Vaccine Research
ISSN: 2423-4923
eISSN: 2383-2819

Design and Expression of VP1 (O IR/P50/2016) Protein in a Prokaryotic System as a Vaccine Candidate against Foot and Mouth Disease

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ARTICLE INFO

Short Communication

VacRes, 2023
Vol. 10, No.2, 44 – 48
Received: 30 12, 2024
Accepted: 01 02, 2025
Pasteur Institute of Iran

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KEYWORDS: Protein Expression, *E. coli*, FMD, Vaccine candidate, Western Blot

ABSTRACT

Introduction: Most of the previous researches about Foot and Mouth Disease vaccine have stated that traditional and inactive vaccines cannot comprehensively control the disease and should be reconsidered. Currently, for this reason, the efforts of researchers are on the production and development of new generation vaccines. FMDV VP1 protein is one of the structural proteins and is crucial for the virus's ability to infect host cells, elicitation of protective immune responses and the determination of serotype specificity. It plays a significant role in the virus's antigenicity and is a target for vaccine development. **Methods:** The coding sequence of FMDV VP1 protein (O IR/P50/2016) was extracted from the NCBI site. Recombinant structure designed during heat shock method was transferred into *Escherichia coli* strain BL21. After culture of transformed bacteria and confirming the presence of the gene by PCR, restriction enzyme digestion and DNA sequencing, the recombinant FMDV VP1 protein was expressed and confirmed by SDS-PAGE and Western blotting. **Results:** The results of the electrophoresis, PCR and enzymatic digestion indicated that the FMDV VP1 protein gene was correctly inserted and cloned into pET-45 plasmid. SDS-PAGE and Western blotting demonstrated the existence of a protein with a molecular weight of 27 kDa. **Conclusion:** The results of the current study indicated that the prokaryotically expressed FMDV VP1 protein can potentially be considered as a replacement for old vaccines or antigens (in diagnostic kits) upon further investigations.

INTRODUCTION

Foot and Mouth disease (FMD) is a widespread, viral, infectious and highly transmissible disease among domestic and wild cloven hooved animals. This disease is the main complication in providing health and animal products, adversely impacting the production of dairy and meat products [1]. Although some countries by the OIE were recognized FMD-free but this disease is endemic in many countries including Iran [2]. Clinical symptoms of the disease are characterized by the onset of fever and vesicular lesions in the tongue and oral mucosa, as well as vesicles in the udder and between the toes, leading to significant weight reduction, diminished lactation output, and stunted growth [3, 4]. The wild host range, high spread of the virus and diversity of serotypes and subtypes (more than 60) that caused by antigenic changes causes epidemics of this disease in the world and this issue has made the control of the disease difficult [5]. FMD Virus (FMDV) serves as the typical Aphthovirus in the family *Picornaviridae* [6].

Viruses in this particular family possess a genome consisting of a single stranded, positive sense, RNA molecule, which is enclosed by a protein shell, commonly referred to as a capsid [7]. This protective capsid typically comprises 60 copies of four distinct virus-encoded structural proteins that are named VP1, VP2, VP3 and VP4 [8]. Immunological studies of FMDV have indicated that among the structural proteins, VP1 (residues 141-160 and 200-213 epitopes) with 213 amino acid (AA) is responsible to stimulate neutralizing antibodies and the G-H loop can attach to the host receptor cell, is located in this protein. Binding to the host cell integrin has been attributed to the conserved part of the GH loop (Arg, Gly and Asp) [9]. This virus, when evaluated in terms of its pathogenic capabilities, is categorized into seven predominant serotypes, which encompass four European- Asian serotypes- namely Asia1, A, O and C- as well as three serotypes originating from south Africa, identified as SAT1, SAT2 and SAT3 divisions [10]. Between these serotypes, serotype A and O has a higher importance in Iran and major damages in recent years, due to the genetic mutation of these two strains in our country has been [11]. In the present, the inactivated vaccine is employed to validate the immunization status of livestock [12]. Inactivated vaccine of FMD, cannot create stable or relatively stable immunity and it is possible that during vaccine production, the virus incompletely deactivated and causes the spread of the disease. Furthermore, inactivated

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[DOI: 10.52547/vacres.10.2.44]

vaccine does not have the ability to elicit a cellular immune response and this is necessary to repeat the vaccination [13, 12]. In addition, animals vaccinated with inactivated vaccine cannot be detected from those that naturally infected. It should be mentioned, there is no cross immunity between serotypes and exposure to one serotype does not confer immunity to other strains.

