

Design and preliminary study of VP1-encoding mRNA-lipid nanoparticles for Enterovirus 71

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ABSTRACT

Background: Hand, foot, and mouth disease (HFMD) remains a major public health concern in the Asia-Pacific region, with Enterovirus 71 (EV71) being a major cause of severe neurological complications in children. The genetic diversity of EV71 highlights the need for adaptable vaccine platforms.

Objective: This study aimed to design and preliminarily evaluate an EV71 VP1-encoding mRNA-lipid nanoparticle (mRNA-LNP) construct as an antigen delivery approach for EV71 vaccine development.

Materials and methods: A codon-optimized VP1 mRNA was synthesized by *in vitro* transcription and formulated into lipid nanoparticles via microfluidic mixing. Physicochemical properties were characterized, and *in vitro* expression was assessed in FreeStyle 293F cells by western blot and immunofluorescence. VP1-specific humoral immune responses were evaluated in BALB/c mice following a prime-boost immunization regimen using ELISA.

Results: The mRNA-LNPs exhibited a mean particle size of 99.24±0.46 nm, a low polydispersity index (0.041±0.004), a slightly negative zeta potential (-20.54±0.98 mV), and an encapsulation efficiency of 92.56±1.53%. *In vitro* studies demonstrated VP1 protein expression following mRNA delivery. Immunization induced VP1-specific IgG responses, with antibody levels significantly increased after prime (1.254±0.775) and boost (4.225±0.322) compared with control groups ($p<0.01$ and $p<0.001$, respectively). Endpoint titers increased from a geometric mean titer of 2,000 (95% CI: 901.50-4,437.00) after priming to 256,000 following booster immunization.

Conclusion: This study demonstrates that EV71 VP1 mRNA-LNPs induce antigen-specific humoral immune responses in mice, providing preliminary evidence of their potential as an antigen delivery approach for EV71 and supporting further studies in vaccine development.

Introduction

Hand, foot, and mouth disease (HFMD) is a common pediatric viral illness and remains a significant public health concern in the Asia-Pacific region. Among causative enteroviruses, Enterovirus 71 (EV71) is of particular importance due to its strong association with severe neurological complications, including aseptic meningitis, brainstem encephalitis, and acute flaccid paralysis.^{1,2}

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Epidemiological studies have demonstrated the continued circulation of genetically diverse EV71 strains, contributing to recurrent outbreaks. In Thailand, subgenotypes B5 and C4 have predominated over the past decade, reflecting ongoing circulation and shifts in subgenotype dominance.^{3,4} This highlights the need for vaccine strategies capable of accommodating regional strain diversity.

Several inactivated EV71 vaccines have been licensed in Asia, including C4-based vaccines in mainland China.^{1,5} In Thailand, an inactivated EV71 vaccine, EntroVac®, has been approved for use in young children and is based on a C4 subgenotype strain.⁶ These vaccines have demonstrated efficacy against EV71-associated HFMD; however, cross-genotype protection remains an important consideration in regions where different EV71 subgenotypes circulate.^{1,5} This consideration is relevant to the Thai setting, where circulating EV71 subgenotypes include both B5 and C4. In addition, serological data in Thai children indicate that a substantial proportion remain susceptible to EV71 infection during early childhood,⁷ supporting the need for effective vaccination strategies. Alternative vaccine approaches, including recombinant protein, peptide-based, and virus-like particle (VLP) vaccines, have demonstrated immunogenicity in experimental models, although most are still under development.^{1,2} However, conventional vaccine strategies generally rely on virus propagation or recombinant protein production, which involve multi-step manufacturing processes and may limit the speed and flexibility of antigen redesign.

In this context, messenger RNA (mRNA) vaccine technology offers a modular and adaptable platform that enables rapid antigen design and cell-free, scalable production,^{8,9} supporting its potential as a flexible platform for EV71 antigen delivery. However, mRNA requires an effective delivery system to protect it from degradation and enable cellular entry. Lipid nanoparticles are widely used for this purpose because they encapsulate mRNA and facilitate intracellular delivery for antigen expression.^{9,10}

EV71 encodes four structural proteins (VP1–VP4), among which VP1 is a major surface-exposed capsid protein involved in receptor binding and viral entry. VP1 contains key neutralizing epitopes,^{1,2,11} and its relatively conserved sequence across subgenotypes supports its potential as a cross-reactive antigen target.⁴

In this study, we designed and evaluated an EV71 VP1-encoding mRNA-LNP construct, integrating antigen design, mRNA synthesis, lipid nanoparticle (LNP) formulation, and evaluation of VP1-specific humoral immune responses in a mouse model. This study provides initial experimental data to support further evaluation and optimization of EV71 mRNA-LNP construct in the context of vaccine development.

