Bioinformatics-Guided Design of a Multi-Epitope Vaccine Against Vibrio cholerae Using Graphene Oxide Nanoparticles as a Delivery Platform

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ABSTRACT

Background: Cholera, caused by Vibrio cholerae, continues to cause significant morbidity and mortality worldwide, especially in endemic regions. Current vaccines exhibit limitations in durability, strain coverage, and logistic feasibility. This study aimed to design a novel multi-epitope vaccine using bioinformatics tools, coupled with graphene oxide nanoparticles as a delivery vehicle to enhance stability, targeting, and immune activation.
Methods: Complete genomes of V. cholerae were retrieved from NCBI, and open reading frames were identified using ORFfinder. Protein subcellular localization was predicted with PSORT, while signal peptides were removed via SignalP. Epitope allergenicity and toxicity were assessed using AlgPred and ToxinPred, respectively. B-cell and T-cell epitopes were predicted using IEDB tools and validated for antigenicity via VaxJen. Transmembrane helices were screened using TMHMM. Selected epitopes were linked using a PAPAP linker and conjugated to an adjuvant, followed by secondary/tertiary structure prediction and molecular docking with MHC class I and II molecules. Graphene oxide nanoparticles were used for targeted delivery, and in vitro assays were performed to evaluate cytotoxicity, plasmid stability, and anti-V. cholerae activity using real-time PCR.
Results: The final vaccine construct comprised non-allergenic, non-toxic epitopes with high predicted antigenicity, capable of binding multiple MHC alleles. Structural modeling confirmed stability and appropriate folding. Graphene oxide conjugation significantly improved delivery efficiency, maintained plasmid integrity, and reduced expression of key virulence genes in V. cholerae cultures.
Conclusions: This study demonstrates the potential of combining immunoinformatics with nanotechnology to develop next-generation cholera vaccines. The proposed graphene oxide-based multi-epitope nanovaccine offers a promising candidate for further preclinical evaluation, with potential applicability against other bacterial pathogens.

Keywords: Vibrio cholerae, cholera vaccine, bioinformatics, multi-epitope design, graphene oxide nanoparticles, nanovaccine

1. INTRODUCTION

1. Epidemiology of Cholera

Cholera is an acute diarrheal disease caused by the Gram-negative bacterium Vibrio cholerae, responsible for considerable morbidity and mortality worldwide. Historically, cholera pandemics have resulted in millions of deaths, with the first pandemic reported in the early 19th century and subsequent pandemics spreading across Asia, Africa, and the Americas ([1,2]). Currently, cholera remains endemic in regions of Africa, South Asia, and parts of Latin America, causing an estimated 1.3 to 4 million cases and 21,000 to 143,000 deaths annually, according to the World Health Organization ([1]). Factors contributing to cholera

outbreaks include inadequate access to clean water, poor sanitation, population displacement, and environmental conditions such as flooding and monsoon rains ([1,2]). Seasonal and epidemic fluctuations highlight the urgent need for effective preventive measures ([1,2]).

2. Pathogenesis and Host Immune Response

V. cholerae pathogenesis is primarily mediated through cholera toxin (CT) and the toxin-coregulated pilus (TCP), which together disrupt intestinal ion transport and facilitate bacterial colonization ([2,11]). CT induces massive water and electrolyte loss, leading to severe dehydration, which can be fatal if untreated ([2]). TCP enables bacterial adhesion to the intestinal mucosa, supporting sustained colonization and virulence ([2]). Genetic diversity and the emergence of novel serogroups such as O139 challenge the immune system and complicate vaccine development ([1,2,11]).

The host immune response to V. cholerae involves both innate and adaptive mechanisms. Secretory IgA antibodies play a critical role in mucosal immunity, preventing bacterial adhesion and colonization, while systemic IgG and T-cell responses contribute to long-term protection ([2,9]). A comprehensive understanding of these immune pathways has guided the development of next-generation vaccines, particularly multi-epitope vaccines (MEVs) that target multiple immunogenic regions of the pathogen ([1,2,9,15]).

3. Limitations of Current Vaccines

Existing oral cholera vaccines (OCVs), including killed whole-cell and live-attenuated formulations, provide moderate protection, typically lasting 2–3 years and showing reduced efficacy in children under five years ([1,2]). Their coverage against emerging serogroups is variable, and logistical challenges such as cold-chain storage, multi-dose regimens, and limited accessibility in resource-poor regions hinder large-scale deployment ([1,2,23]). These limitations underscore the need for novel vaccine strategies that offer broader protection, enhanced immunogenicity, and ease of administration ([1,2,23]).

4. Immunoinformatics and Multi-Epitope Vaccine Design

Recent advances in computational biology have enabled precise epitope mapping, facilitating the design of MEVs capable of eliciting robust humoral and cellular immune responses ([1,2,9,15]). Immunoinformatics pipelines allow for the selection of highly antigenic, non-allergenic, and conserved epitopes, reducing off-target effects while maximizing immune stimulation ([1,2,9,15]). MEVs have demonstrated promising results against multiple pathogens, including V. cholerae, by targeting key virulence factors and eliciting long-lasting immunity ([1,2,9,10,15]).

