Identification of MHC Class II Immunopeptidomes from \textit{Shigella flexneri} 2a-infected Macrophages as Potential Vaccine Candidates

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\section*{Abstract}

\textbf{BACKGROUND:} \textit{Shigella} is a Gram-negative rod-shaped intracellular bacterial pathogen that causes bacterial dysentery or shigellosis among children under five years old. Antibiotics have been less effective in treating shigellosis due to the multi-drug resistance of \textit{Shigella}. Therefore, an effective vaccine is urgently needed to prevent this disease. The present study aims to determine the peptides presented by major histocompatibility complex (MHC) class II molecules of \textit{Shigella}-infected macrophages using mass spectrometry-based immunopeptidomics approaches. The MHC class II-associated peptides derived from \textit{Shigella}-infected macrophages are candidates for developing subunit-based \textit{Shigella} vaccine.

\textbf{METHODS:} THP-1-derived macrophages were infected with \textit{Shigella flexneri} 2a at the multiplicity of infection equal to 10. The lysate was immunoprecipitated and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The sequences retrieved were analyzed using bioinformatics tools.

\textbf{RESULTS:} The \textit{Shigella}-infected THP-1-derived macrophages contained sample peptides from source proteins of almost all subcellular localizations. Eight peptides from \textit{S. flexneri} 2a-infected macrophages were predicted to be localized at the outer membrane proteins (OMPs) of \textit{S. flexneri} 2a by the PSORTb server. Two of the OMP-associated peptides were predicted as antigenic, non-allergenic, and non-toxic by respective bioinformatics tools.

\textbf{CONCLUSION:} The findings in this study showed two selected OMPs have great potential for vaccine development against shigellosis.

\textbf{KEYWORDS:} immunopeptidomics, mass spectrometry, vaccine development, \textit{Shigella}, MHC peptides

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\section*{Introduction}

Shigellosis is a diarrheal disease caused by \textit{Shigella}, a rod-shaped Gram-negative bacterial pathogen.\textsuperscript{(1,2)} This bacteria is among the major contributor to the burden of the world and one of the most common causes of diarrhea.\textsuperscript{(3,4)} Annually, approximately 165 million cases of shigellosis are reported in many countries, with the majority of cases occurring in children under the age of five.\textsuperscript{(5)} \textit{Shigella flexneri, Shigella sonnei, Shigella dysenteriae}, and \textit{Shigella boydii} are the four dominant species of \textit{Shigella}. \textit{S. flexneri} is the most common species responsible for the endemic disease accounting for over 60\% of shigellosis incidences in developing countries.\textsuperscript{(6)}

Poor sanitation and weak water treatment lead to microbial contamination in the water supply.\textsuperscript{(7)} \textit{Shigella} illness is generally spread by ingesting contaminated water...
Shigellosis has a low infective dosage of 10 to 100 bacilli, which is enough to cause severe diarrhea and dehydration. Treatment with antibiotics is advised to lessen the severity of the condition. However, considering the risk of resistant bacteria, mass drug administration cannot be used to control shigellosis. Implementing an appropriate vaccine could serve long-term and consistent immunity against shigellosis.

Among all cellular components of human immune cells, macrophages are among the first to contact Shigella during pathogenesis. In addition, macrophages play a crucial role during Shigella infection by engulfing and eliminating pathogens and connecting the innate with the adaptive immune reaction throughout the antigen presentation. Macrophages also give an inflammatory response against Shigella infection. Given their variability and effect on immune responses, macrophages were selected as research subjects in this study.

The peptides presented by the major histocompatibility complex (MHC) class II of macrophages can activate not only T-cells, but also B-cells, and phagocytic cells. Because of this capability, MHC class II-associated peptides were targeted as candidates for subunit-based Shigella vaccine development. The present study applied the mass spectrometry (MS)-based immunopeptidomics approach to identify the peptides presented by MHC class II of S. flexneri 2a-infected THP-1-derived macrophages. Several bioinformatic tools were used to determine the most suitable peptides for vaccine candidates, originating from outer membrane proteins (OMPs), conserved with all the Shigella species, antigenic, non-allergenic, and non-toxic. The identified peptides are beneficial for the candidates to develop a subunit-based Shigella vaccine.