Materials and methods

mRNA construct design and template preparation

The full-length VP1 coding sequence of EV71 subgenotype B5 isolate SiICRC04/TH/2012 (GenBank accession No. KX372317) was used as the antigen template.³ The mRNA construct comprised a 5' untranslated region (UTR) containing a Kozak sequence, a tissue plasminogen activator (tPA) signal peptide, the VP1 coding sequence, and a 3' UTR with a poly(A) tail.

The VP1 sequence was reverse-translated and codon-optimized for human expression using mRNAid (version 1.0.0). Rare codon usage was evaluated using the GeneScript Rare Codon Analysis Tool, and RNA secondary structure was predicted using RNAfold to estimate thermodynamic stability.

The optimized VP1 coding sequence was synthesized as double-stranded DNA (Gene Universal Inc., USA) and cloned into the Linearized Template Vector (BspQ I), supplied with the Cloning Kit for mRNA Template, using an In-Fusion cloning system (Takara Bio Inc., Japan). Recombinant plasmids were transformed into the *Escherichia coli* Stbl2 strain, and correct insertion was confirmed by colony PCR and Sanger sequencing. Plasmids were purified and linearized with the BspQI restriction enzyme to generate a DNA template for *in vitro* transcription.

In vitro transcription and mRNA characterization

In vitro transcription (IVT) was performed using a CleanCap-based IVT system (TriLink BioTechnologies, USA) with N1-methylpseudouridine-5'-triphosphate substituted for UTP and co-transcriptional capping. Template DNA was removed by DNase I treatment (New England Biolabs, USA), and mRNA was purified using an RNA Cleanup Kit (New England Biolabs, USA).

mRNA concentration was determined using a RiboGreen-based assay (Invitrogen, USA). Residual DNA contamination was assessed by PCR amplification of the VP1 region, with linearized plasmid DNA used as a positive control. mRNA size and integrity were analyzed using a Bioanalyzer system (Agilent Technologies, USA). Sequence accuracy was confirmed by reverse transcription followed by Sanger sequencing.

Preparation and characterization of mRNA-LNPs

mRNA was encapsulated into lipid nanoparticles (LNPs) using GenVoy-ILM reagents and a NanoAssemblr Ignite microfluidic mixing platform (Precision Nanosystems, Canada). Lipids were diluted in ethanol, and mRNA was diluted in acetate buffer (pH 4.0) to achieve a lipid-to-mRNA molar ratio of 4:1 (ionizable lipid: nucleotide). The solutions were combined at a 3:1 (v/v) ratio with a total flow rate of 12 mL/min. Formulated mRNA-LNPs were diluted in phosphate-buffered saline (PBS, pH 7.4) and concentrated using centrifugal filters. Empty LNPs, used as a formulation

control for immunofluorescence analysis and animal immunization, were prepared using the same microfluidic mixing procedure as the mRNA-LNPs, but without mRNA.

Particle size, polydispersity index (PDI), and zeta potential of the mRNA-LNP formulation were measured by dynamic light scattering using a Zetasizer instrument (Malvern Panalytical, UK). Samples were diluted in PBS (200-fold for particle size and PDI, 30-fold for zeta potential) and measured at 25 °C. Encapsulation efficiency (%EE) was determined using a RiboGreen assay in the presence or absence of 1% Triton X-100 to distinguish total and unencapsulated mRNA. Fluorescence was measured, and %EE was calculated as:

$$\%EE = [(total\ mRNA-free\ mRNA)/total\ mRNA] \times 100$$

All characterization measurements of the mRNA-LNP formulation were performed in triplicate.

