common food-borne pathogen that causes human gastroenteritis in the United States. Consumption of contaminated poultry products is considered as the major source of human *Campylobacter* infection. An effective vaccine would be a promising alternative to antibiotic supplements to curb *C. jejuni* colonization in poultry gastrointestinal (**GI**) tract. However, the genetic diversity among the *C. jejuni* isolates makes vaccine production more challenging. Despite many attempts, an effective *Campylobacter* vaccine is not yet available. This study aimed to identify suitable candidates to develop a subunit vaccine against *C. jejuni*, which could reduce colonization in the GI tract of the poultry. In the current study, 4 *C. jejuni* strains were isolated from retail chicken meat and poultry litter samples and their genomes were sequenced utilizing next-generation sequencing technology. The genomic sequences of *C. jejuni* strains were screened to identify potential antigens utilizing the reverse vaccinology approach. In silico genome analysis predicted 3 conserved potential vaccine candidates (phospholipase A [***PldA***], TonB dependent vitamin B12 transporter [***BtuB***], and cytolethal distending toxin subunit B [***CdtB***]) suitable for the development of a vaccine. Furthermore, the expression of predicted genes during host-pathogen interaction was analyzed by an infection study using an avian macrophage-like immortalized cell line (**HD11**). The HD11 was infected with *C. jejuni* strains, and the RT-qPCR assay was performed to determine the expression of the predicted genes. The expression difference was analyzed using ΔΔCt methods. The results indicate that all 3 predicted genes, *PldA, BtuB*, and *CdtB,* were upregulated in 4 tested *C. jejuni* strains irrespective of their sources of isolation. In conclusion, in silico prediction and gene expression analysis during host-pathogen interactions identified 3 potential vaccine candidates for *C. jejuni*.

Key words: reverse vaccinology, *Campylobacter jejuni*, host-pathogen interaction, RT-qPCR, poultry

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INTRODUCTION

Food-borne illness caused by *Campylobacter* is one of the major causes of human gastroenteritis (Kaakoush et al., 2015). Epidemiologic studies suggest that the majority of human infections are related to *Campylobacter jejuni* (***C. jejuni***) (Suzuki and Yamamoto, 2009; Skarp et al., 2016). In the United States, 1.3 million people are infected with *Campylobacter* annually, and the economic impact due to disease outbreaks is predicted to be around $6.9 billion annually (Tack et al., 2019; Scharff, 2020). Approximately half of food-borne *Campylobacter* outbreaks in human are associated with the consumption of contaminated poultry products (Friedman et al., 2004; Wilson et al., 2008; EFSA, 2011; Tack et al., 2019). Campylobacter colonizes up to 6 to 10 $\log_{10}$ CFU/g in GI tract of broilers (Sahin et al., 2001; Newell and Fearnley, 2003; Singh Dhillon et al., 2006) and their colonization does not cause any harm to birds (Beery et al., 1988; Gormley et al., 2014). This makes poultry a major reservoir host and the primary source of *Campylobacter* infection in humans (Fravalo et al., 2021). The quantitative risk assessment model by Romero-Barrios et al. (2013) had estimated that a 3 $\log_{10}$ CFU/g *C. jejuni* reduction in the cecal content of broiler could reduce the risk of human infection from consumption of chicken meat by 100%. The conventional approach of controlling pathogens utilizing antimicrobial products has become less effective due to widespread antibiotic

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resistance. Therefore, there is a critical need for an alternative intervention strategy to curb *C. jejuni* colonization in poultry to minimize human infection. The development of an effective vaccine might be a promising alternative for reducing *C. jejuni* colonization in poultry.

Previously, multiple strategies have been employed to develop a vaccine against *C. jejuni*. However, there was variation in the protection between experiments and limited cross-protection against heterogeneous challenge conditions were observed in the previously tested vaccine candidates (Ziprin et al., 2002; Wyszyńska et al., 2004; Neal-McKinney et al., 2014; Chintoan-Uta et al., 2015; Hodgins et al., 2015; Chintoan-Uta et al., 2016; Nothaft et al., 2016). Despite many attempts, an effective *Campylobacter* vaccine which can reduce colonization in ceca of poultry during heterogeneous challenge condition remains elusive. In order to tackle this problem, we utilized reverse vaccinology (**RV**) technology, a novel and emerging vaccine development strategy (Rappuoli, 2000), along with a host-pathogen interaction model via cell infection to identify conserved antigens among *C. jejuni* and *Campylobacter coli*, which can be potentially developed into vaccines. RV uses genomic sequences to identify potential antigens for vaccine production in silico (Sette and Rappuoli, 2010), reducing both the time and cost of vaccine development. This technique has been previously used to predict potential antigen candidates effectively for several pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa, Helicobacter pylori, Brucella melitensis*) (Oprea and Antohe, 2013; Talukdar et al., 2014; Naz et al., 2015; Rashid et al., 2017; Vishnu et al., 2017) and developed a vaccine against *Neisseria meningitidis* serogroup B (Rappuoli, 2000).