**Methods**

The MHC class II immunopeptidomes from the Shigella-infected macrophages and the bioinformatic analyses was identified to determine the candidate of Shigella vaccine. Briefly, immunoprecipitation was performed on the lysate of Shigella-infected macrophages followed with the performance of liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the sequences of MHC immunopeptidomes (Figure 1). To identify the dominant peptide presented by MHC class II of Shigella-infected macrophages, the peptide sequences retrieved from these techniques were examined using multiple bioinformatics tools. Several criteria were established to propose the peptide as a promising candidate for Shigella vaccine development.

**Bacterial Culture**

A mild clinical strain of S. flexneri 2a (SH062) was acquired from the laboratory of Assoc. Prof. Dr. Kirmpal Kaur Banga Singh at the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia. The methods were carried out following the guidelines approved by The Human Research Ethics Committee of Universiti Sains Malaysia (No. USMKK/PPP/JEPeM [248.3(10)]). The bacteria was thawed from -80°C storage, cultured on Luria–Bertani (LB) agar (Merck, Darmstadt, Germany), and incubated at 37°C overnight. A single colony of S. flexneri 2a from LB agar was transferred into 10 mL LB broth (Merck, Darmstadt, Germany).
Germany) and incubated overnight at 37°C with 200 rpm agitation. A hundred microliters of overnight bacterial suspension was transferred into 10 mL LB broth and incubated at 37°C with 200 rpm agitation until the bacteria grew to the mid-log phase (OD600 = 0.5; ultraviolet-visible spectrophotometer Agilent 8453, California, USA) for the infection assay. One milliliter of this culture, to be used for infection experiments, was centrifuged at 1,800 ×g for 5 minutes (Centrifuge 5415 R, Eppendorf, Darmstadt, Germany). The supernatant was discarded to ensure all media was removed completely. The cells were washed thrice with sterile phosphate buffered saline (PBS) (Nacalai Tesque, Kyoto, Japan) and the cell pellet was reconstituted in PBS to OD600 = 0.5 (UV-Visible spectrophotometer Agilent 8453, California, USA).

**Cell Culture**

In the present study, THP-1 cell lines were used as the model of human macrophages. The THP-1 cells were cultured in the same condition as explained earlier.(12) These cells were grown in Roswell Park Memorial Institute 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 1% penicillin–streptomycin (Nacalai Tesque, Kyoto, Japan), 10% fetal bovine serum (Tico Europe, Amstelveen, Netherlands), and 0.05 mM β-mercaptoethanol (Gibco, Massachusetts, USA). The cells were incubated at standard growth condition, 37°C with 5% CO₂ incubator (NuAire NU-4750E, Plymouth, USA).

Before performing the infection assay, THP-1 cells were stimulated to become elongated, adherent and macrophage-like cells by the adding of 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (InvivoGen, Pak Shek Kok, Hong Kong). For this purpose, THP-1 cells (2 × 10⁶ cells/mL) in complete culture media containing 100 ng/mL PMA were seeded in 24-well plate (1 mL/well) (Nest, Jiangsu, China). After 72 hours of PMA stimulation at standard growth conditions (NuAire NU-4750E, Plymouth, USA), PMA-containing medium was removed, washed once with pre-warmed PBS (Nacalai Tesque, Kyoto, Japan) and replaced with 1 mL cell culture medium without PMA and antibiotic. In order to allow the cells to rest after the differentiation phase, these cells were incubated again under the standard growth conditions for 24 hours. The differentiated cells, THP-1 derived macrophages, were ready to for the infection studies.

**Infection Assay and Cell Lysis**

The infection assay was performed based on the previous protocol.(10) The S. flexneri 2a was infecting THP-1-derived macrophages (2 × 10⁶ cells/mL) at the multiplicity of infection (MOI) equal to 10. As a negative control, non-infected cells were used. The mixture of S. flexneri 2a with THP-1-derived macrophages was centrifuged at 700 ×g (Kubota Plate Spin II, Osaka, Japan) at room temperature for 10 min to bring the bacteria to get close contact with the THP-1-derived macrophages and maximize the bacterial adherence to this cells. This mixture was incubated for one hour at standard growth conditions (NuAire NU-4750E, Plymouth, USA) to allow the infection to proceed. Then, the media was removed, washed twice with PBS and replaced with 1.5 mL fresh culture media containing 100 µg/mL gentamicin (Mims, Tokyo, Japan) to eliminate extracellular bacteria.

