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Protective Efficacy of 49.8 kDa Pili Protein Epitopes of Shigella flexneri Against Bacterial Infection in Enterocytes

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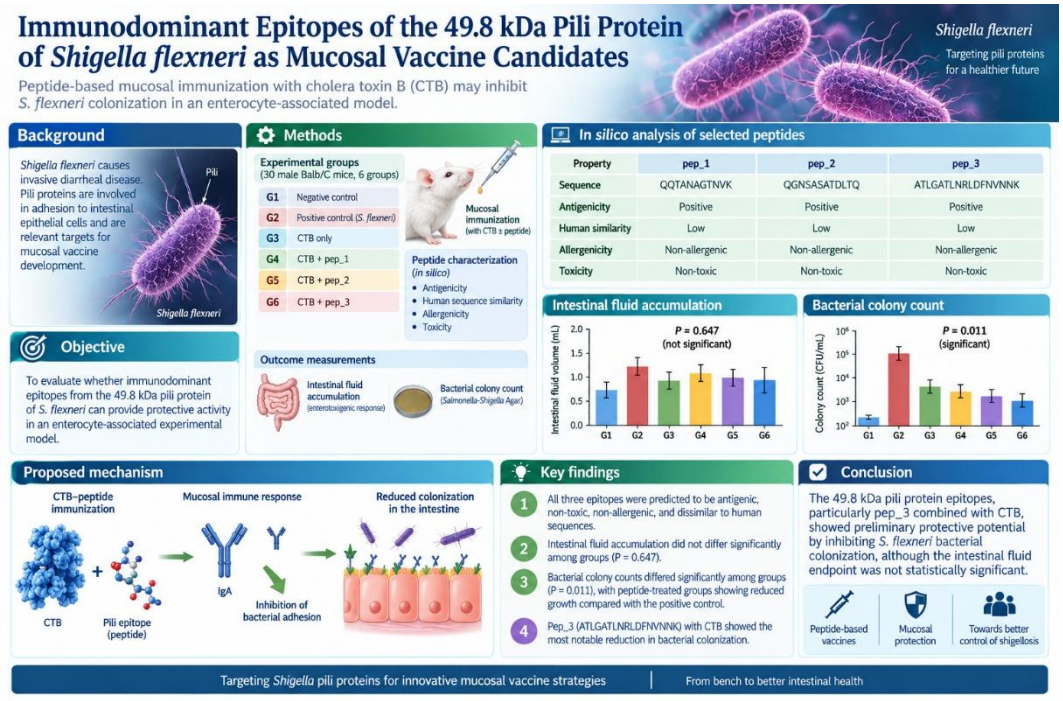
Graphical Abstract



Abstract: *Shigella flexneri* remains an important cause of invasive diarrheal disease, and adhesin-associated pili proteins are relevant targets for mucosal vaccine exploration. Objective: This study evaluated whether immunodominant epitopes derived from the 49.8 kDa pili protein of *S. flexneri* could provide protective activity against bacterial infection in an enterocyte-associated experimental model. Thirty male Balb/C mice were assigned to six groups: negative control, positive control, cholera toxin B subunit (CTB) only, CTB plus pep_1, CTB plus pep_2, and CTB plus pep_3. The three peptides were characterized in silico for antigenicity, human sequence similarity, allergenicity, and toxicity. Protective activity was assessed by intestinal fluid accumulation and bacterial colony growth on Salmonella-Shigella Agar. All three epitopes were predicted to be antigenic, non-toxic, non-allergenic, and dissimilar to human receptor sequences. Intestinal fluid accumulation varied among groups, but the difference was not statistically significant (P = 0.647). Bacterial colony counts differed significantly among groups (P = 0.011), with peptide-treated groups showing reduced colony growth compared with the positive control pattern. The 49.8 kDa

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pili protein epitopes, particularly pep_3 (ATLGATLNRLDFNVNNK) combined with CTB, showed preliminary protective potential by inhibiting *S. flexneri* bacterial colonization, although the intestinal fluid endpoint was not statistically significant.