Considering these problems, efforts to develop new vaccines including recombinant such as subunit, synthetic, peptide vaccine, DNA vaccine and vector vaccines are necessary [14, 15]. Bacterial expression systems for the production of the protein, allows the researchers to produce a large amount of protein in a short period of time [16]. The methodology of this microorganism is simple and economic cell culture, coupled with rapid and high-density growth, alongside established transcriptional and translational processes (with a defined genetic framework), have significantly enhanced the applicability of these microorganisms [17]. Due to the fact that using the eukaryotic system is costly and time-consuming, and considering that this system is used for proteins that require post translation modifications such as glycosylation [18], we decided to use the prokaryotic system to express VP1 protein (non-glycosylated [19]). Considering the aforementioned points, this study aims to clone and express the structural and main protein of the FMDV related to serotype O, which has caused significant outbreak in our country, in a prokaryotic system such as *E. coli* (due to its advantages). This effort is intended to pave the way for the development of a recombinant vaccine for FMD.

MATERIALS AND METHODS

Design and Construction of pET45- VP1 Recombinant Plasmid

The VP1 gene sequence from FMDV serotype O (GenBank accession no. QKS42884.1) was retrieved from the NCBI database. This reference sequence was aligned with sequences from Iranian serotype O isolates using MEGA X software to identify a conserved region. A gene fragment encoding amino acids 127–332 of the VP1 capsid protein was selected for synthesis. The nucleotide sequence was designed to incorporate a *KpnI* restriction site at the 5' end and an *XhoI* site at the 3' end to facilitate directional cloning. The sequence was subsequently codon-optimized for high-level expression in *Escherichia coli* using the NovoProlab Website (https://www.novoprolabs.com/tools/codon-optimization). The optimized sequence was synthesized commercially and cloned into the pET-45b(+) expression vector (Novagen) between the *KpnI* and *XhoI* sites by Biomatik Corporation (Canada). The resulting construct, pET45-VP1, encodes an N-terminal hexahistidine (His_6)-tag fusion protein and carries an ampicillin resistance gene for selection. For plasmid propagation, the construct was transformed into chemically competent *E. coli* BL21 (DE3) cells (Thermo Fisher Scientific, USA) via heat shock transformation according to the manufacturer's protocol. Transformants were selected on LB agar plates supplemented with 100 µg/mL ampicillin [20].

Extraction, linearization and sequencing of recombinant pET45- VP1 plasmid

The recombinant pET45-VP1 plasmid was extracted from transformed *E. coli* using the Transgene Biotech Easy Pure Plasmid Miniprep Kit (China). Initial confirmation of the VP1 gene insertion was performed via PCR using gene-specific primers.For sequencing, the plasmid was linearized with SacI restriction enzyme (Thermo Fisher Scientific, USA, Lot No: 00898186) according to the manufacturer's instructions. The nucleotide sequence of the insert was verified by Sanger sequencing using universal T7 promoter and T7 terminator primers. Sequencing was performed by Gene Fanavaran Company (Tehran, Iran). The resulting chromatograms were aligned and compared against the original designed VP1 sequence to confirm the absence of any unintended mutations.

PCR analysis of transformed BL21 (DE3) and selection multi copy colonies

Positive transformants were selected and grown in LB medium supplemented with 50 µg/mL ampicillin. To verify the presence of the recombinant VP1 gene insert, colony PCR was performed in a 25 µL reaction volume using universal T7 promoter and T7 terminator primers that flank the multiple cloning site of the pET-45b(+) vector. The thermal cycling conditions (ABI, Korea) were as follows: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min. The PCR products were then analyzed by electrophoresis on a 1% (w/v) agarose gel, alongside a 100 bp DNA ladder (Fermentas, USA), to confirm the amplification of a fragment of the expected size.