In vitro expression analysis

FreeStyle 293F cells were cultured in suspension and used for *in vitro* expression analysis. Expression of EV71 VP1 from the IVT mRNA was examined by western blot following transfection reagent-mediated delivery and by immunofluorescence analysis following mRNA-LNP treatment.

For western blot analysis, cells were seeded at 2×10^6 cells per well and incubated with 2.0 µg of IVT mRNA using an RNA transfection reagent (Takara Bio, Japan) for 72 hrs. Culture supernatants were collected, concentrated, and analyzed by western blot using a mouse monoclonal anti-EV71 VP1 antibody (clone 1A2-C1, GeneTex, USA), followed by an HRP-conjugated secondary antibody (DakoCytomation, Denmark). Immunoreactive bands were detected using a chemiluminescent substrate (Thermo Fisher Scientific, USA). Untreated cells served as negative controls.

For immunofluorescence analysis, cells were seeded at 1×10^6 cells per well and assigned to four groups: untreated negative control, empty LNPs, unformulated EV71 VP1 mRNA, and EV71 VP1 mRNA-LNPs. The EV71 VP1 mRNA-LNPs group was treated with 8.0 µg of mRNA in LNP formulation (72.4 µL), the empty LNPs group was treated with an equivalent volume of empty LNPs (72.4 µL), and the unformulated EV71 VP1 mRNA group was treated with 8.0 µg of mRNA without LNP formulation. After incubation for 72 hrs, cells were fixed with 40% methanol in acetone at -20 °C overnight and stained with the same primary antibody, followed by staining with a FITC-conjugated secondary antibody (DakoCytomation, Denmark). Nuclei were counterstained with DAPI, and corresponding bright-field and fluorescence images were acquired using a fluorescence microscope (Nikon Instruments, USA).

Animal immunization and sample collection

Female BALB/c mice (6-8 weeks old) were

obtained from Nomura Siam International Co., Ltd. and housed under specific-pathogen-free conditions. All animal procedures were conducted in accordance with institutional guidelines for animal care and use and were approved by the Thammasat University Animal Care and Use Committee (approval No. 017/2025).

Mice were randomly assigned to three groups (N=6 per group): EV71 VP1 mRNA-LNPs group, empty LNPs control group, and PBS control group. Animals were immunized intramuscularly with 5 µg mRNA in a total volume of 50 µL on day 0 (prime) and day 28 (boost). Control groups received either empty LNPs as a formulation control or PBS. Blood samples were collected prior to immunization, at 4 weeks post-prime via submandibular vein puncture, and at 4 weeks post-boost via cardiac puncture under thiopental anesthesia. Mice were euthanized following terminal blood collection. Serum samples were separated and stored at -80 °C until analysis.

Measurement of EV71 VP1-specific IgG

VP1-specific IgG responses were measured by ELISA. Plates were coated with recombinant EV71 VP1 protein (0.1 µg/well; Antibody System, France) and blocked with 3% bovine serum albumin in PBS. Serum samples were diluted 1:1000 and incubated on coated plates. Bound antibodies were detected using an HRP-conjugated rabbit anti-mouse secondary antibody (1:2000), followed by color development with TMB substrate (SeraCare Life Sciences, USA). Absorbance was measured at 450 nm with a reference wavelength of 620 nm.

Endpoint IgG titers were determined for sera from mice immunized with EV71 VP1 mRNA-LNPs using the same ELISA protocol with twofold serial dilutions (1:250-1:8,192,000). Titers were defined as the reciprocal of the highest dilution exceeding the cutoff value, calculated from pre-immunization sera using a prediction-limit method at 99% confidence level.¹²

Statistical analysis

Physicochemical properties of the mRNA-LNP formulation, including particle size, PDI, zeta potential, and encapsulation efficiency, are presented as mean±standard deviation (SD). EV71 VP1-specific IgG levels (OD450) are presented as mean±SD, whereas endpoint antibody titers are presented as geometric mean titers (GMT) with 95% confidence intervals. Statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, USA). Differences among groups were analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Changes over time were analyzed using repeated-measures ANOVA with Tukey's multiple comparisons test. A $p < 0.05$ was considered statistically significant.