Keywords: bacterial colonies; enterocyte; epitope; intestinal fluid; protective efficacy; Shigella flexneri

INTRODUCTION

Shigellosis is an invasive diarrheal disease caused by Shigella spp. and remains a major public health concern, particularly among young children in low- and middle-income settings.[1-3] S. flexneri is one of the most important species in endemic settings and is strongly associated with inflammatory enteric disease, transmission in crowded environments, and the continuing need for preventive interventions.[1-3] Despite decades of vaccine research, a broadly available licensed Shigella vaccine is still not in routine public health use, making additional antigen discovery and preclinical evaluation scientifically relevant.[16-20]

Shigella pathogenesis depends on epithelial invasion, manipulation of host-cell signaling, intracellular survival, inflammatory tissue injury, and bacterial surface structures that support early host-pathogen interactions.[4-7] Adhesion is a key early event in infection, and bacterial pili, fimbrial structures, and other surface organelles can mediate attachment, immune recognition, and colonization in Gram-negative pathogens.[8-11] Therefore, proteins associated with pili or adhesin function remain biologically plausible targets for vaccine-oriented antigen screening.

Protection at the intestinal mucosa requires more than systemic antibody responses. Secretory IgA contributes to immune exclusion, neutralization of enteric pathogens, preservation of epithelial barrier integrity, and control of inflammatory injury during Shigella infection.[12-15] For this reason, experimental readouts such as intestinal fluid accumulation and bacterial colony recovery can provide functional evidence of whether a candidate antigen reduces fluid secretion or limits enterocyte-associated bacterial colonization.

Epitope-based and peptide-based vaccine strategies offer a focused way to test defined immunogenic regions while reducing unnecessary antigenic complexity.[21-24] However, short peptides often require appropriate adjuvant or carrier strategies, and cholera toxin B subunit (CTB) has long been explored as a mucosal adjuvant or antigen carrier.[25,26] Previous work on 49.8 kDa S. flexneri pili protein peptides reported intestinal immune activation and identified antigenic epitope sequences relevant to shigellosis vaccine development.[27,28] Building on that background, this study aimed to evaluate the protective efficacy of three 49.8 kDa pili protein epitopes against S. flexneri infection using in silico safety characterization, intestinal fluid measurement, and bacterial colony assessment.

MATERIALS AND METHODS

Research design and experimental animals

This experimental study used 30 male Balb/C mice (Mus musculus), aged 6-8 weeks. The animals were randomly assigned into six groups, with five mice per group. The animals were obtained from and maintained at the Experimental Animal Laboratory, Faculty of Medicine, Brawijaya University, Malang, Indonesia. Reporting details were strengthened with reference to ARRIVE 2.0 principles where applicable.[34]

The antigenic peptides derived from the 49.8 kDa pili protein of S. flexneri were pep_1 (QSSTGTNSQSDLDS), pep_2 (DTTITKAETKTVTKNQVVDTPVTTDAAK), and pep_3 (ATLGATLNRLDFNVNNK). CTB was used as an adjuvant for immunization.[25,26] The six treatment groups were negative control, positive control, CTB only, CTB plus pep_1, CTB plus pep_2, and CTB plus pep_3.

In silico characterization of epitopes

Before ex vivo protective efficacy testing, the three peptide epitopes were characterized in silico. Antigenicity was predicted using the VaxiJen v2.0 server, and

sequence similarity was evaluated using BLASTp to assess homology with human proteins and minimize cross-reactivity risk.[29-31] Allergenicity was assessed using AllerTOP v2.0, and peptide toxicity was evaluated using ToxinPred.[32,33]

Isolation of mucosal fluid

Intestinal mucosal fluid was isolated with minor modifications from the submitted protocol. A 15 cm intestinal segment was excised and opened longitudinally to expose the mucosal surface. The mucus layer was collected by longitudinal scraping using a sterile spatula and transferred into sterile tubes containing phosphate-buffered saline (PBS). The suspension was vortexed and centrifuged at 12,000 rpm at 4 degrees Celsius for 10 minutes. The pellet was resuspended in PBS, followed by dialysis against PBS. The resulting mucosal fluid, presumed to contain sIgA, was used for subsequent protective efficacy assays.