Currently, 3 studies have utilized RV to predict potential vaccine candidates against *C. jejuni* (Meunier et al., 2016; Jain et al., 2019; Gupta and Kumar, 2020). Two of them utilized *C. jejuni* strain NCTC 11168 (human isolate) to predict the epitope-based vaccine intended to prevent human infection (Jain et al., 2019; Gupta and Kumar, 2020); however, selected candidates were not subjected to the development of the vaccine. Additionally, Meunier et al. (2016) intended to develop an avian *C. jejuni* vaccine using *C. jejuni* strain 81 to 176 (human isolate) to predict antigenic proteins and was tested in the broiler; but, the tested candidates were unable to provide the desired protection. Utilizing human isolates to predict the vaccine candidates for poultry might be less effective due to the genomic variation and low homology in the proteins among the clinical human isolates and chicken isolates (Epping et al., 2021; Truccollo et al., 2021; Audu et al., 2022). Thus, the aim of this study was to develop an effective vaccine that can reduce colonization of *C. jejuni* in ceca of poultry to reduce human food-borne illness. The objective was to predict potential *C. jejuni* vaccine candidates by utilizing the information of genome sequences of multiple *C. jejuni* strains isolated from the different sources of broiler chicken via in silico analysis and further analyze the expression of candidate genes during host-pathogen interactions to determine the pathogenic role of the predicted antigens.

MATERIALS AND METHODS

C. jejuni *Genome Sequence and Protein Prediction*

In this study, we utilized 4 *C. jejuni* genome sequences, which we had previously isolated from various poultry sources (Poudel et al., 2022a) and sequenced by Illumina and Oxford Nanopore techniques (Poudel et al., 2022b). Among them, 3 *C. jejuni* strains (MS2005, MS2058, and MS2074) were isolated from retail meat samples in Mississippi, and strain MS2167 was isolated from broiler feces. The information of genome sequences of 4 C. jejuni used in this study was available in NCBI GenBank (CP084080, CP084082, CP084084, and CP084085 (Poudel et al., 2022b).

Antigenic Protein Prediction

Vaxign (http://www.violinet.org/vaxign/; He et al., 2010) was used to find the subcellular localization, transmembrane helices, and adhesion probability of all predicted proteins for each strain. Vaxign utilizes pSORTb 2.0 to predict subcellular localization (Gardy et al., 2005), optimized HMMTOP based on a general hidden Markov model to predict transmembrane helix topology (Kall et al., 2007), and SPAAN to calculate the adhesion probability of protein (Sachdeva et al., 2005).

The proteins were filtered by the following criteria: 1) surface exposed protein either present in the outer membrane or extracellular matrix, 2) adhesion probability score $\geq$ 0.5, and 3) no more than one transmembrane helixes (Monterrubio-López et al., 2015; Meunier et al., 2016). The surface-exposed proteins were selected because the host immune system targets these proteins to recognize and expel the pathogen. Adhesion probability of protein was considered an important factor for the vaccine candidate selection. During the bacterial invasion, the adhesion structure encounters the host immune system, and immunity against the adhesion structure helps to prevent further colonization and infection. Protein sequences having multiple transmembrane helixes were removed because they are difficult to express and purify (Monterrubio-López et al., 2015).

VaxiJen v2.0 (http://www.ddgpharmfac.net/VaxiJen/VaxiJen/VaxiJen.html; Doytchinova and Flower, 2007) was used to predict the antigenicity of the filtered proteins. VaxiJen software, based on RV, utilized a machine learning approach to predict antigenic protein sequence. This software utilizes the physicochemical properties to predict protein's antigenicity from the submitted amino acid sequences (Dalsass et al., 2019). A protein with an antigen score of $\geq$ 0.5 was selected for further analysis (Doytchinova and Flower, 2007). Proteins with an antigen score $\geq$ 0.5 are more likely

recognized by the host immune system, making them potential vaccine candidates (Doytchinova and Flower, 2007; Monterrubio-Lopez et al., 2015), and were selected for further analysis.

Protein Conservation Prediction

To select vaccine candidates with the broadest spectrum, the predicted antigen proteins were aligned to genomes of 206 *C. jejuni* strains (including 18 *C. jejuni* from poultry strains; Supplemental Table S1), 18 *C. jejuni* strains isolated only from poultry (Supplemental Table S2), and 34 *C. coli* strains (Supplemental Table S3) available from NCBI using tblastn from BLAST+ v 2.2.31 (Camacho et al., 2009) via Geneious prime v2020.2.2 (Integrated Bioinformatic Solution, Auckland, North Island, New Zealand). Proteins with a pairwise identity ≥50% and minimum query coverage ≥ 80% were considered highly conserved and kept for further analysis. In addition, the candidate proteins were blast against *Gallus gallus* (taxid:9031) protein database from NCBI (https://blast.ncbi.nlm.nih.gov/) using blastp to identify any candidate proteins too similar (pairwise identity ≥80%) to a host protein.

Prediction of B-Epitope

BCPreds (http://ailab.ist.psu.edu/bcpred/) was used to predict B-cell epitopes for the selected candidate proteins. B-cell epitopes were selected utilizing 2 different algorithms, the amino acid pair method and string kernels. BCPreds predict antigenic linear nonoverlapping epitopes from the sequence of preselected amino acid sequences. All the preselected protein sequences from VaxiJen were analyzed, and epitopes with a score of > 0.8 (specificity > 80%) were considered B-epitope. In order to test the individual antigenicity of selected epitomes, they were further accessed utilizing VaxiJen v2.0 software. Epitope sequences having antigenic scores >0.5 were selected, and B-epitope density was calculated utilizing antigenic predicted epitopes (bcpreds score > 0.8 and VaxiJen >0.5) for the preselected protein sequence.