Under the standard growth condition, the macrophage-bacterium mixture was incubated for 5 h. The wells were washed twice with ice-cold 1× phosphate-buffered saline (PBS) to ensure that the medium was removed entirely. The infected THP-1-derived macrophages were scraped-off in the ice-cold 1× PBS using a cell scraper and transferred to an ice-cold 2 mL microcentrifuge tube. The harvested cell suspension was centrifuged at 600 ×g for 5 min, washed, and resuspended with 100 µL ice-cold lysis buffer composed of 50 mM Tris (pH 8.0) (Fisher BioReagents, Pennsylvania, USA), 150 mM sodium chloride (Bio Basic, Ontario, Canada), 2 mM ethylene glycol tetraacetic acid (Biobasic, Ontario, Canada), 2 mM ethylenediaminetetraacetic acid (Sigma-Aldrich, St. Louis, USA), and 0.3% NP-40. The suspension was incubated on ice for 30 min and centrifuged at 14,000 ×g for 10 min. The supernatant was transferred to a new 1.5 mL microcentrifuge tube and stored at -80°C until further use.

**Immunoprecipitation and LC-MS/MS**

MHC class II molecules were immunoprecipitated using 10 µg/mL purified anti-human HLA-DR, DP, and DQ (Biolegend, Vancouver, Canada) complexed with Dynabeads™ Protein G (Thermo Fisher Scientific, Massachusetts, USA). A 100 µL lysate containing peptide antigen was added and incubated for 1 h at room temperature. The MHC class II-peptide complexes were eluted using 30 µL of 10% acetic acid. The peptides were purified from high-molecular-weight components by ultrafiltration using a 10 kDa cutoff membrane filter (Vivaspin 6, GE Life Sciences, Amersham, UK). Twenty microliters of eluted peptide were pass through a C18 precolumn.

The peptides were analyzed using the Shimadzu prominence nano high-performance liquid chromatography (Shimadzu, Kyoto, Japan) method through electrospray...
ionization mass spectrometry coupled with a 5600 TripleTOF mass spectrometer (Sciex, Massachusetts, USA). Subsequently, the solution was diluted with a 1:10 matrix (α-cyano-4-hydroxycinnamic acid, 10 mg/mL) and stippled onto a stainless steel Opti-TOF 384-well plate. The stippled samples were assayed by using the first run of standard LC-MS. The assay was continued to operate the second run of MS/MS, which focused on the 15 most intense peaks of the first MS (excluding peaks identified as trypsin). The laser was set in MS mode to fire 400 times per spot and in MS/MS mode 2000 times per spot. The applied laser intensity was 2800 J (MS) and 3900 J (MS/MS). A mass range of 400 to 4000 amu was used with a focus mass of 2100 amu.

De novo sequencing of LC-MS/MS-derived sequences was automatically performed by the DeNovo Explorer™ version 3.6 software (Applied Biosystems, Massachusetts, USA). The spectra were analyzed to identify proteins of interest using the Mascot sequence matching software (Matrix Science) with the SwissProt database. The taxonomy was used to proteobacteria accessed in September 2019 with 198,235 sequences.

Bioinformatic Analyses
The background was removed from the data sets and assessed for their length (-mer). The localization of peptides was predicted using subcellular localization prediction software, PSORTb version 3.0.2 (https://www.psort.org/psortb/). (13) The identified peptides that were predicted OMPs by the PSORTb were screened on the non-redundant protein database using the BLASTp tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Then, the selected peptide sequences were screened for conservancy with proteins from S. sonnei, S. boydii, and S. dysentery. Epitopes were also screened for their conservation with similarity to human proteins. Then, the peptide sequences were predicted for the B-cell epitope using BepiPred 2.0 (http://www.cbs.dtu.dk/services/BepiPred/instructions.php) (14) and ABCPred (http://crdd.osdd.net/raghava/abcpred/) (15) servers. The prediction was performed using both servers concurrently, with the threshold set at 0.45. The B-cell epitopes resulting from the two algorithms were assembled, and the overlapping regions were selected as predicted B-cell epitopes. (16)

The T-cell epitopes of the selected peptide sequences were predicted using MHCpred (http://www.ddg-pharmfac.net/mhcpred/MHCpred/). (17) For the classification of peptides into binders and non-binders, a 500 nM binding threshold was decided, with an IC<sub>50</sub> binding value <500 nM as binders. Thus, IC<sub>50</sub> values less than 500 nM were used in this study to ensure a higher affinity.