Measurement of intestinal fluid volume

Following epitope immunization, mouse intestines were excised and cut into 10 cm segments. Both ends of each segment were tightly ligated with surgical thread. Each segment was injected with 100 µL of S. flexneri suspension (10^6 CFU/mL) and immersed in Roswell Park Memorial Institute (RPMI) medium. Samples were incubated at 37 degrees Celsius under continuous agitation using a shaker water bath at 60 rpm for 3 hours. Each intestinal segment was weighed before and after bacterial exposure. Intestinal damage was assessed by calculating the difference in intestinal fluid weight after exposure compared with before exposure.

Quantification of bacterial colonization

Intestinal segments of 10 cm were exposed to S. flexneri that had been pre-coated with mucosal sIgA samples. The segments were incubated for 3 hours at 36 degrees Celsius and subsequently rinsed with sterile PBS. The tissue was homogenized using a Potter homogenizer in an equal volume of sterile PBS. A 100 µL aliquot of homogenate was plated onto SSA medium and incubated for 18-24 hours at 37 degrees Celsius. Colonies showing characteristic S. flexneri morphology were quantified using a colony counter.

Statistical analysis

Data were analyzed using ANOVA and the Kruskal-Wallis test. Results are presented as mean values and standard deviations. A P value less than 0.05 was interpreted as statistically significant.

RESULTS

In silico characterization

The in silico characterization showed that all three epitopes were antigenic, had no similarity to human receptors, were predicted to be non-toxic, and were predicted to be non-allergenic (Tables 1 and 2). These results support further ex vivo protective efficacy testing, while recognizing that computational screening does not replace experimental safety and immunogenicity assessment.[29-33]

Table 1. Antigenicity and similarity analysis of 49.8 kDa pili protein epitopes

Peptide	Antigenicity score	Antigenicity interpretation	Similarity score	Similarity interpretation
pep_1 (Q-S)	1.6531	Antigen (>0.4)	<40	No similarity
pep_2 (D-K)	0.8225	Antigen (>0.4)	<40	No similarity
pep_3 (A-K)	1.2407	Antigen (>0.4)	<40	No similarity

Table 2. Toxicity and allergenicity analysis of 49.8 kDa pili protein epitopes

Peptide	Score	Toxicity	Allergenicity
pep_1 (Q-S)	-0.77	Non-toxin	Non-allergen
pep_2 (D-K)	-1.55	Non-toxin	Non-allergen
pep_3 (A-K)	-1.20	Non-toxin	Non-allergen

Intestinal fluid accumulation

The volume of intestinal fluid in mice challenged with S. flexneri was used as an indicator of intestinal damage after bacterial exposure. Variations in intestinal fluid weight were observed across the experimental groups (Figure 1). However, the between-group comparison was not statistically significant (P = 0.647).