Results

Design and preparation of EV71 VP1 mRNA construct

The EV71 VP1 mRNA construct was designed

based on a Thai subgenotype B5 isolate (SiCRC04/TH/2012), as schematically illustrated in Figure 1. Codon optimization increased the codon adaptation index (CAI) to >0.9. Secondary structure prediction using RNAfold yielded a minimum free energy of -421.13 kcal/mol, suggesting stable folding of the optimized mRNA transcript.

The optimized VP1 coding sequence was successfully cloned into the Linearized Template Vector (BspQ I), and correct insertion was confirmed by colony PCR and Sanger sequencing. The resulting plasmid was efficiently linearized, yielding a DNA template suitable for *in vitro* transcription.

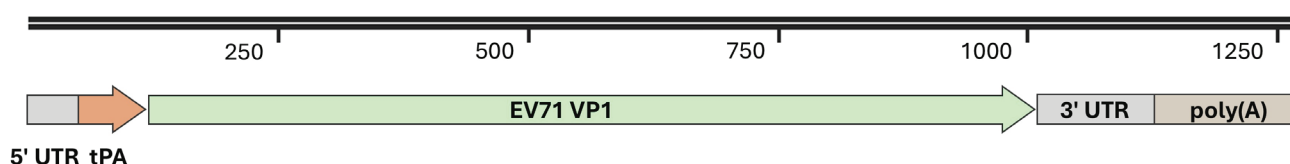


Figure 1. Schematic representation of the EV71 VP1 mRNA construct, including the 5' untranslated region (UTR) with a Kozak sequence, the tissue plasminogen activator (tPA) signal peptide, the VP1 coding sequence, the 3' UTR, and the poly(A) tail.

Characterization of *in vitro*-transcribed EV71 VP1 mRNA

Capped, N1-methylpseudouridine-modified ($m^1\psi$) EV71 VP1 mRNA was successfully synthesized by IVT. The reaction yielded 862 μ g of mRNA from 140 μ L, corresponding to a concentration of 6.1 mg/mL.

PCR analysis of DNase-treated samples showed no detectable amplification (Figure 2A), indicating removal

of residual plasmid DNA. RNA integrity analysis using the Agilent TapeStation system showed a single band at approximately 1,192 nt, consistent with the expected transcript length including the poly(A) tail (Figure 2B). Sequence verification by reverse transcription followed by Sanger sequencing confirmed concordance with the designed VP1 coding sequence.