Expression of recombinant VP1 protein

A single, well-isolated transformed colony was selected and used to inoculate 20 mL of LB broth supplemented with 50 µg/mL ampicillin. This starter culture was incubated overnight at 37°C with shaking. Subsequently, 1 mL of this pre-culture was used to inoculate 100 mL of fresh LB-ampicillin medium in a baffled Erlenmeyer flask. The culture was grown at 37°C with vigorous shaking (200-250 rpm) until the optical density at 600 nm (OD_{600}) reached approximately 0.6. Protein expression was then induced by the addition of IPTG to a final concentration of 0.4 mM. Induction was carried out for 5 h at 37°C with continuous shaking. Following induction, the bacterial cells were harvested by centrifugation at 4°C for 15 min at a minimum of 6000 × *g*. The resulting cell pellet was stored at -20°C for subsequent protein purification steps.

SDS- PAGE and Western Blotting

Recombinant protein expression was confirmed by SDS-PAGE and Western blot analysis. Cell lysates were resolved on a 12% SDS-PAGE gel at 100 V for 3 h. For immunoblotting, proteins were electrophoretically transferred to a PVDF membrane (Bio-Rad, Cat. #162-0177). The membrane was then blocked for 1 h at room temperature with 5% (w/v) bovine serum albumin (BSA; Sigma, Germany) in Tris-Buffered Saline containing 0.1% Tween-20 (TBST). Following blocking, the membrane was incubated overnight at 4°C with gentle agitation in a 1:700 dilution of a horseradish peroxidase (HRP)-conjugated anti-His tag monoclonal antibody (Sina Biotech, Iran, Cat. #SB-019661) prepared in blocking buffer. The membrane was subsequently washed three times with TBST. Target protein bands were visualized using an Enhanced Chemiluminescence (ECL) substrate (Thermo Fisher, USA) and imaged with a digital imaging system.

RESULTS

Bioinformatics Studies

After designing the construct containing the VP1 protein coding gene and codon optimization, the gene with the

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nucleotides in Table 1 was sent to Gene Universal (Canada) for cloning in pET-45 vector. The recombinant pET-45 vector designed in SnapGene software version 8.0.2 is shown in Fig. 1.

Table 1. Nucleotide Sequence of the VP1 Construct (The capital letters are cleavage sites related to XhoI and KpnI at the beginning and end of the sequence and the red letters are termination codon)

Gene Sequence	GGTACCggcgaaagcgcggatccggtgaccgcgaccgtggaaaactatggcggcgaaacccaggtgcagcgccgccagcataccgatgtgagctttattctggatcgct ttgtgaaagtgaccccgcgcgatcagattaacgtgctggatctgatgcagattccggcgcataccctggtgggcgcgctgctgcgcaccgcgacctattattttgcggatctggaa gtggcggtgaaacataaaggcaacctgacctgggtgccgaacggcgcgccggaaaccgcgctggataacaccaccaacccgaccgcgtatcataaagcgccgctgacccgc ctggcgctgccgtataccgcgccgcatcgcgtgctggcgaccgtgtataacggcgattgcaaatatggcgaaagccgcgcgaccaacgtgcgcggcgatctgcaggtgctgg cgcagaaagcggcgcgcaccctgccgaccagctttaactatggcgcgattaaagcgacctgggtgaccgaactgctgtatcgcatgaaaaaagcggaaacctattgcccgcgc ccgctgctggcgattcatccgagcgaaccgaaacataaccagatgattgtggcgcatgtgtaaCTCGAG