Selection of Vaccine Candidates

In order to remove the predicted homologous protein among 4 different *C. jejuni* strains (MS2005, MS2058, MS2074, and MS2167) and identify the previously tested vaccine candidate multiple alignment was done. A multiple alignment tool MAFFT (Multiple Alignment using Fast Fourier Transform) was utilized to align the predicted protein sequence obtained from the *C. jejuni* strains using Geneious prime v2020.2.2 (Integrated Bioinformatic Solution, Auckland, North Island, New Zealand). Protein sequences with >80% pairwise identity and >80% minimum query coverage were categorized as homologous protein sequence. Blastp analysis was performed to identify the name and function of the homologous proteins with a reference protein database of NCBI database (https://blast.ncbi.nlm.nih.gov/) with selected organism bacteria so that previously characterized and tested vaccine candidates can be removed from the final selection.

Gene Expression Analysis During Host-Pathogen Interaction

The expression analysis of the predicted gene candidates during host-pathogen interaction provides the crucial information about the role of candidate genes during infection. The expression levels of those candidate genes might be associated with the efficacy of developed vaccine. Therefore, the expression analysis of predicted potential antigenic vaccine candidates from in silico was performed during the host-pathogen interaction assay, utilizing following methods.

Bacterial Strain Selection and Culture Preparation

For the host-pathogen interaction assay, we selected 4 different *C. jejuni* strains, including MS2191, MS2074, ATCC29428, and ATCC33560, which were previously isolated from 4 different sources (broiler cloacal swab, retail chicken meat, human clinical, and bovine feces isolate, respectively; Poudel et al., 2022a) to test how the predicted candidate genes express in different strains of *C. jejuni* isolated from different sources during host-pathogen interaction. For the bacterial growth, the stock of the *C. jejuni* strains was cultured in double-strength blood-free Bolton broth (2 × **BF-BB)** (Oxoid, ThermoFisher Scientific, Waltham, MA) at 42°C for 48 h under microaerobic conditions (85% nitrogen, 10% carbon dioxide, and 5% oxygen) using Mart anaerobic jar with an Anoxomat II System (Mart Microbiology B. V., Lichtenvoorde, Oost Gelre, Netherlands). For the infection of the cells, the bacterial cell pellet was resuspended in Advanced Dulbecco's Modified Eagle Medium (Gibco Advanced DMEM, ThermoFisher Scientific, Grand Island, NY), supplemented with 1% fetal bovine serum (**FBS**) and OD_{600} was adjusted to 0.2 (~8 $\log_{10}$ CFU/mL).

Cell Infection Model The avian macrophage-like immortalized cell line (**HD11**) was used for the host-pathogen infection study of the *C. jejuni* strains. The HD11 cell line was suspended in an Advanced Dulbecco's Modified Eagle Medium supplemented with varying levels of 2.5 to 10% FBS (Gibco Fetal Bovine Serum, ThermoFisher Scientific, Grand Island, NY), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco Pen strep, ThermoFisher Scientific, Grand Island, NY), and 2 mM GlutaMax (Gibco GlutaMax-I, ThermoFisher Scientific, Grand Island, NY). The cell line was grown at 37°C with 5% CO_2 and an 80 to 90% confluency (6.0 $\log_{10}$ cells/well) in 6-well tissue culture plates. Each of the *C. jejuni* strains, approximately at 8.3 $\log_{10}$ CFU/mL, was suspended in Advanced DMEM with 1% FBS and without antibiotics. Each *C. jejuni* suspension was used to infect a monolayer of the HD11 cells, to make the multiplicity of infection of 100 (Larson et al., 2008; Peng et al., 2018;

Pogacar et al., 2020). Culture plates consisting of both *C. jejuni* and HD11 cells were incubated at 37°C in a 5% CO_2 air atmosphere incubator (Forma Series 3 Water Jacketed CO_2 Incubator, ThermoFisher, Marietta, OH) for 3 h. After infection and incubation, the nonattached bacteria and media were aspirated, and the wells still containing the attached bacteria and cells were washed 3 times using 1 mL of PBS. After washing, approximately 6.5 $\log_{10}$ CFU *C. jejuni* strains were attached (*C. jejuni* MS2191 [6.70 $\log_{10}$ CFU/well]; *C. jejuni* MS2074 [6.31 $\log_{10}$ CFU/well]; *C. jejuni* ATCC29428 [6.82 $\log_{10}$ CFU/well]; *C. jejuni* ATCC33560 [6.30 $\log_{10}$ CFU/well]). The cell pellet of the infected HD11 cells from each well was collected utilizing the cell scraper and stored at -80°C for further analysis.

RNA Extraction and cDNA Synthesis The total RNA was extracted from the infected cell pellets containing both prokaryotic and eukaryotic cells using Zymo Quick-Miniprep Plus (Zymo Research, Irvine, CA) with bead beating using ZR Bashing Beads (Zymo Bashing Beads). On column DNase I treatment was done following the manufacturer's protocol to eliminate the genomic DNA contamination. RNA quality, quantity, and purity were assessed via 1% gel electrophoresis and NanoDrop One spectrophotometer (Thermo Scientific, Wilmington, MA). A total of 1000 ng of the extracted RNA from the infected cells was reversely transcribed to first-stand cDNA using SuperScript VILO MasterMix (ThermoFisher Scientific, Carlsbad, CA). Similarly, prokaryotic RNA was extracted from bacterial cultures of all 4 *C. jejuni* strains suspended in cell culture medium without HD11 cell line (control; untreated group), and a total of 20 ng of prokaryotic RNA was used for the first-strand cDNA synthesis.