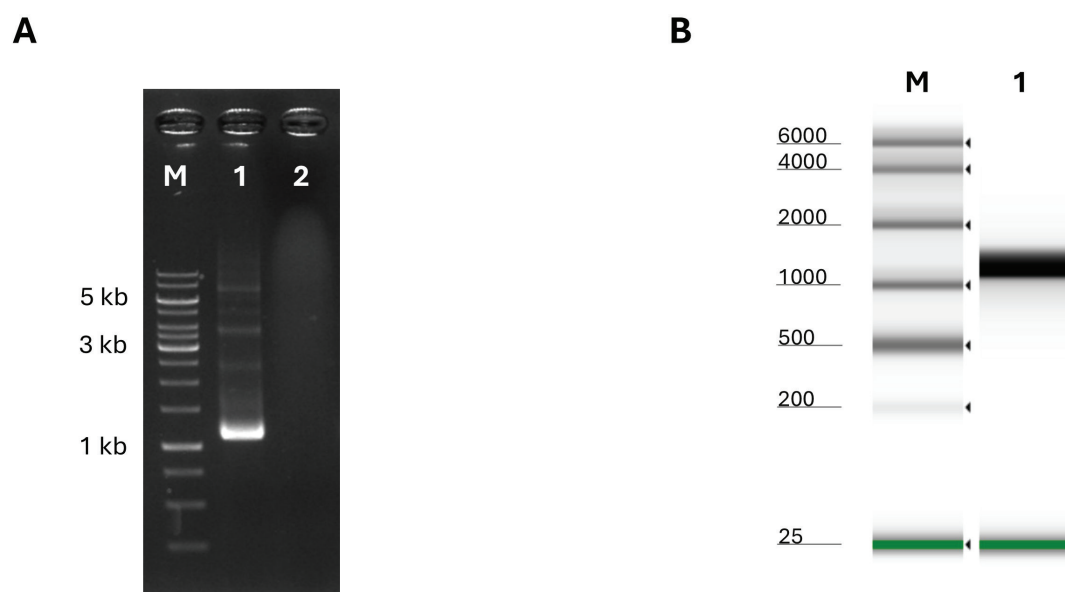


Figure 2. Quality assessment of *in vitro*-transcribed EV71 VP1 mRNA. A: PCR analysis of DNase-treated mRNA samples confirming removal of residual plasmid DNA; Lane M: DNA ladder (1 kb), Lane 1: PCR from linearized pDNA (positive control), Lane 2: PCR from DNase-treated mRNA preparation; B: RNA integrity analysis using Agilent TapeStation, showing a single band corresponding to the expected transcript size, Lane M: DNA ladder (1 kb), Lane 1: EV71 VP1 mRNA (~1,192 nt).

Preparation and characterization of EV71 VP1 mRNA-LNPs

The *in vitro*-transcribed EV71 VP1 mRNA was encapsulated in lipid nanoparticles using a microfluidic mixing approach, yielding a clear, homogeneous formulation.

The mRNA-LNPs exhibited a mean hydrodynamic diameter of 99.24 ± 0.46 nm, with a low polydispersity index (0.041 ± 0.004) and a slightly negative zeta potential (-20.54 ± 0.98 mV). The encapsulation efficiency was $92.56 \pm 1.53\%$ (Table 1).

Table 1. Physicochemical characteristics of EV71 VP1 mRNA-LNP formulation.

Formulation	Size (nm)	PDI	Zeta potential (mV)	%EE
EV71 VP1 mRNA-LNPs	99.24±0.46	0.041±0.004	-20.54±0.98	92.56±1.53

In vitro expression of EV71 VP1 following mRNA transfection and mRNA-LNP treatment

VP1 expression was first examined by Western blot after transfection reagent-mediated delivery of EV71 VP1 mRNA into FreeStyle 293F cells. A VP1-specific

immunoreactive band was detected in the transfected sample, but not in the non-transfected control, indicating that the designed mRNA could direct VP1 protein expression. The band migrated at an apparent molecular weight of approximately 45-55 kDa (Figure 3).

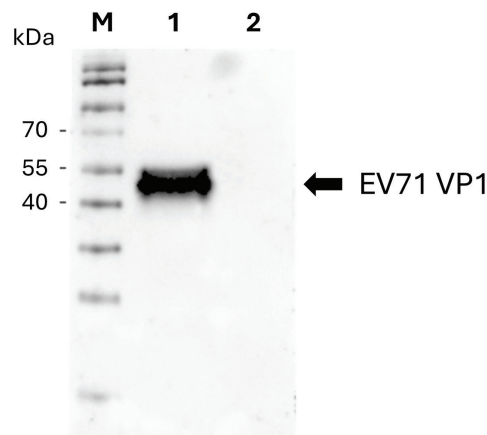


Figure 3. Western blot analysis of EV71 VP1 expression in FreeStyle 293F cells. VP1 expression was examined in culture supernatants after transfection reagent-mediated delivery of EV71 VP1 mRNA. Lane M: protein ladder, Lane 1: transfected sample, Lane 2: non-transfected control. The arrow indicates the VP1-specific immunoreactive band at approximately 45-55 kDa.

Immunofluorescence analysis was then performed to assess VP1 expression following mRNA-LNP treatment. VP1-specific FITC fluorescence was observed in EV71 VP1 mRNA-LNP-treated cells, whereas no specific

fluorescence was detected in untreated cells, empty LNP-treated cells, or cells treated with unformulated EV71 VP1 mRNA. DAPI and bright-field images confirmed the presence of cells in the examined fields (Figure 4).