The final sequences of the peptides were tested to predict antigenicity, allergenicity, and toxicity. Antigenicity prediction was performed using an online antigen prediction server, VaxiJen version 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). (18) The threshold was set at 0.4 against bacterial species. (19) AllergenFP version 1.0 (http://ddg-pharmfac.net/AllergenFP/) (20) was used to define the vaccine candidates as allergen or non-allergen. The toxicity of the peptides were predicted using ToxinPred (http://www.imtech.res.in/raghava/toxinpred/). (21)

Results

MHC-associated Peptide Sequence Identification
The Shigella-infected THP-1-derived macrophages were lysed, and LC-MS/MS was used to isolate the MHC class II molecules and analyze the HLA-associated peptides. A total of 793 unique peptide sequences were identified from the data set. The background was first removed from the data sets, yielding 647 peptide sequences relative to the control. The peptide sequences with the length between 9- to 25-mer were determined, giving 472 peptide sequences selected for further analyses. Figure 2 showed the length distribution between 9- to 25-mer peptides of S. flexneri 2a-infected macrophages.

Protein Localization, BLASTp Screening, and Conservation Prediction
Gram-negative bacteria have five major subcellular localization sites: cytoplasm, periplasm, inner membrane, outer membrane, and extracellular space. (22) The subcellular localization of each peptide sequence was predicted using PSORTb. Figure 3 showed the distribution of subcellular localization of peptide sequences identified using this tool. Peptide sequences predicted as OMPs were selected for further analysis. All peptides localized at the nuclear, periplasmic, inner membrane, extracellular, or cytoplasmic proteins were filtered out. The tool classified eight peptides as OMPs.

The detailed information of the peptides was obtained using BLASTp. The gene bank accession number, protein name, and position of the peptide in the full-length protein were discovered from this server. The protein conservation against S. sonnei, S. boydii, and S. dysentery was also obtained using BLASTp. All peptide sequences were conserved with all Shigella species. The conservation

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analysis against the human proteome homology showed no significance (Table 1).

**B-cell and T-cell Epitope Determination**

The eight peptide sequences that originated from the outer membranes were recognized for the eligibility to become B-cell epitopes using BepiPred and ABCPred. Based on Table 2, two peptide sequences were determined as non-B-cell epitope (SVVLTHAGK and TAAEEIAAVK) and were thus removed from the selection list. Then, the binding affinity between MHC class II and the T-cells was determined to ensure the eligibility of the peptides as T-cell epitopes. Five peptide sequences showed at least one sequence fragment with IC$_{50}$$<500$ nM, indicating the high and intermediate binding between these peptides with MHC class II (Table 2). The peptide sequence QLRGSSLHPSDVAK was removed from the list because it was determined as a non-binder. The final five peptide sequences were selected for the subsequent analyses.

**Allergenicity and Toxicity Prediction**

An efficient immune response is not only based on the positive recognition of peptides with significant affinity by MHC molecules but also the antigenicity status. Analyses with the VaxiJen v2.0 server presented that the peptide sequence EHKEEGATKRT scored the highest antigenicity (2.1459), followed by RFLIDEMEACIANLPLEVLCGR (0.6351) (Table 3). Three of the selected peptides showed a VaxiJen score of less than 0.4 (FTRDGNFLSYSSYHSQK, ILLAVNKAEGMK, and MSLSIADQNK), indicating non-antigenicity.

