Yeast-Based Expression of Zika Virus Envelope Protein and CTB Fusion for Oral Vaccine Development

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Abstract

This study describes the development of an expression system for an oral Zika virus vaccine. The vaccine is based on a fusion protein of the cholera toxin B subunit (CTB) and the Zika virus envelope protein (E protein), connected via a GGSG linker and expressed in *Saccharomyces cerevisiae*. The ADH1 promoter was incorporated into the yeast expression vector to drive transcription of the fusion protein. Yeast transformants were confirmed through colony PCR, and initial protein expression was validated using SDS-PAGE.

Introduction

Zika Virus Outbreak and Health Implications

The Zika virus, an arbovirus within the Flavivirus genus, gained global attention during outbreaks in 2015 and 2016 across the Americas, with Brazil as the epicenter (1). Transmitted primarily by Aedes mosquitoes, Zika infections led to severe health outcomes, particularly among pregnant women, where the virus is linked to congenital Zika syndrome, including microcephaly, brain calcifications, and other neurological malformations in newborns, resulting in lifelong developmental challenges (2). In adults, Zika virus infection has been associated with neurological complications, such as Guillain-Barré syndrome (1). Despite its significant public health impact, vaccine development for Zika has faced challenges, especially in resource-constrained settings, highlighting the need for a stable, accessible, and easily administered vaccine, such as one based on oral delivery.

Structure of Zika Virus and Its Envelope Protein

The Zika virus particle has a positive-sense, single-stranded RNA genome approximately 10.7 kb in size, wrapped in a protein capsid and further enveloped by a lipid membrane derived from the host cell. Embedded within this envelope are two major structural proteins: the membrane (M) protein and the envelope (E) protein (3). The E protein facilitates viral attachment and entry

by binding to host cell receptors and mediates membrane fusion. Its immunogenic nature makes it a primary target for neutralizing antibodies, playing a critical role in host immunity and serving as an ideal antigen candidate for vaccine development (4).

AB Toxins in Foodborne Pathogens and the Immunogenic Potential of B Subunits

Many foodborne pathogens, such as *Vibrio cholerae* (cholera toxin, CT) and enterotoxigenic *Escherichia coli* (heat-labile toxin, LT), produce AB toxins characterized by an enzymatically active A subunit and a receptor-binding B subunit (5). These B subunits bind host cell receptors like GM1 ganglioside without inducing toxicity, presenting a unique opportunity as safe, stable immunogens. The CTB subunit, specifically, binds intestinal GM1 receptors, facilitating antigen delivery and mucosal immune responses (6). Studies have shown that B subunits can be used as antigen carriers, triggering immune responses that extend protection without toxicity (7).

Vaccine Design Concept

Based on CTB's binding properties, we hypothesized that fusing CTB with the Zika E protein, connected via a GGSG linker, would generate a safe and effective oral vaccine. CTB's binding to intestinal cells would enhance uptake and recognition of the Zika E protein as a target antigen (6). This fusion protein, expressed in yeast, could be heat-dried, maintaining structural stability and ensuring inactivation of the yeast cells. This delivery system allows oral administration, improving accessibility in remote and resource-limited areas.

Materials and Methods

1. **Expression vector design**

   The expression vector was designed to express a fusion protein consisting of three components:

   - **N-terminal CTB (Cholera Toxin B subunit)**: This portion facilitates receptor binding on intestinal cells, directing the fusion protein toward mucosal immune sites. (Portions highlighted in yellow)
   - **GGSG Linker**: A flexible linker placed between the CTB and Zika E protein sequences to maintain independent folding and functionality of each protein domain.(Portions highlighted in blue)

- **Zika Virus Envelope Protein (E protein)**: The primary antigen that elicits a protective immune response against Zika virus infection.(Portions highlighted in orange)

The sequence for the fusion protein is:

MAQSSRICHGVQNPCVIISNLSKSNQNKSPFSVSLKTHQHPRAYPISSSWGLKKSGMTL
IGSELRPLKVMSSVSAGGSGMTPQNITDLCAEYHNTQIYTLNDKIFSYTESLAGKREMAII
TFKNGAIFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISM
ANGTGGSGIRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTV
SNMAEVRSYCYEASISDMASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGL
FGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENRA
KVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLP
WHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAK
GRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCK
VPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHW
HRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFG
GMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