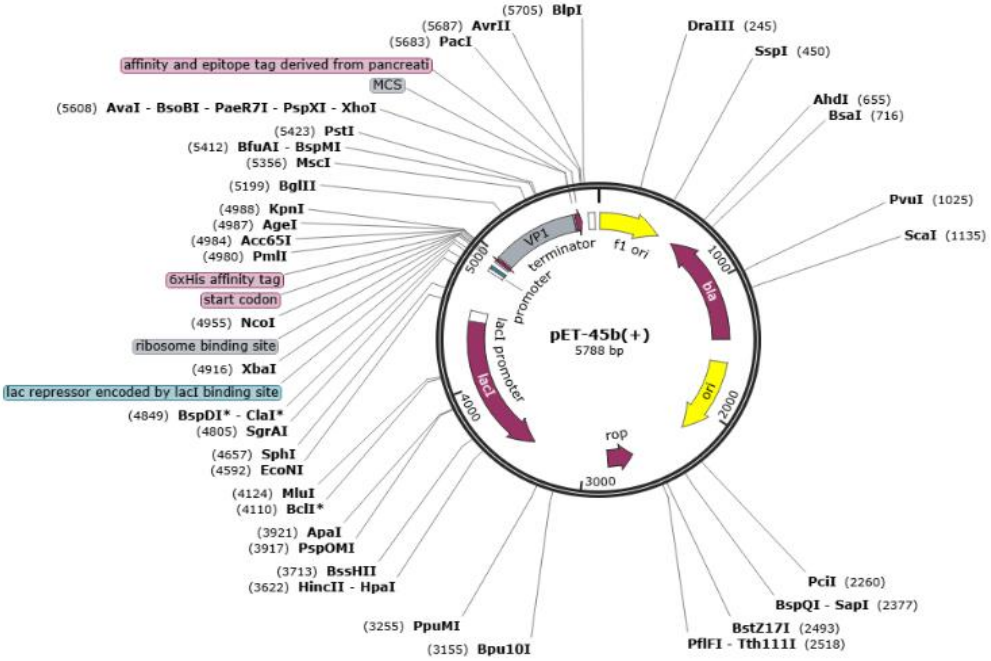

Fig. 1. Schematic representation of the recombinant pET45-VP1 construct.

Production and Linearization of pET45- VP1 Plasmid

After the synthesis of pET45- VP1 recombinant plasmid, it was successfully transformed into *E. coli* BL21 competent cells. Following extraction of the plasmid, analysis by agarose gel electrophoresis indicated the linearization of the plasmid by SacI (Fermentas, USA) restriction enzyme (Fig.2A). PCR analysis using T7 primers confirmed that the gene of interest was inserted into the correct position (Fig. 2B).

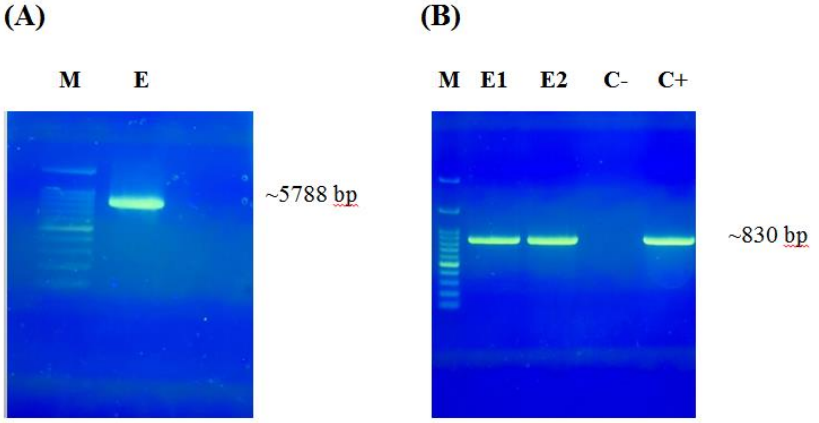

Fig. 2. (A) Results of restriction enzyme (M: Marker 1kb, E: Linearized extracted pET45-VP1 recombinant plasmid). (B) PCR results of the inserted gene. M: 100 bp plus DNA ladder; E1 and E2 PCR product; C-: negative control; C+: positive control.

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[DOI: 10.52547/vacres.10.2.44]

Protein Analysis of the Recombinant VP1

In order to evaluate the expression levels of the structural VP1 protein, samples were subjected to analysis via SDS-PAGE and Western blotting techniques. SDS-PAGE analysis showed an estimated molecular weight of 27 kDa and validated the synthesis of the VP1 protein by the modified *E. coli* (BL21) cells (Fig. 3A). The molecular weight of the construct was calculated by www.aatbio.com. The detection of VP1 protein by the anti-His tag antibody was confirmed by Western blotting (Fig. 3B) and the findings indicated that protein expression occurs at 4-5 hours subsequent to the administration of IPTG.