RT-qPCR RT-qPCR assay was conducted using QuantStudio 3 (Applied Biosystems, Waltham, MA) and the following program: an initial denaturation step at 95°C for 2 min, followed by 40 cycles at 95°C for 5 sec and 60°C for 20 sec. The mixture of each assay contained 1 μL of diluted cDNA (ranged 5−15 times), 5 μL of 2 × PowerTrack SYBR Green Master Mix (ThermoFisher Scientific, Carlsbad, CA), 0.25 μL of each forward and reverse primer with 10 μM concentration, and 3.5 μL of nuclease-free water to make a final volume of 10 μL.

Normalization of Prokaryotic RNA Amount in Total RNA Sample Due to the limited and varied bacterial RNA content among the infected host cells, the first-strand cDNA product of pure *C. jejuni* extracted RNA (described in "RNA Extraction and cDNA Synthesis") was used to generate a standard curve of *16S rRNA* gene for estimating the prokaryotic RNA content in each extracted total RNA in the treated samples (RNA obtained from infected cells). The standard curve of *16S rRNA* gene was conducted with 10-fold serial dilutions of first-strand cDNA product utilizing prokaryotic RNA obtained from *C. jejuni* strains and *Campylobacter*-specific *16S rRNA* primer pair via the RT-qPCR assay. In order to estimate the bacterial RNA content in treated samples, cycle threshold value (**Ct-value**) of the *16S rRNA* gene was obtained from the first-strand cDNA product from each treated sample. The actual bacterial RNA amounts for each treated sample were estimated using the equation obtained from the standard curve via RT-qPCR. The estimated bacterial RNA amount present in the treated samples ranged between 0.03 and 0.06 ng/μL. In order to make a similar input amount of RNA from both treated and untreated samples, untreated samples were further diluted based on the predicted amount of RNA present in the treated samples. The normalized samples were used for analyzing the expression level of the vaccine candidate genes.

Candidate Gene Expression The gene-specific primers for each gene of interest were designed using Geneious prime v2020.2.2 (Integrated Bioinformatic Solution, Auckland, North Island, New Zealand). The information about primer and primer sequences are listed in Table 1. In order to verify the amplicon size and primer specificity, the standard RT-PCR was conducted using Eppendorf Master cycler ep gradient system (Eppendorf, Enfield, CT). The condition used for the RT-PCR was an initial heat step 95°C for 2 min, followed by 35 cycling steps: 95°C for 30 sec and 60°C for 30 sec, 72°C for 45 sec, followed by a final extension step at 72°C for 5 min. The correctly amplified PCR product was cleaned up using GeneJET PCR purification Kit (ThermoFisher Scientific, Waltham, MA) and cloned into pGEM-T Easy vector (Promega, Madison, WI) following the manufactures protocol. Five independent recombinant plasmid DNAs for each gene of interest were isolated and sent to Eurofins Genomics LLC (Louisville, KY) for Sanger sequencing, and sequencing results were aligned with the original target gene sequence using

Table 1. List of primers used in RT-qPCR analysis.

Transcript Target transcript	Orientation	Primer Sequence (5′−3′)	Length (nt)	Tm (°C)	Amplicon size (bp)
CdtB	Forward	TCCTGTAATTGCATAATCAAGAGTCC	26	61.4	206
	Reverse	CAGATGTAGGAGCAATTATCACAGC	25	62.9	
PldA	Forward	AAGCAATGGCAAGGGAGATGAG	22	62.7	201
	Reverse	TCATCGCCCAAATACGCTAAATTC	24	61.6	
BtuB	Forward	TACGCAATGTAATCAGCATAGAAGG	25	61.3	217
	Reverse	GTTACACCATGGGAATTATCAAGAAC	26	61.4	
Campylobacter 16S[1]	Forward	GATGAAGCTTTTAGCTTGCTAGAAGTGG	28	65.0	165
	Reverse	GTCTCATCCTACACCGAAAAACTTTCC	27	65.0	

[1]Accession number: LS483362.1.

Geneious prime v2020.2.2 (Integrated Bioinformatic Solution, Auckland, North Island, New Zealand) to confirm the specificity of each designed primer pair.

The expression changes of predicted vaccine candidate genes in the bacteria-cell infection study were analyzed using RT-qPCR, with the assay mixture and condition as described previously. For each tested *C. jejuni* strain, 3 biological replicates were analyzed, and 3 technical replicates were conducted in RT-qPCR assay for each sample. The expression change of the interested transcript was determined based on the ΔΔCt method (Livak and Schmittgen, 2001). *Campylobacter*-specific *16S rRNA* gene was used as a reference gene for normalization. The paired Student *t* test was used to compare the control (RNA obtained from *C. jejuni* not involved in cell-infection) and treatment (RNA obtained from *C. jejuni*, which was involved in cell-infection) using GraphPad prism 9 (GraphPad Software, San Diego, CA).