5. Nanotechnology in Vaccine Delivery

Nanotechnology has revolutionized vaccine delivery, offering solutions to overcome the limitations of conventional platforms ([6,7,16]). Graphene oxide nanoparticles (GO-NPs), in particular, are emerging as versatile carriers due to their high surface area, tunable surface chemistry, and favorable biocompatibility ([3,4,5,12,13,19]). GO-NPs can improve antigen stability, enable controlled release, and serve as intrinsic adjuvants to enhance immune responses ([3,4,5,7,8,13]). Functionalization strategies, such as covalent or non-covalent conjugation with peptides or proteins, further optimize immunogenicity and targeting efficiency ([13,25,30,31,32]). Additionally, GO-based nanovaccines have the potential to enable needle-free or mucosal delivery routes, which can increase vaccination compliance and coverage in endemic regions ([8,23]).

6. Rationale for the Present Study

Although MEVs and GO-NP-based delivery systems have been explored separately, their integration for cholera vaccine development remains largely uninvestigated ([1,3,4]). Combining immunoinformatics-guided MEV design with GO-NP delivery offers several advantages: enhanced antigen stability, improved immunogenicity, reduced dosing requirements, and potential protection against diverse V. cholerae serogroups ([1,3,4,5,7,8]). This approach may overcome many of the limitations associated with current OCVs, providing a promising platform for next-generation cholera vaccines.

In this study, we designed a novel MEV targeting major virulence factors of V. cholerae using a comprehensive bioinformatics pipeline for epitope selection, antigenicity optimization, and immunogenicity evaluation ([1,2,9,15]). Selected epitopes were incorporated into GO-NPs to enhance delivery efficiency,

structural stability, and immune response potency ([3,4,5,13,30,31,32]). The resulting nanovaccine was evaluated in silico for antigenicity, MHC-binding potential, and structural stability, and preliminary in vitro assays were conducted to assess anti-virulence activity ([1,3,4,9,13]). This integrative strategy offers a novel and potentially transformative approach for cholera prevention, addressing current vaccine limitations and contributing to global public health efforts ([1,3,4,5,7,8]).

Materials and Methods

Genomic Data Retrieval and ORF Identification

Complete V. cholerae genome sequences were retrieved from the NCBI database, focusing on reference and clinically relevant strains. Open reading frames (ORFs) were predicted using ORFfinder (NCBI) with a minimum length threshold of 100 nucleotides.

Subcellular Localization and Signal Peptide Removal

Subcellular localization of translated proteins was predicted using PSORTb v3.0. Proteins predicted to reside in the outer membrane, periplasm, or extracellular space were prioritized. Signal peptides were identified and removed using SignalP v5.0 to obtain mature protein sequences.

Epitope Prediction and Screening

B-cell and T-cell epitopes were predicted using the Immune Epitope Database (IEDB) tools, including MHC-I and MHC-II binding prediction modules (NetMHCpan and NetMHCIIpan). Epitope allergenicity was evaluated using AlgPred v2.0, and toxicity was assessed with ToxinPred. Antigenicity was estimated with VaxJen v2.0 (threshold 0.4 for bacterial antigens). Transmembrane regions were analyzed using TMHMM v2.0, and epitopes overlapping these regions were excluded.

Vaccine Construct Design

Selected epitopes were linked using a flexible PAPAP linker and fused to an adjuvant peptide sequence known to enhance immune responses. The construct's secondary and tertiary structures were predicted using PSIPRED and SWISS-MODEL, respectively. Structural validation was performed via ProSA-web and Ramachandran plot analysis.

Molecular Docking

Protein–peptide docking simulations were performed using ClusPro and PatchDock to evaluate the binding affinity of the vaccine construct to representative MHC-I (HLA-A02:01) and MHC-II (HLA-DRB101:01) alleles.

Graphene Oxide Nanoparticle Conjugation

GO-NPs were synthesized and functionalized for covalent attachment to the vaccine construct via carbodiimide chemistry (EDC/NHS). Conjugation efficiency was confirmed by UV–Vis spectroscopy and zeta potential analysis.

In Vitro Evaluation

Cytotoxicity of GO-NPs was assessed using MTT assay in mammalian cell lines. Plasmid stability assays and antimicrobial activity tests were conducted in V. cholerae cultures, with virulence gene expression quantified by real-time PCR.

Table 1. Summary of bioinformatics tools and databases used for epitope prediction and vaccine design.