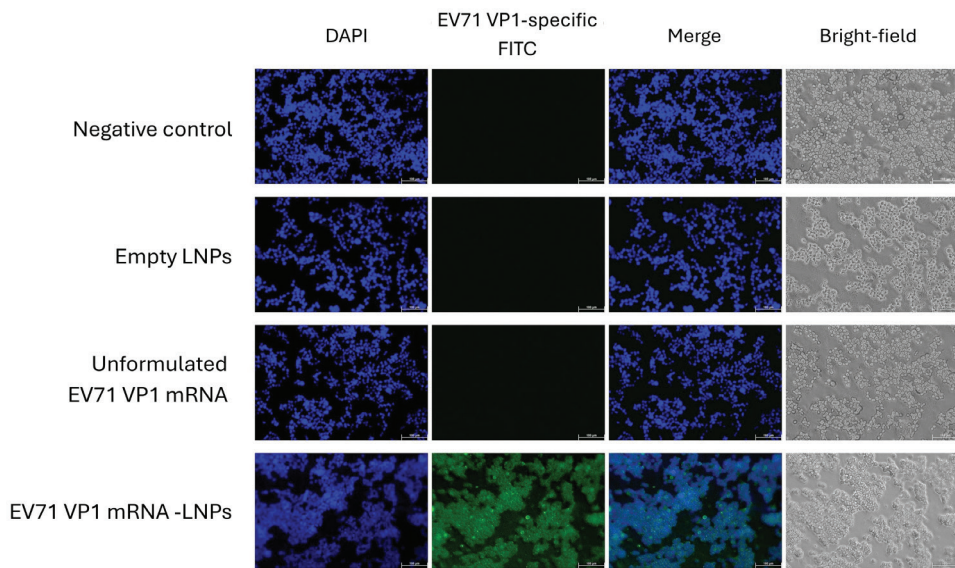


Figure 4. Immunofluorescence analysis of EV71 VP1 expression in FreeStyle 293F cells following mRNA-LNP treatment. Four groups were examined: untreated negative control, empty LNPs, unformulated EV71 VP1 mRNA, and EV71 VP1 mRNA-LNPs. FITC signals indicate EV71 VP1-specific staining, DAPI indicates nuclei, and merged FITC/DAPI and bright-field images are shown. VP1-specific FITC fluorescence was observed only in the EV71 VP1 mRNA-LNPs group.

EV71 VP1-specific humoral immune responses in mice

EV71 VP1-specific IgG responses were measured by ELISA using sera collected prior to immunization, at 4 weeks post-prime, and at 4 weeks post-boost. Antibody responses in the EV71 VP1 mRNA-LNPs group were compared with those in the empty LNP formulation control and PBS control groups. One mouse in the PBS group died during the study and was excluded from analysis.

At the pre-immune time point, absorbance values were low and comparable among groups (EV71 VP1 mRNA-LNPs: 0.020 ± 0.008 , empty LNPs 0.015 ± 0.002 , PBS: 0.015 ± 0.001), indicating the absence of pre-existing VP1-specific antibodies. Following the prime dose, mice immunized with EV71 VP1 mRNA-LNPs developed detectable antibody

responses (1.254 ± 0.775), which were significantly higher than those in both control groups (empty LNPs: 0.015 ± 0.002 , PBS: 0.017 ± 0.002 , $p < 0.01$). Antibody levels further increased after booster immunization (4.225 ± 0.322) and remained significantly higher than controls ($p < 0.001$). In contrast, mice receiving empty LNPs showed background-level signals comparable to PBS throughout the study (Figure 5A).

Endpoint titer analysis showed a geometric mean titer (GMT) of 2,000 (95% CI: 901.50-4,437.00) after the prime dose. Following booster immunization, GMT increased to 256,000, with all samples showing titers at this level (Figure 5B). Endpoint titers at both the post-prime and post-boost time points were significantly higher than those at the pre-immune time point ($p < 0.05$ and $p < 0.001$, respectively).