RESULTS

In Silico Prediction of Vaccine Candidate

Of the 7,132 proteins (1,820, 1,933, 1,629, and 1,759 from MS2005, MS2058, MS2074, and MS2167, respectively), 30 proteins passed the selection criteria for Vaxigen (Supplemental Table S4). *C. jejuni* strains MS2005 and MS2058 possess 1,820 and 1,933 protein-coding sequences (**CD**), respectively and from each strain, 7 protein sequences meet the applied selection criteria of localization, adhesion probability, and transmembrane helixes. *C. jejuni* strain MS2074 consists of 1,629 CDs and 10 proteins, which meet the selection criteria. *C. jejuni* strain MS2167 consists of 1,750 CDs and possesses 6 protein sequences that meet the selection criteria (Supplemental Table S4). The antigenicity of the preselected proteins was tested using VaxiJen v2.0. The antigenic score for *C. jejuni* strains MS2005, MS2058, MS2074, and MS2167 ranged from 0.36 to 0.72 (Supplemental Table S4). Of the 30 preselected protein sequences, 27 were classified as antigenic (VaxiJen antigenic probability > 0.5).

The sequences of vaccine candidate proteins were aligned against the *Gallus gallus* proteins to find candidates with high similarity to the host; none of the selected proteins were found homologous to the host (Supplemental Table S4). The candidate sequences were also aligned to genomes of *C. jejuni* and *C. coli* strains available from NCBI. Poorly conserved candidates (<80% present among the *C. jejuni* and *C. coli* strains) were removed from future analysis. Strains MS2005, MS2058, and MS2167 consist of 5 highly conserved protein sequences (TonB dependent vitamin B12 transporter [**BtuB**], major outer membrane porin [**MoMP**] or PorA, Phospholipase A (**PldA**), flagellar capping protein [**FliD**], and Cytolethal distending toxin subunit B [**CdtB**]). Strain MS2074 consists of 6 highly conserved proteins (BtuB, MoMP, or PorA, PldA, FliD, flagellar hook protein [**FlgE**], and CdtB; Table 2 and Supplemental Table S4). Although extracellular CdtB protein was poorly conserved (66.5% present) among all *C. jejuni* strains, it was considered a potential candidate because it was highly conserved (88.89%) among the poultry specific *C. jejuni* strains and (91.18%) among *C. coli* strains, so protein CdtB was added back to conserved list. A total of 21 proteins were obtained while analyzing the 4 *C. jejuni* strains MS2005, MS2058, MS2074, and MS2167 (Table 2).

Antigenic protein sequences obtained were analyzed for the number and density B-epitopes. The conserved protein sequences from *C. jejuni* strain MS2005, MS2058, MS2074, and MS2167 contain BCPred B-epitopes ranging from 2 to 13 and density of 0.008 to 0.020.

Table 2. List of potential vaccine candidates selected from the analysis of *C. jejuni* using different bioinformatic software.

Annotated protein ID	Protein size (aa)	Localization	Adhesin probability	Antigen probability	Sharing (%) among *C. jejuni*	Sharing (%) among *C. jejuni* strains from poultry origin	Sharing (%) among *C. coli*	MAFFT alignment	Protein name
MS2005_GHPAHEKB_01319	329	OMP	0.659	0.566	97.09	94.44	100	98.80%	Phospholipase A
MS2058_ICDGEIKM_01045	329	OMP	0.646	0.553	97.57	94.44	100		
MS2074_CJDKBBOE_01490	329	OMP	0.670	0.580	97.09	94.44	100		
MS2167_CDNINIFE_01244	329	OMP	0.659	0.566	97.09	94.44	100		
MS2074_CJDKBBOE_01412	418	OMP	0.744	0.622	99.03	100	100	81.4%	MoMP or PorA
MS2058_ICDGEIKM_00883	424	OMP	0.664	0.631	99.03	100	100		
MS2167_CDNINIFE_01337	425	OMP	0.722	0.642	99.03	100	100		
MS2005_GHPAHEKB_01469	431	OMP	0.863	0.642	99.03	100	100		
MS2074_CJDKBBOE_00243	545	Extracellular	0.852	0.653	100	100	100		FlgE
MS2167_CDNINIFE_00321	642	Extracellular	0.801	0.724	99.51	100	100	97%	FliD
MS2005_GHPAHEKB_00382	643	Extracellular	0.799	0.690	99.51	100	100		
MS2058_ICDGEIKM_00093	643	Extracellular	0.799	0.690	99.51	100	100		
MS2074_CJDKBBOE_00715	643	Extracellular	0.774	0.690	99.15	100	100		
MS2074_CJDKBBOE_00067	706	OMP	0.652	0.614	96.60	100	100	94.8%	BtuB
MS2058_ICDGEIKM_01297	715	OMP	0.665	0.610	96.60	88.89	100		
MS2005_GHPAHEKB_01062	718	OMP	0.689	0.608	96.60	88.89	100		
MS2167_CDNINIFE_00988	718	OMP	0.686	0.607	96.60	88.89	100		
MS2005_GHPAHEKB_00842	265	Extracellular	0.654	0.5867	66.50	88.89	91.18	100%	CdtB
MS2058_ICDGEIKM_01516	265	Extracellular	0.654	0.5867	66.50	88.89	91.18		
MS2074_CJDKBBOE_00273	265	Extracellular	0.654	0.5867	66.50	88.89	91.18		
MS2167_CDNINIFE_00770	265	Extracellular	0.654	0.5867	66.50	88.89	91.18		

Abbreviation: OMP, outer membrane protein.