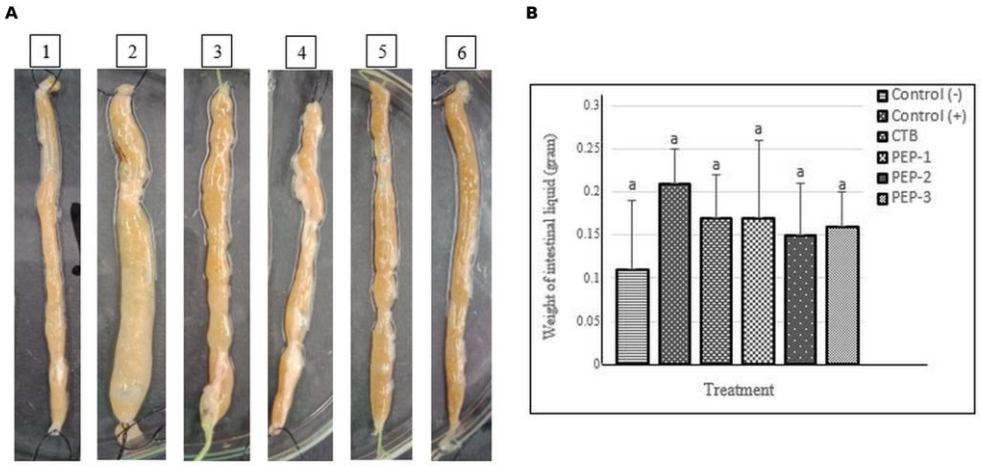

Figure 1. Intestinal morphology and intestinal fluid weight after S. flexneri challenge. A: intestinal morphology in the diarrhea treatment model; B: comparative analysis of intestinal fluid weight across groups. Group 1: control negative/placebo; Group 2: control positive/bacterial infection; Group 3: CTB only; Group 4: PEP-1 (CTB plus pep_1); Group 5: PEP-2 (CTB plus pep_2); Group 6: PEP-3 (CTB plus pep_3). The submitted analysis reported P = 0.647.

Bacterial colony growth

Bacterial colony growth on SSA medium was used as an indicator of protective capacity against S. flexneri proliferation. The treatment groups showed distinct bacterial growth patterns compared with the positive control group (Figure 2). The submitted statistical analysis showed a significant difference among groups (P = 0.011).

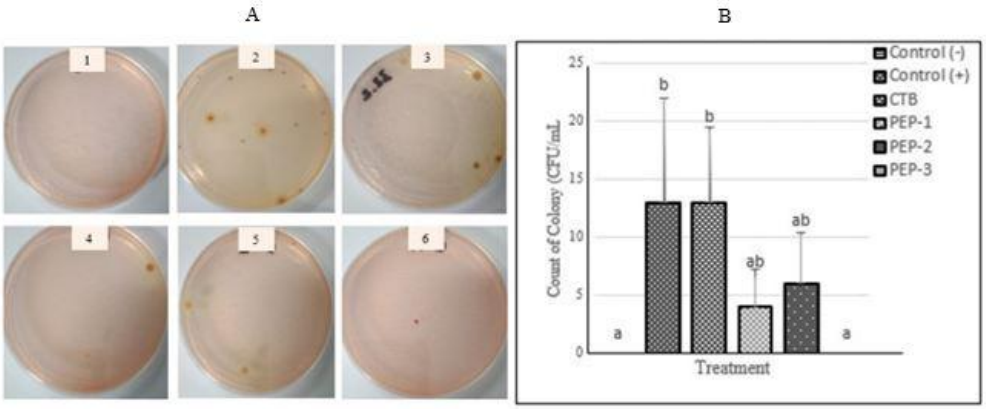

Figure 2. Observation of S. flexneri bacterial colonies on SSA medium. A: bacterial colony growth on SSA culture media; B: statistical analysis of bacterial colony counts. Group 1: control negative/placebo; Group 2: control positive/bacterial infection; Group 3: CTB only; Group 4: PEP-1 (CTB plus pep_1); Group 5: PEP-2 (CTB plus pep_2); Group 6: PEP-3 (CTB plus pep_3). The submitted analysis reported P = 0.011.