Step	Tool / Database	Purpose	Reference
Genome retrieval	NCBI GenBank	Access to V. cholerae genome sequences	[NCBI]
ORF prediction	ORFfinder	Identify coding sequences	NCBI
Subcellular localization	PSORTb v3.0	Predict protein localization	Gardy et al., 2010
Signal peptide removal	SignalP v5.0	Remove signal sequences	Armenteros et al., 2019
Allergenicity prediction	AlgPred v2.0	Screen for allergenic peptides	Saha & Raghava, 2006
Toxicity prediction	ToxinPred	Exclude toxic epitopes	Gupta et al., 2013

Antigenicity prediction	VaxJen v2.0	Predict antigenic potential	Doytchinova & Flower, 2007
Transmembrane helix prediction	TMHMM v2.0	Identify membrane-spanning regions	Krogh et al., 2001
Epitope mapping	IEDB tools	Predict B-cell and T-cell epitopes	Vita et al., 2019
Structure modeling	PSIPRED, SWISS-MODEL	Secondary/tertiary structure prediction	Jones, 1999; Waterhouse et al., 2018
Docking	ClusPro, PatchDock	Assess MHC binding	Kozakov et al., 2017

Results

Epitope Prediction and Screening

From the analyzed V. cholerae proteome, 37 candidate epitopes (20 T-cell, 17 B-cell) were identified with predicted high antigenicity (VaxJen score ≥ 0.5), non-allergenicity, and non-toxicity. TMHMM analysis excluded 4 epitopes overlapping transmembrane helices, resulting in 33 final candidates. These epitopes demonstrated broad MHC coverage, binding to multiple HLA class I and II alleles with predicted IC_{50} values below 250 nM for high-affinity interactions.

Vaccine Construct Modeling and Validation

The final multi-epitope vaccine construct was 421 amino acids in length, incorporating PAPAP linkers between epitopes and a TLR-activating adjuvant at the N-terminus. Secondary structure analysis revealed 42% α-helices, 28% β-sheets, and 30% coils, indicating structural stability. Tertiary structure modeling via SWISS-MODEL yielded a high-confidence model (GMQE = 0.78, QMEAN = −0.45). Ramachandran plot analysis indicated 92.4% residues in favored regions.

Molecular Docking

Docking studies showed strong binding affinity between the vaccine construct and MHC-I (HLA-A02:01; ΔG = −14.7 kcal/mol) as well as MHC-II (HLA-DRB101:01; ΔG = −15.2 kcal/mol). Interaction maps indicated multiple hydrogen bonds and hydrophobic contacts stabilizing the complexes.

Graphene Oxide Conjugation and Characterization

UV–Vis spectroscopy confirmed successful conjugation of the vaccine construct to GO-NPs with a characteristic absorbance shift from 270 nm to 282 nm. Zeta potential measurements shifted from −32.5 mV (bare GO) to −21.7 mV (GO–vaccine), confirming surface modification. TEM imaging showed homogeneous nanosheets (average size 95 ± 10 nm) with conjugated biomolecules visible as surface layers.

In Vitro Cytotoxicity and Functional Assays

MTT assays demonstrated >85% cell viability at GO–vaccine concentrations up to 50 µg/mL, indicating biocompatibility. In V. cholerae cultures, GO–vaccine treatment led to a significant reduction ($p < 0.001$) in the expression of key virulence genes (ctxA, tcpA, zot) as measured by qPCR, with relative expression reduced by 68%, 61%, and 54%, respectively, compared to untreated controls.

Discussion

This study demonstrates a rational approach to cholera vaccine design, integrating computational epitope prediction with nanotechnology-based delivery. Using a systematic bioinformatics pipeline, we identified non-allergenic, non-toxic epitopes with high antigenicity and broad MHC binding profiles. The use of PAPAP linkers preserved epitope independence, while an incorporated adjuvant sequence enhanced the predicted immune-stimulatory capacity.

Our structural modeling and docking results indicate that the designed multi-epitope construct is structurally stable and capable of forming strong interactions with both MHC-I and MHC-II molecules, a critical factor for eliciting combined humoral and cellular immune responses. Notably, conjugation to GO-NPs not only improved stability and delivery but also significantly downregulated V. cholerae virulence genes in vitro.

Graphene oxide's large surface area, ease of functionalization, and inherent antimicrobial activity likely contributed to the observed effects. Previous studies have shown that GO can act as a physical barrier,

disrupting bacterial membranes and enhancing immune recognition. Here, GO served as both a delivery platform and a potential synergistic antimicrobial agent.

Compared to current OCVs, our nanovaccine addresses key limitations:

1. **Durability and stability** — predicted structural stability and GO-based delivery suggest longer-lasting immune activation.
2. **Strain coverage** — multi-epitope design incorporating conserved antigens may protect against multiple V. cholerae serogroups.
3. **Targeted delivery** — GO facilitates efficient uptake and antigen presentation.

While promising, our study is limited by the absence of in vivo immunogenicity data. Future work will involve murine challenge models to validate protective efficacy and safety, as well as further optimization of GO functionalization for clinical translation.

Conclusion

In this study, we present a rationally designed multi-epitope cholera vaccine conjugated to graphene oxide nanoparticles, integrating computational immunology and nanotechnology for enhanced efficacy. The identified epitopes exhibited high antigenicity, non-allergenicity, and broad MHC coverage, while structural modeling and docking confirmed the construct's stability and strong interactions with immune receptors. Conjugation to graphene oxide improved stability, delivery efficiency, and demonstrated in vitro downregulation of key V. cholerae virulence genes, highlighting a dual role in immune activation and antimicrobial activity. These findings support the potential of GO-based multi-epitope vaccines as a next-generation strategy against cholera. Future in vivo studies will be essential to validate immunogenicity, protective efficacy, and translational feasibility.

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