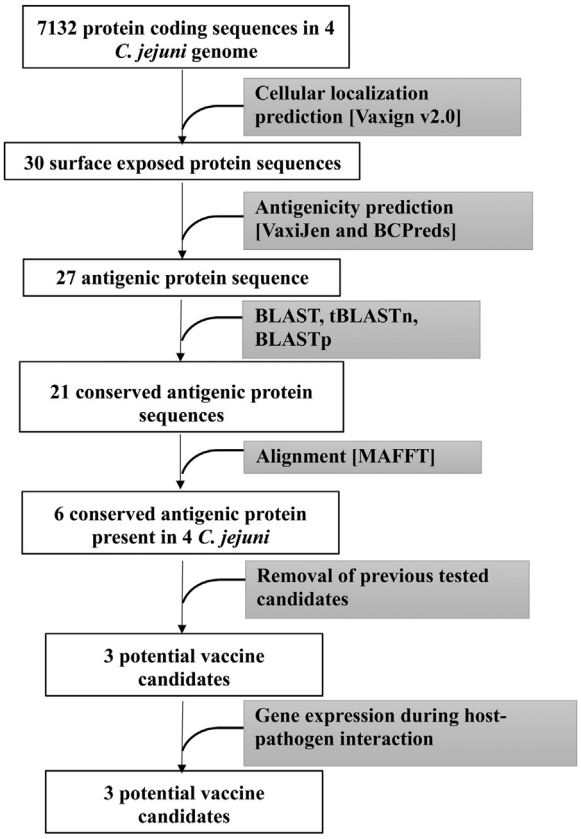

Figure 1. A flow chart summarizing the methodology utilized in this study to identify the potential antigenic vaccine candidates against *Campylobacter jejuni*.

For *C. jejuni* strains, MS2005, MS2058, MS2074, and MS2167 consist of amino acid pair antigenic B-epitope numbers between 3 and 11 and density between 0.009 and 0.015. The epitope number and density are summarized in Supplemental Table S4.

Removal of Similar and Previously Tested Protein

Antigenic protein sequences predicted from 4 different *C. jejuni* strains were aligned using MAFFT alignment. From 21 conserved antigenic proteins, 6 highly conserved candidates were selected: 3 extracellular proteins (FlgE, FliD, and CdtB), and 3 outer membrane proteins (PldA, MoMP, and BtuB) (Table 2). The proteins FlgE, FliD, and MoMP were removed from further analysis due to a lack of cross-protection and short-lived protection during previous in-vivo experiments (de Zoete et al., 2007; Isalm et al., 2010; Meunier et al., 2017). Finally, 3 potential vaccine candidates, PldA, BtuB, and CdtB were obtained (Table 2 and Figure 1).

Expression Analysis of Selected Vaccine Candidates

The expression analysis during host-pathogen interaction helps to understand the immunogenicity of the in silico predicted candidates. Therefore, the expression analysis of predicted potential antigenic vaccine candidates from in silico was performed through the host-pathogen interaction assay. The results of gene expression analysis during host-pathogen interaction were summarized in Figures 2–4. During the infection of HD11 cells by *C. jejuni* strains, there was significant upregulation ($P \leq 0.05$) of all 3 vaccine candidate genes suggesting that these genes might have a significant impact on the bacterial pathogenesis. The *PldA* gene, which plays a major role in the hemolytic activity, was significantly upregulated by 3.41- to 65.41-fold in all 4 *C. jejuni* strains (MS2191, MS2074, ATCC33560, ATCC29428). The expression of *PldA* gene was highest in *C. jejuni* strain MS2191 isolated from cloacal swab of broiler followed by strain ATCC29428 (human clinical isolate), ATCC33560 (bovine feces isolate), and MS2074 (chicken meat isolate). Compared to other isolates, *C. jejuni* isolated from chicken meat showed the lowest expression of *PldA* gene during interaction with the host-cell (Figure 2). *BtuB* gene, an outer membrane vitamin B12 transporter, was upregulated by 4.03- to 105.4-fold in all 4 tested *C. jejuni* strains. The expression of *BtuB* gene was highest in *C. jejuni* strain ATCC29428 compared to strains isolated from the other sources. Similar to *PldA, BtuB* expression was also lower in

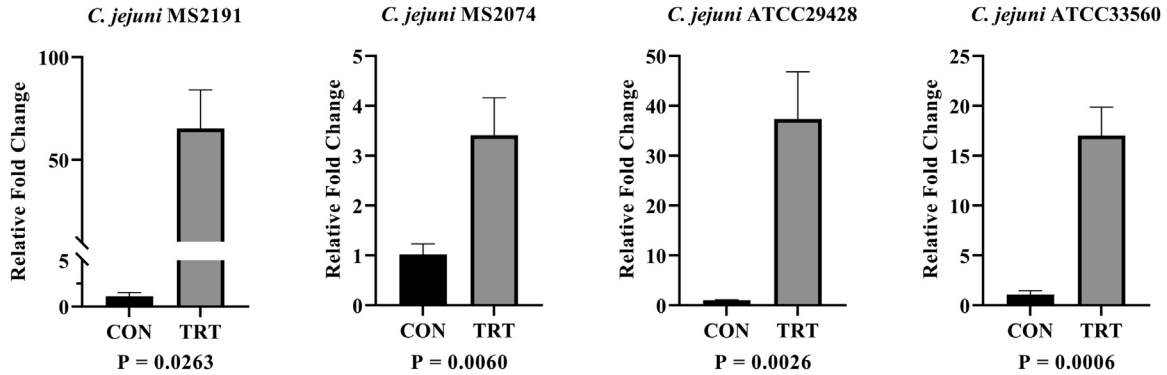

Figure 2. Expression analysis of *PldA* gene in 4 *C. jejuni* strains (A) MS2191, (B) MS2074, (C) ATCC29428, and (D) ATCC33560, during host-pathogen infection study via RT-qPCR. Relative fold change in vaccine candidate gene *PldA* during the infection of HD11 cells. Each vertical bar represents mean relative fold change ± standard error of mean (n =3). The expression change of transcript was determined based on ΔΔCt methods. Abbreviations: CON, untreated group; TRT, treatment group.