DISCUSSION

This study evaluated three immunodominant epitopes derived from the 49.8 kDa pili protein of S. flexneri using a combination of computational screening and ex vivo protective efficacy testing. The computational results indicated that pep_1, pep_2, and pep_3 were antigenic, had low human sequence similarity, and were predicted to

be non-toxic and non-allergenic. These findings are consistent with the logic of epitope-based vaccine design, in which antigenic potential must be balanced with early safety screening before functional testing.[21-24,29-33]

The biological rationale for focusing on pili-derived epitopes is plausible. Shigella disease involves epithelial invasion and inflammatory tissue damage, and surface-associated adhesins or pili-related structures can contribute to early host-pathogen contact.[4-11] A peptide derived from such a structure may therefore contribute to immune recognition that interferes with bacterial attachment, colonization, or downstream epithelial injury. However, the present data remain preliminary because the work evaluated functional endpoints rather than directly measuring binding inhibition, epithelial invasion, cytokine profiles, or quantitative mucosal antibody titers in the same assay set.

The intestinal fluid endpoint showed a downward pattern in some peptide-treated groups but did not reach statistical significance ($P = 0.647$). This result should be interpreted cautiously. It suggests that the peptides did not produce a statistically clear reduction in fluid accumulation under the submitted experimental conditions, even though the direction of the pattern may be biologically interesting. The conclusion should therefore avoid overstating protection based on this endpoint alone.

The bacterial colony endpoint provided stronger evidence of functional activity. Colony counts differed significantly among groups ($P = 0.011$), and the peptide-treated groups showed reduced bacterial growth compared with the positive control pattern. This supports the interpretation that the epitopes, particularly pep_3 combined with CTB, may inhibit S. flexneri colonization in the intestinal model. The finding is consistent with previous work showing that mucosal antibodies can reduce Shigella epithelial injury and preserve barrier integrity.[12-15]

The use of CTB is also relevant to interpretation. CTB has been studied as an oral mucosal adjuvant and antigen carrier, and it may enhance antigen delivery and mucosal immune responses.[25,26] Therefore, the observed protective pattern should be understood as the effect of peptide administration in combination with CTB, not as peptide-only efficacy. Future work should include peptide-only controls, CTB-matched controls, direct sIgA measurement after challenge, epithelial adhesion or invasion assays, and quantitative histopathology.

This study has several limitations. The sample size was small, with five animals per group. The manuscript did not provide complete animal ethics approval information, randomization implementation details, blinding procedures, or sample-size justification. The intestinal fluid endpoint was not statistically significant, and the available figures did not provide exact numeric summary values for all plotted bars. In addition, computational predictions are screening tools and should not be interpreted as definitive evidence of safety or immunogenicity. These limitations do not invalidate the observed colony-count pattern, but they mean the findings should be presented as preliminary preclinical evidence requiring confirmation.[34]

CONCLUSION

The three immunodominant epitopes derived from the 49.8 kDa pili protein of S. flexneri were predicted to be antigenic, non-toxic, non-allergenic, and dissimilar to human receptor sequences. In the ex vivo intestinal model, the epitopes reduced intestinal fluid accumulation descriptively, but this endpoint was not statistically significant ($P = 0.647$). Bacterial colonization differed significantly among groups ($P = 0.011$), with pep_3 (ATLGATLNRLDFNVNNK) combined with CTB showing the strongest inhibitory pattern in the submitted figure. These results support the preliminary potential of 49.8 kDa pili protein epitopes as Shigella vaccine candidates, while further studies are needed to confirm mucosal immune mechanisms, dose-response effects, and reproducibility.

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AUTHORS CONTRIBUTIONS

Khoirul Anam, Siti Raudah, Muhammad Fahmi Aminuddin, Yuliati, Agustina Tri Endharti, and Sumarno Reto Prawiro contributed to the conception, experimental work, analysis, manuscript preparation, and critical revision of the study. All authors reviewed and approved the manuscript.

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DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AI STATEMENT

Generative artificial intelligence was used to support language. No AI tool was used to generate original research data, perform experiments, or alter study results.

DISCLOSURE STATEMENT

The authors declare no conflict of interest. The data are original and have not been published in another journal.

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