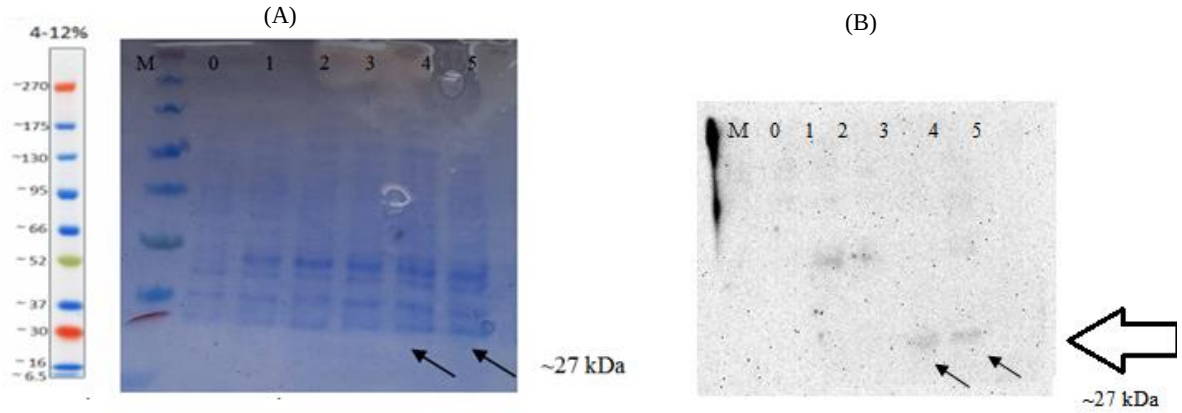

Fig 5. (A) SDS PAGE of the recombinant VP1 protein expressed by the *E. coli* (BL21) at different hours (0, 1, 2, 3, 4, 5h). (B) Western blot analysis using anti-his antibody.

DISCUSSION

The FMD virus remains a significant pathogen worldwide and poses a threat to the livestock industry. This virus intermittently spreads from endemic regions to zones where the disease does not occur or happens rarely [21]. Due to the drawbacks of conventional and traditional vaccines, FMD continues to be widespread in many regions of the world. This underscores the urgent requirement for the progress of a novel recombinant vaccine [22, 23].The antigenic regions related to FMDV are present on structural proteins that three antigenic zones belong to VP1 protein. Consequently, VP1 that encoded by the 1D region of the genome, is the most important protein in the structure of the FMDV, capable of eliciting an immune response in susceptible hosts [24, 25]. The G-H loop located on the VP1 protein is where the virus binds to the host cell receptor and plays an important role in virus immunogens [26]. For this purpose, in our study, the nucleotide sequence of the VP1 gene type O IR/P50/2016 was used to prepare and evaluated the subunit recombinant vaccine. Due to the fact that subunit vaccines do not contain the genome of the virus, the possibility of replication and as a result of mutation in the host is zero and side effects of these vaccines are less than the complete virion [27]. Considering that the prokaryotic system has many advantages such as rapid growth, well characterized genetics and cost effectiveness [17], were decided to use this system to produce recombinant protein.

So far, various studies have demonstrated the capability of this system to express of different viral proteins and VP1 can induce immune response. Shi *et al.* (2006) indicated that in murine models, the FMD virus VP1 protein was capable of prompting both humoral and cellular immune responses and accompany VP1 protein with bovine IFN-gamma can enhance the immune response [28]. Yang *et al.* (2008) concluded VP1 protein not only induces cellular and humoral immunity, but also causes the target animal to have complete protection against the virus [29]. Jung *et al.* (2013) showed that VP1-GST antigen is suitable for application in vaccine formulation and the advancement of serological diagnostic assays, and it has been established that large-scale production in Escherichia coli is feasible [30]. Motamedi *et al.* (2014) exhibited the pET-28-VP1 cassette was expressed in *E. coli* and this recombinant construct needs more studies to be able to use as a recombinant vaccine against FMD virus type O/IRN/2010 in future [31]. Liu *et al.* (2017) represented the recombinant proteins (VP1, VP1-gp120, VP1-E2) were properly expressed in the SF9 insect cells and had good the right immune response [19]. Tang et al (2023) demonstrated that the VP1 protein isolated from FMDV carriers and expressed in the *E. coli* system can immunize mice against the virus [32]. In conclusion, preliminary outcomes showed that the expressed structural VP1 protein (FMD O IR/P50/2016) can be produced at an acceptable level in the *E. coli* expression system. In conclusion, the findings of this study position this protein as a candidate for a new FMD vaccine which its potential to enhance disease control in endemic regions remains to be evaluated. Its additional utility as a specific antigen for diagnostic assays further underscores its value and warrants comprehensive investigation in future studies.

ACKNOWLEDGEMENT

Finally, I would like to thank the management (Dr Aghaeipour Kolyani) and staff of Behesht Aein laboratory for financial support and assistance in research.

CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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