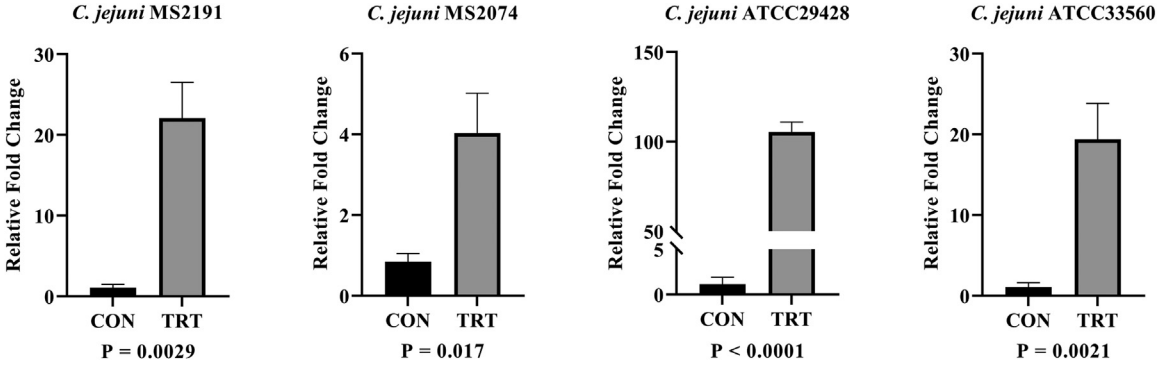

Figure 3. Expression analysis of *BtuB* gene in 4 *C. jejuni* strains (A) MS2191, (B) MS2074, (C) ATCC29428, and (D) ATCC33560, during host-pathogen infection study via RT-qPCR. Relative fold change in vaccine candidate gene *BtuB* during the infection of HD11 cells. Each vertical bar represents mean relative fold change ± standard error of mean (n =3). The expression change of transcript was determined based on ΔΔCt methods. Abbreviations: CON, untreated group; TRT, treatment group.

C. jejuni strain MS2074 isolated from chicken meat samples compared to other strains (Figure 3). *CdtB*, an extracellular protein responsible for producing toxins, was found to have a higher variation in the expression among the isolates; its upregulation ranged from 3.15- to 590.04-fold among tested strains. The expression of *CdtB* gene was much higher in *C. jejuni* strain ATCC33560 compared to other strains tested, whereas the expression level was similar other 3 strains (MS2074, MS2191, and ATCC29428) ranging between 3.15 and 8.550 (Figure 4).

DISCUSSION

Reverse Vaccinology Approach

Despite the utmost necessity to control *C. jejuni* colonization in poultry to reduce human infection, there are no effective antibiotic alternative strategies for reducing colonization in the gastrointestinal tract of poultry. Previous attempts to make an effective vaccine to reduce colonization of *C. jejuni* in poultry have been unsuccessful (Pumtang-on et al., 2021). The utilization of conventional strategies for developing the vaccine against *C. jejuni* in poultry has not led to a successful vaccine that can produce both immunogenicity and reduce colonization of bacteria in the ceca of poultry. Reverse vaccinology might be a suitable strategy for the development of a vaccine against *C. jejuni*. RV is a new vaccine developmental strategy that helps in the rapid identification and refinement of the antigenic vaccine candidates using the genome sequence of microorganisms (Moxon et al., 2019). Previously, the vaccine candidate predicted using RV technology was directly utilized for immunogenicity testing via vaccine development (Rappuoli, 2000; Meunier et al., 2016; Meunier et al., 2017). Even though RV technology helps us identify and narrow down the potential vaccine candidate, the possibility of obtaining a successful vaccine is low (Meunier et al., 2016; Meunier et al., 2017). Developing the vaccine without knowing how these candidate genes express and function during the interaction with the host might have potentially increased the risk of vaccine candidate failure, increasing the cost, and the time for developing a vaccine, ultimately leading to low efficacy for vaccine development. To circumvent this issue, we introduced an additional screening step, that is, gene expression analysis during host-pathogen interaction, to further validate the vaccine candidates after in silico prediction. The gene expression analysis during host-pathogen interactions helps to understand the role of the gene during the pathogenesis of bacteria, as well as to quantify the expression level of genes, helping to determine the best suitable candidate for the development of the