2. **DNA Work**

The CTB gene, a non-toxic component of cholera toxin, was synthesized and codon-optimized for expression in *Saccharomyces cerevisiae*. Due to regulatory restrictions on obtaining *Vibrio cholerae* strains, the CTB gene was custom-ordered from **Bioneer**. The confirmed sequence is as follows:

atgactccacagaacataaccgacttatgcgcggagtatcataatacgcagatctacactctcaacgacaaaatcttctcat
acacggagtcactcgcaggtaagagggaaatggccattatcacattcaaaaacggagcgatcttccaggtcgaggtccct
ggaagccagcacatcgactcgcagaagaaggcaatagagagaatgaaagacacgctaagaatagcctacctcacgg
aggcaaaggtagagaaattgtgcgtttggaacaacaagacaccacacgccatagcggctatcagcatggctaacggaa
cggg aggatcagga

Zika Virus E Protein Gene Sourcing

The Zika virus envelope protein (E protein) plays a key role in viral attachment and entry into host cells and serves as a primary antigen for neutralizing antibody responses. The E protein sequence was sourced from the **NCBI GenBank database** (Accession: KU497555.1) and codon-optimized for yeast expression. The referenced nucleotide sequence is as follows:

atcaggtgcataggagtcagcaatagggactttgtggaaggtatgtcaggtgggacttgggttgatgttgtcttggaaca
tgggggttgtgtcaccgtaatggcacaggacaaaccgactgtcgacatagagctggttacaacaacagtcagcaacatg
gcggaggtaagatcctactgctatgaggcatcaatatcagacatggcttcggacagccgctgcccaacacaaggtgaag
cctaccttgacaagcaatcagacactcaatatgtctgcaaaagaacgttagtggacagaggctggggaaatggatgtgga
ctttttggcaaagggagcctggtgacatgcgctaagtttgcatgctccaagaaaatgaccgggaagagcatccagccaga
gaatctggagtaccggataatgctgtcagttcatggctcccagcacagtgggatgatcgttaatgacacaggacatgaaac
tgatgagaatagagcgaaggttgagataacgcccaattcaccaagagccgaagccaccctgggggttttggaagctta
ggacttgattgtgaaccgaggacaggccttgacttttcagatttgtattacttgactatgaataacaagcactggttggttcaca
aggagtggttccacgacattccattaccttggcacgctggggcagacaccggaactccacactggaacaacaaagaag
cactggtagagttcaaggacgcacatgccaaaaggcaaactgtcgtggttctagggactcaagaaggagcagttcacac
ggcccttgctggagctctggaggctgagatggatggtgcaaagggaaggctgtcctctggccacttgaaatgtcgcctgaa
aatggataaacttagattgaagggcgtgtcatactccttgtgtaccgcagcgttcacattcaccaagatcccggctgaaaca
ctgcacgggacagtcacagtggaggtacagtacgcagggacagatggaccttgcaaggttccagctcagatggcggtg
gacatgcaaactctgaccccagttgggaggttgataaccgctaaccccgtaatcactgaaagcactgagaactctaagat
gatgctggaacttgatccaccatttggggactcttacattgtcataggagtcggggagaagaagatcacccaccactggca
caggagtggcagcaccattggaaaagcatttgaagccactgtgagaggtgccaagagaatggcagtcttgggagacac
agcctgggactttggatcagttggaggcgctctcaactcattgggcaagggcatccatcaaatttttggagcagctttcaaatc
attgtttggaggaatgtcctggttctcacaaattctcattggaacgttgctgatgtggttgggtctgaacacaaagaatggatct
atttcccttatgtgcttggccttaggggagtgttgatcttcttatccacagccgtctctgct

Fusion Gene Construction

The CTB and Zika E genes were connected using a GGSG linker (sequence: `ggaggatcagga`). PCR was used to amplify the CTB and Zika E gene segments while simultaneously introducing the GGSG linker at the junction site. The final CTB-linker-E fusion construct was confirmed via sequencing before cloning into the yeast expression vector.

Yeast Expression Vector (pICE-ADE2-HphMx)

The expression vector pICE-ADE2-HphMx was obtained from Addgene for use in *Saccharomyces cerevisiae*. However, the vector lacked a yeast-compatible promoter upstream of the expression site, necessitating the insertion of a suitable promoter.

Insertion of ADH Promoter

To enable efficient transcription of the CTB-Zika E fusion gene in yeast, the **ADH1 promoter** was selected. The ADH1 promoter sequence was amplified by PCR from wild yeast (*Saccharomyces cerevisiae*) genomic DNA using primers designed to include restriction sites for cloning. The primer sequences were as follows:

ADH1p_XhoI-F (Forward Primer):

`CCGCTCGAGattaaaacaagaagaggg`

ADH1p_R (Reverse Primer):

`CATtgtatatgagatagttgattg`

PCR Amplification of ADH1 Promoter

PCR amplification was performed using wild yeast genomic DNA as the template. The reaction conditions included high-fidelity polymerase to ensure accuracy, and the amplified product was purified and verified via agarose gel electrophoresis.