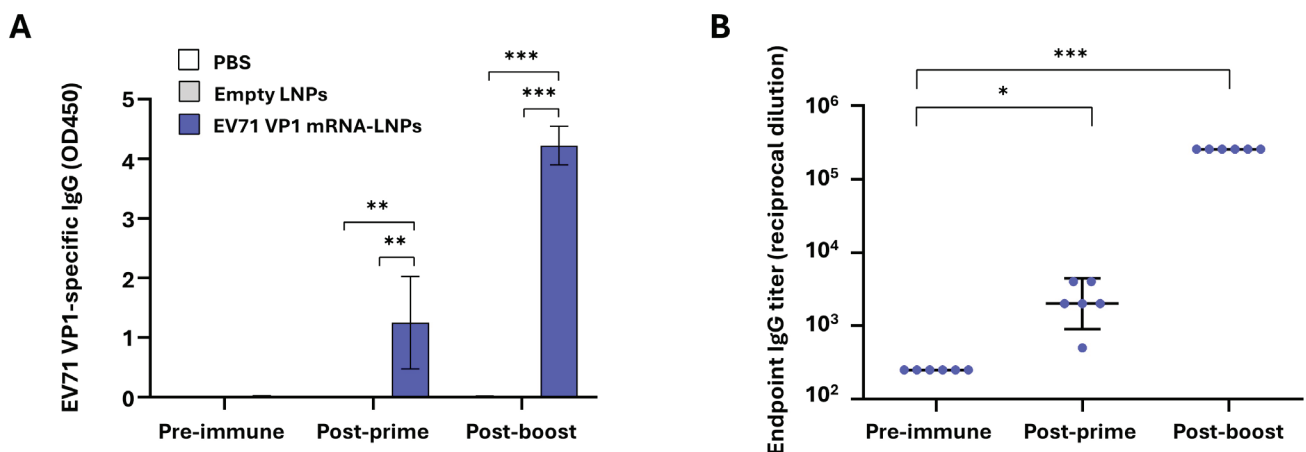


Figure 5. Humoral immune responses induced by EV71 VP1 mRNA-LNP immunization in mice. A: EV71 VP1-specific IgG responses measured by ELISA at pre-immune, 4 weeks post-prime, and 4 weeks post-boost. Data are presented as mean $\pm$ SD. B: Endpoint IgG titers against EV71 VP1. Data are expressed as geometric mean titers (GMT) with 95% confidence intervals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Discussion

This study demonstrates the development of an EV71 VP1-encoding mRNA-LNP construct and its ability to induce antigen-specific humoral immune responses in a mouse model. The integrated workflow, from mRNA design to *in vitro* expression and *in vivo* humoral immune responses, supports its use for EV71 antigen delivery. While existing vaccines and experimental approaches rely on multi-step manufacturing and protein-based antigen production, the mRNA-LNP system demonstrated here enables streamlined design and antigen expression, which may facilitate adaptation to EV71 subgenotypes with genetic diversity.

The selection of a VP1 sequence derived from a circulating Thai subgenotype B5 strain supports the epidemiological relevance of this construct. Subgenotype B5 has been associated with multiple HFMD outbreaks in Thailand and remains in circulation.^{3,4,13} The selected sequence retains the SP70 region, a conserved neutralizing epitope within the surface-exposed GH loop, a major antigenic site.^{11,14} The preservation of this region supports its

role in antibody recognition and suggests its potential relevance across EV71 subgenotypes. In addition, the presence of the SP2 region associated with T-cell responses suggests broader immunological relevance.¹⁵

The mRNA construct incorporated key structural elements and modified nucleosides consistent with current mRNA vaccine design strategies to enhance translational efficiency while reducing innate immune sensing at the RNA level.^{9,16} The predicted secondary structure and minimum free energy suggest a thermodynamically stable transcript, and RNA structure has been reported to influence mRNA stability and translational efficiency.¹⁷ Such structural optimization may support sustained antigen expression, thereby influencing downstream immune responses. The observed yield, absence of detectable residual DNA, and presence of a single dominant transcript band support the quality of the IVT mRNA preparation used for downstream LNP formulation. Given that IVT reactions can generate byproducts, including dsRNA, abortive RNAs, and RNA:DNA hybrids, stringent quality control is important before formulation.¹⁸

The formulated mRNA-LNPs exhibited physicochemical properties, including particle size, polydispersity index, zeta potential, and encapsulation efficiency, within ranges associated with stable and effective delivery systems. Particle sizes around 100 nm are associated with lymphatic transport and uptake by antigen-presenting cells, which may facilitate antigen delivery and presentation. The slightly negative surface charge may also influence cellular uptake, although its *in vivo* impact remains context dependent.^{10,19,20}

In vitro expression analyses confirmed that the designed EV71 VP1 mRNA directed VP1 expression in FreeStyle 293F cells. The apparent molecular weight shift observed by western blot may reflect altered electrophoretic mobility or post-translational processing during ER/Golgi trafficking following secretory pathway targeting by the incorporated tPA signal sequence.^{21,22} The exact cause of this shift warrants further investigation. Immunofluorescence analysis showed VP1-specific FITC fluorescence only in EV71 VP1 mRNA-LNP-treated cells, but not in untreated cells, empty LNP-treated cells, or cells treated with unformulated EV71 VP1 mRNA. These findings support the functional role of the LNP formulation in facilitating mRNA delivery and enabling detectable antigen expression under the conditions tested.