Allergenicity prediction of each selected peptide was performed using the AllergenFP version1.0. The server classified EHKEEGATKRT and RFLIDEMEACIANLPLEVLCGR as non-allergenic and the remaining epitopes as a possible human allergen. Then, all the selected epitopes were found as non-toxic to humans. The epitopes comprising the high antigenicity score and classified as non-allergenic and non-toxic to humans were
Table 1. MHC class II-associated peptides derived from OMPs of *S. flexneri* 2a.

| Peptide Sequences     | Length | Gene Bank Accession Number | Protein Name                                      | Length | Positions | Protein Sequence Homology among S. *serotypes (%) | Human Proteome Homology (%) |
|-----------------------|--------|-----------------------------|---------------------------------------------------|--------|-----------|---------------------------------------------------|----------------------------|
| EHKEEGATKRT           | 11     | NP_706439.1                 | Outer membrane receptor FepA                      | 746    | 136–146   | S. *sonnei*: 99.33 S. *boydii*: 99.33 S. *dysentery*: 99.33 | No significance            |
| FTRDGNFLSYSSYHSGSQK   | 19     | NP_706863.2                 | Outer membrane usher FimD-like protein            | 851    | 336–354   | S. *sonnei*: 100 S. *boydii*: 99.54 S. *dysentery*: 99.31 | No significance            |
| ILLAVNKAEGMK          | 12     | OXB27458.1                  | FhuE receptor                                     | 450    | 342–353   | S. *sonnei*: 99.03 S. *boydii*: 99.03 S. *dysentery*: 99.03 | No significance            |
| MSLSIADQNK            | 10     | WP_1286391 26.1             | RNA polymerase sigma factor RpoD (613 peptide length) | 613    | 442–451   | S. *sonnei*: 100 S. *boydii*: 85.71 S. *dysentery*: 100 | No significance            |
| QLRGSSLHPSDVAK        | 15     | WP_1566525 26.1             | YfiR family protein                               | 172    | 25–39     | S. *sonnei*: 85.71 S. *boydii*: 100 S. *dysentery*: 100 | No significance            |
| RFLIDEMEACIANLPLEVLCGR| 22     | OXB29755.1                  | Murein hydrolase activator EnvC                   | 415    | 92–113    | S. *sonnei*: 98.51 S. *boydii*: 97.7 S. *dysentery*: 100 | No significance            |
| SVVLTHAGK             | 9      | WP_1123176 75.1             | L-threonine dehydrogenase                         | 383    | 195–203   | S. *sonnei*: 100 S. *boydii*: 100 S. *dysentery*: 100 | No significance            |
| TAAEIAAVK             | 9      | WP_0162455 72.1             | type 4b pilus protein PilO2                       | 431    | 217–225   | S. *sonnei*: 100 S. *boydii*: 100 S. *dysentery*: 100 | No significance            |
Table 2. Number of B-cell and T-cell epitopes presented on the peptide sequences.

| Peptide Sequences | Number of B-cell Epitopes | Number of T-cell Epitopes |
|------------------|--------------------------|--------------------------|
|                  | BepiPred 2.0 and ABCPred (Threshold Set at 0.45) | MHCPred | IC₅₀ Value (nM) |
|                  | EHKEEGATKRT | EHKEEGATKRT | EHKEEGATKRT | 495.45 |
|                  | GNFLSYSSYHSQSQK | GNFLSYSSYHSQSQK | GNFLSYSSYHSQSQK | 424.62 |
|                  | ILLAVNKAEGMK | ILLAVNKAEGMK | ILLAVNKAEGMK | 297.17 |
|                  | MSLSIADQNK | MSLSIADQNK | MSLSIADQNK | 393.55 |
|                  | QLRGSSLLHPSDVAK | QLRGSSLLHPSDVAK | QLRGSSLLHPSDVAK | 34.28 |
|                  | RFLIDEMEACIANLPLEVLCGR | RFLIDEMEACIANLPLEVLCGR | RFLIDEMEACIANLPLEVLCGR | 34.28 |

B-cell epitopes were predicted using BepiPred and ABCPred, and the peptide-binding affinity of MHC class II DRB1*0101 was predicted using MHCPred sever with a threshold <500 nM.

considered possible epitopes that induce a strong immune response. These peptides included EHKEEGATKRT and RFLIDEMEACIANLPLEVLCGR (Table 3).