Vector and Insert Preparation

The recombinant gene (ADH1 promoter - CTB - linker - Zika E with BamHI) and the **pICE-ADE2-HphMx vector** were prepared for ligation using the following steps:

Enzyme Digestion:

- Both the gene insert and the vector were digested with **XhoI** and **BamHI** restriction enzymes to generate compatible sticky ends.
- The digestion reaction was performed under optimal conditions to ensure complete digestion of both DNA samples.

Purification:

- The digested vector and insert were purified using a PCR purification kit to remove enzymes, small fragments, and impurities.
- The purified DNA concentrations were measured using a nanodrop spectrophotometer.

Ligation:

- The purified insert and vector were ligated using **T4 DNA ligase** in a reaction optimized for molar ratios of insert to vector.
- The ligation mixture was incubated at 16°C overnight to ensure efficient ligation.

Transformation and Selection in E. coli(DH5α)

- The ligated plasmid was introduced into chemically competent *E. coli* DH5α cells using the heat-shock method.
- Transformed cells were plated on LB agar plates containing **ampicillin** (100 µg/mL) to select for transformants carrying the recombinant plasmid.

Screening for Positive Clones:

- Individual colonies were picked and subjected to **colony PCR** to confirm the presence of the insert in the plasmid.
- PCR products were analyzed via agarose gel electrophoresis, and colonies showing the expected band size were selected for further analysis.

Sequencing Confirmation:

- Positive clones were sequenced to verify the correct assembly of the ADH1 promoter, CTB-linker, and Zika E gene construct within the pICE-ADE2-HphMx vector.

Yeast Transformation and Selection

The confirmed plasmid was transformed into *Saccharomyces cerevisiae* using the lithium acetate (LiAc) method.

Transformed yeast cells were plated on hygromycin-containing selective media.

Positive yeast transformants were confirmed by colony PCR, using primers specific to the ADH1-CTB-linker-Zika E construct.

3. **Protein Expression Analysis**

Liquid Culture:

- Positive yeast colonies were cultured in selective liquid media at 30°C with shaking.

Protein Extraction:

- Cells were harvested and lysed mechanically using glass beads to extract soluble proteins.

SDS-PAGE

- Protein lysates were analyzed by SDS-PAGE to detect the expected molecular weight band for the CTB-Zika E fusion protein.

Discussion

The developed yeast-based system demonstrates the feasibility of producing the CTB-Zika E fusion protein as a candidate for an oral Zika vaccine. The colony PCR results confirm the presence of the construct in both *E. coli* and yeast transformants. Initial SDS-PAGE analysis validates that the fusion protein is being expressed in *Saccharomyces cerevisiae*.

Further work will focus on the detailed characterization of the expressed protein. Specifically, Western blot analysis using antibodies specific to CTB and Zika E protein is planned for confirming protein identity and integrity. Additionally, scaled-up protein production and

formulation into a heat-stable oral vaccine format will be explored. These steps will ensure the functional viability and scalability of the vaccine for further testing and application.

While antibody unavailability has delayed Western blot validation, the observed SDS-PAGE band provides strong preliminary evidence of successful expression. Future characterization will help establish the immunogenic potential of the fusion protein and its application in animal models for further evaluation.

Keywords

Zika virus, CTB, yeast expression, oral vaccine, SDS-PAGE, colony PCR

References

1. Musso, D., & Gubler, D. J. (2016). Zika Virus. *Clinical Microbiology Reviews*, 29(3), 487-524.
2. Mlakar, J., Korva, M., Tul, N., Popović, M., Poljšak-Prijatelj, M., Mraz, J., ... & Avšič Županc, T. (2016). Zika virus associated with microcephaly. *New England Journal of Medicine*, 374(10), 951-958.
3. Lazear, H. M., & Diamond, M. S. (2016). Zika virus: New clinical syndromes and its emergence in the Western Hemisphere. *Journal of Virology*, 90(10), 4864-4875.
4. Barba-Spaeth, G., Dejnirattisai, W., Rouvinski, A., Vaney, M. C., Medits, K., Sharma, A., ... & Rey, F. A. (2016). Structural basis of potent Zika–dengue virus antibody cross-neutralization. *Nature*, 536(7614), 48-53.
5. Sánchez, J., & Holmgren, J. (2011). Cholera toxin – A foe & a friend. *The Indian Journal of Medical Research*, 133(2), 153-163.
6. Lavelle, E. C., & Ward, R. W. (2022). Mucosal vaccines — fortifying the frontiers. *Nature Reviews Immunology*, 22, 236–250.
7. Stratmann, T. (2015). Cholera toxin subunit B as adjuvant—An accelerator in protective immunity and a break in autoimmunity. *Vaccines*, 3(3), 579-596.