Immunization with EV71 VP1 mRNA-LNPs induced VP1-specific IgG responses, particularly following a prime-boost regimen. This pattern is consistent with observations from previous EV71 vaccine studies^{23,24} and may reflect affinity maturation processes within germinal centers, leading to the generation of higher-affinity antibody responses.²⁵ Booster immunization has been shown to influence B-cell selection dynamics within germinal centers, contributing to improved antibody magnitude and quality.^{26,27}

Empty LNPs were included as a formulation control to account for background signals or nonspecific effects associated with the LNP formulation in the absence of the EV71 VP1 mRNA payload. The background-level VP1-specific IgG responses observed in the empty LNPs group, comparable to PBS, support that the antibody responses in the EV71 VP1 mRNA-LNPs group were associated with VP1 expression from the mRNA payload. More broadly, the immunogenicity of mRNA-LNP vaccines is not limited to antibody responses. Innate immune sensing associated with mRNA vaccination has been implicated in vaccine adjuvanticity and may influence downstream adaptive immune responses.²⁸ In addition to humoral responses, mRNA vaccines enable antigen presentation via both MHC class I and class II pathways, thereby supporting the activation of CD8⁺ and CD4⁺ T cells.⁹ In EV71 infection, T-cell-mediated immunity contributes to viral clearance and protective responses.²⁹ A recent study reported that an mRNA-based EV71 vaccine can induce both neutralizing antibodies and Th1-biased cellular responses, providing protection in preclinical

models.³⁰ Further investigation is required to evaluate these immunological mechanisms in the context of the present construct.

Overall, this study provides a proof-of-concept demonstration of a VP1-based mRNA-LNP construct for EV71 and lays the groundwork for further investigation. From a translational perspective, this approach may support antigen adaptation and scalable production, thereby facilitating iterative updates to match regionally circulating EV71 subgenotypes. However, comprehensive evaluation of neutralizing activity, cross-genotype protection, and cellular immunity will be required to define its protective potential.

Limitations

This study has several limitations. Immunogenicity was evaluated in a small cohort of mice and should be considered preliminary. Only binding antibody responses were assessed; neutralizing activity, cellular immune responses, antibody durability, and cross-genotype neutralization were not evaluated. In addition, the animal study did not include an unformulated EV71 VP1 mRNA group; therefore, direct comparison of *in vivo* immunogenicity between unformulated mRNA and mRNA-LNPs was not performed. Future studies should address these limitations to better define the protective potential of the VP1-encoding mRNA-LNPs evaluated here.

Conclusion

This study demonstrates the development and preliminary evaluation of an EV71 VP1-encoding mRNA-LNP construct. VP1 expression was confirmed in FreeStyle 293F cells, and immunization induced VP1-specific IgG responses in mice, indicating antigen-specific humoral responses. These findings provide proof of concept for an EV71 mRNA-LNP approach and support further studies to evaluate neutralizing activity, cellular immunity, and protective efficacy.

Ethical approval

This study was approved by the Thammasat University Animal Care and Use Committee (ethical approval No. 017/2025).

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Conflict of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Parnuphan Panyajai: conceptualization, methodology, formal analysis, project administration, writing-review and editing; **Rujiraporn Pitaksalee:** methodology, investigation, writing original draft, visualization; **Apichai Prachasuphap:** methodology, investigation, formal analysis;

Kodcharad Jongpitisub: investigation; **Pantida Treeyoung:** investigation; **Sirawit Wet-osot:** investigation; **Nalinee Saengtong:** investigation; **Nadthanan Pinyosukhee:** methodology, investigation; **Rattanawadee Wichajarn:** investigation; **Kritsamon Sophondilok:** investigation; **Anicha Luengchaichaweng:** investigation; **Panadda Dhepakson:** supervision.

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