### Discussion

In the pathogenesis of shigellosis, the interaction between macrophages and *Shigella* is crucial. Given the crucial roles of macrophages in host defense, acquiring valuable candidates for the *Shigella* vaccine is critically essential for future immunoprevention development. The discovery of pathogen-derived MHC-binding peptides has been made possible by immunopeptidomes. The present study identified the range of peptides eluted from *S. flexneri* 2a-infected macrophages, and unique *S. flexneri* 2a-derived peptides were discovered. THP-1-derived macrophages, as a model of human macrophages, were used in this investigation to determine the peptide sequences presented by MHC class II molecules of macrophages infected with clinical strains of *S. flexneri* 2a.

The peptide presented by MHC class II is displayed on the cell surface and is identified by helper T-cells or CD4⁺ T-cells. Helper T-cells trigger appropriate immunological responses, such as cytotoxic T-cell activation, localized inflammation caused by phagocyte recruitment, or contributing to the activation of a humoral immune response via B-cell activation.(23) The multifunctional criteria of helper T-cell increase the interest to study MHC class II-associated peptides.

Table 3. Prediction of antigenicity, allergenicity, and toxicity of selected peptide sequences.

| Peptide Sequences | Antigenicity | Allergenicity | Toxicity |
|------------------|--------------|---------------|----------|
|                  | VaxiJen v2.0 (Threshold 0.4) | AllergenFP 1.0 | ToxinPred |
|                  | Score | Prediction | Score | Prediction | Score | Prediction |
| EHKEEGATKRT | 2.1459 | Antigen | Non-allergen | −0.93 | Non-toxin |
| FTRDGNSYSSYHSQSQK | 0.2445 | Non-antigen | Allergen | −0.97 | Non-toxin |
| ILLAVNKAEGMK | 0.161 | Non-antigen | Non-allergen | −1.15 | Non-toxin |
| MSLSIADQNK | 0.3158 | Non-antigen | Allergen | −0.82 | Non-toxin |
| RFLIDEMEACIANLPLEVLCGR | 0.6351 | Antigen | Non-allergen | −0.18 | Non-toxin |
In the present study, we used a bioinformatics approach in peptide selection. The prediction of normalized peptide subcellular localization was the initial stage in the selection procedure. For a protein to be accessible to immune surveillance, it must be physically exterior to the microbial organism or at the very least present on its surface, rather than being hidden away to avoid detection by the immune system.(24) Therefore, the peptides localized at the OMPs were selected in the present study. Bacterial OMPs are important for bacterial survival and growth in their host environments. The OMPs have a high potential to be vaccine candidates against infections because the host immune system can easily recognize the OMPs as foreign substances to initiate host immune defense mechanisms against bacterial infection.(25) A similar approach based on the immunopeptidomics study and predicting subcellular localization of the peptide sequences as the outer membrane has also been applied in the other studies to identify vaccine candidates.(26,27)

Five peptide sequences were predicted to be eligible as B-cell and T-cell epitopes, thus being eligible to activate humoral and cellular immune responses. Two of the peptides were highly antigenic, indicating the capability to induce immune responses. The allergenicity prediction is another crucial step before designing a vaccine. Most vaccines shift the immune reaction to the allergic response by activating type II T-helper cells, and immunoglobulin E. Allergenicity analysis in the present study suggested two peptides as non-allergenic, with the final selection of EHKEEGATKR and RFLIDEMEACIANLPLEVLCGR as the candidate for subunit-based Shigella vaccine development. Both peptide sequences have been predicted to be conserved among other Shigella species and immunogenic, making them ideal candidates for developing a broad-spectrum vaccination.

**Conclusion**

In summary, two target sequences from OMPs of *S. flexneri* 2a were selected through mass spectrometry-based immunopeptidomics and bioinformatics analyses. The bioinformatic predictions showed the selected sequences were conserved among the *Shigella* strains with particular *S. boydii*, *S. dysentery*, and *S. sonnei* and therefore suggested they are potentially effective against all the strains of *Shigella* spp. The candidate peptides can be experimentally validated using in vitro and in vivo analyses.

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**Authors Contribution**

CYL and KKBS were involved in planning and supervised supervising the work. NRMS, CMKHI, and MM processed the experimental data, performed the analysis, drafted the manuscript, and designed the figures. CHL prepared the project administration.

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