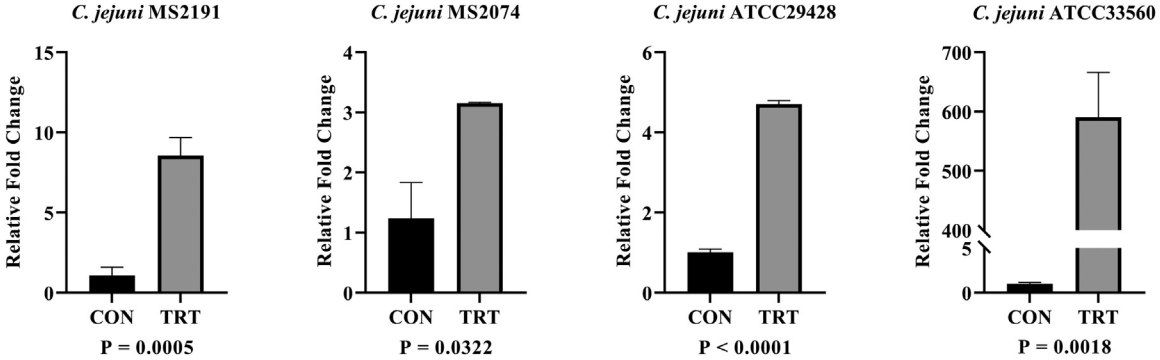

Figure 4. Expression analysis of *CdtB* gene in 4 *C. jejuni* strains (A) MS2191, (B) MS2074, (C) ATCC29428, and (D) ATCC33560, during host-pathogen infection study via RT-qPCR. Relative fold change in vaccine candidate gene *CdtB* during the infection of HD11 cells. Each vertical bar represents mean relative fold change ± standard error of mean (n =3). The expression change of transcript was determined based on ΔΔCt methods. Abbreviations: CON, untreated group; TRT, treatment group.

vaccine. Therefore, in the current study, we combined the in silico and gene expression analysis during host-pathogen interaction assay to identify potential vaccine candidates.

In the current study, to select the universal vaccine candidate against *C. jejuni*, the genomes of multiple *C. jejuni* strains obtained from different poultry sources were analyzed to predict the conserved surface-exposed antigenic proteins. Furthermore, we utilized 4 *C. jejuni* strains obtained from 4 different sources (broiler cloacal swab, retail chicken meat, human clinical, and bovine feces isolate) for analyzing vaccine candidate expression during host-pathogen interaction to identify the candidate that significantly upregulated in all tested strains so that the selected candidate would provide protection against *C. jejuni* originated from a broad host range (wild animals, birds, food animals, and pet animals). Since poultry can get infected from different sources, *C. jejuni* can get colonized in the birds' gastrointestinal tract, ultimately leading to *C. jejuni* exposure to humans. Finally, combining the in silico approach and gene expression analysis, we identified 3 potential vaccine candidates: BtuB (an outer membrane protein), CdtB, and PldA (extracellular proteins).

Candidate Protein Description

BtuB is a barrel-shaped outer-membrane protein responsible for the transportation of vitamin B12, an essential nutrient and cofactor necessary for numerous microbial metabolic pathways (Fang et al., 2017). Outer membrane BtuB protein is directly connected to the TonB-dependent system in the inner membrane of the cell. Recently, the TonB-dependent transporters (TBDTs) have gained attention in developing the vaccine for gram-negative bacteria, and this transporter has all the essential characteristics for successful vaccine development (Wang et al., 2021) These finding collectively suggest that conserved antigen BtuB could be further used to develop a universal vaccine against genetically diverse *C. jejuni* strains.

Cytolethal-distending toxin consists of 3 subunits CdtA, CdtB, and CdtC, which help in the production of toxins and the pathogenesis of *Campylobacter*. CdtB was identified as the potential antigen candidate for the construction of subunit vaccines, and it was predicted to be located extracellularly. Along with *Campylobacter*, this toxin was produced by other enteric and nonenteric bacterial pathogens (*Escherichia coli, Shigella spp.*, Actinobacillus actinomycetemcomitans, and *Haemophilus ducreyi*) (Svensson et al., 2002; Thelestam and Frisan, 2004; Boesze-Battaglia et al., 2017; Meza-Segura et al., 2017). Furthermore, the prevalence of toxin-producing gene *CdtB* was present in greater than 90% of poultry isolates (Talukder et al., 2008; Findik et al., 2011; Nouri Gharajalar et al., 2020).

The third antigenic protein identified in this study was Phospholipase A (PldA), which plays a major role in hemolytic activity and helps bacterial colonization (Ziprin et al., 1999; Ziprin et al., 2002). Although PldA had not been previously tested as a potential vaccine candidate via developing a vaccine, this antigen was also previously identified as the potential vaccine candidate by in silico analysis utilizing the *C. jejuni* 81 to 176 strain (Meunier et al., 2016). Finally, this gene is yet to be tested as a vaccine candidate.

These findings collectively suggest that conserved antigenic proteins BtuB, CdtB, and PldA could be further used to develop a universal vaccine against genetically diverse *C. jejuni* strains. Although these proteins selected here were predicted and identified as antigenic through in silico analysis and gene expression analysis during host-pathogen interaction, we still need to determine the efficacy of these vaccine candidates during in vivo experiments.

CONCLUSION

In conclusion, this research identifies 3 potential antigenic vaccine candidates which have the potential to develop effective vaccines against *C. jejuni* and *C. coli* in poultry. We expect that these selected candidates may help reduce *C. jejuni* and *C. coli* colonization in the poultry gastrointestinal tract and subsequently help reduce *C. jejuni* and *C. coli* infections in humans. Furthermore, this study added a valuable step for screening the vaccine candidates utilizing RV technology, which can be utilized for selecting the vaccine candidate for other pathogens.

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Availability of Data: The authors confirm that the data supporting the finding of this study are available within the article and its additional materials.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2023.102592.

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