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Engineering foot-and-mouth disease virus serotype O IND R2/1975 for one-step purification by immobilized metal affinity chromatography

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ABSTRACT

Immobilized metal affinity chromatography (IMAC) allows for the efficient protein purification via metal affinity tag such as hexa-histidine (His_6) sequence. To develop a new chromatography strategy for the purification and concentration of foot-and-mouth disease virus (FMDV) particles, we inserted the His_6-tag at the earlier reported site in the VP1 G-H loop of the FMD virus serotype O vaccine strain IND R2/1975. Display of the His_6-tag on the capsid surface, endowed the virus with an increased affinity for immobilized nickel ions. We demonstrated that the His_6-tagged FMDV could be produced to high titre and purified from the infected BHK-21 cell lysates by IMAC efficiently. Further, a 1150-fold reduction in protein contaminant level and an 8400-fold reduction in DNA contaminant level were achieved in the IMAC purification of His_6-tagged FMDV. Through various functional assays it has been found that the tagged virus retains its functionality and infectivity similar to the non-tagged virus. The affinity purification of the His_6-tagged FMDV may offer a feasible, alternative approach to the current methods of FMDV antigen purification, concentration and process scalability.

1. Introduction

Foot-and-mouth disease (FMD) continues to remain a globally important livestock disease affecting the cloven-hoofed animals. The disease is endemic in many parts of the world, especially in developing countries where it imposes trade barrier on livestock and their products [1]. The causative agent FMD virus (FMDV) is the prototype member of the genus *Aphthovirus* within the Picornaviridae family [2]. The positive sense single stranded RNA genome of FMDV (~8.2 kb) consists of a large open reading frame that is flanked by highly structured 5′ and 3′ un-translated regions (UTRs) which are indispensable for FMDV genome replication [3]. The monocistronic viral genome encodes a single polyprotein that gets cleaved subsequently into 12 proteins by virus encoded proteinases that are required for virus replication and assembly (Ryan et al., 1989). The capsid precursor polyprotein (P1) is processed into four structural proteins VP4, VP2, VP3 and VP1. A single molecule of these four structural proteins assemble initially to form a protomer, five protomers assemble to form a pentamer, and 12 such pentamers assemble to form the viral capsid that encloses the genomic RNA [4]. The capsid proteins VP2, VP3 and VP1 form the outer capsid surface, whilst the VP4 protein remains at the inner surface of the virion capsid. The surface exposed FMDV capsid proteins play a key role in the antigenic determinants of the FMDV. Further, the capsid surface also interacts with various cell-surface receptor molecules prior to the cell entry of FMDV by clathrin- or caveola-mediated endocytosis pathways [5,6]. Especially, a prominent capsid surface exposed loop connecting the βG and βH strands of the VP1 capsid protein (known as G-H loop) contains a highly conserved 'arginine–glycine–aspartate' (RGD) motif, which interacts with α_V-integrin family of cell surface receptors [7,8].

The current vaccine against FMD is a chemically inactivated whole virus antigen, formulated with a suitable adjuvant. Systematic vaccination programmes with this inactivated vaccine have successfully reduced the number of FMD outbreaks in enzootic countries. Furthermore, vaccination programmes with inactivated vaccine also resulted in the elimination of FMD in Western Europe

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by the year 1989 [9–11]. For the preparation of FMD vaccine, pure viral preparations are necessary since the contaminants in vaccine could evoke undesirable hypersensitivity response [12]. Further, FMD vaccine should be free from contaminating viral non-structural proteins (NSPs), as the presence of NSPs could lead to difficulties in distinguishing infected from repeatedly vaccinated animals using currently approved diagnostic assays [13]. Therefore, several methods have been reported for the concentration and purification of FMDV such as poly-ethylene glycol precipitation [14,15], ultrafiltration [16], and aqueous two phase partition [16]. However, these techniques exhibit poor specificity and the viral particles remain accompanied by considerable amount of contaminants [12].

In order to aid the virus purification and concentration strategies, here, we reported the characterisation of a hexa-histidine (His_6)-tagged FMDV serotype O Indian vaccine strain IND R2/1975 constructed by reverse genetics techniques and the subsequent use of the His_6-tagged virus in one-step purification by immobilized metal affinity chromatography (IMAC). Inspired from the work of Seago et al. [17], we inserted the His_6-tag into the G-H loop of VP1 protein and successfully rescued the infectious His_6-taged FMDV without affecting the infectivity titre and biological properties. Furthermore, we demonstrated that the one-step IMAC purification of His_6-tagged FMDV is simple and fast, with a high yield of infectious viral particles essentially free from protein and DNA contaminants.

2. Materials and methods

2.1. Cell line and virus

FMDV susceptible cell line BHK-21 was propagated in Glasgow minimum essential medium (GMEM) (Sigma, USA) supplemented with 10% foetal bovine serum (FBS). Cell culture adapted FMDV strain O IND R2/1975 at passage five was obtained from the national FMD virus repository maintained at Project Directorate on Foot-and-mouth disease (PD-FMD), Mukteswar, India. In addition, FMDV serotypes A and Asia 1 vaccine strains, A IND 40/2000 and Asia1 IND 63/1972, respectively (both at passage five) were also obtained from PD-FMD virus repository. Virus infected BHK-21 cell culture supernatant served as a source of viral RNA for the synthesis of full-length cDNA clone.

2.2. Construction of the FMDV O IND R2/1975 full-length cDNA clone and its His6-tagged derivative

Viral RNA was extracted from the infected cell culture supernatant using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and used for the full-genome cDNA synthesis. All the primers with restriction enzyme (RE) sites (Table 1) were designed based on the genomic sequence of FMDV O IND R2/1975 at passage five (unpublished data). The cDNA synthesis was performed with Thermoscript™ Reverse Transcriptase (Invitrogen, USA) enzyme and specific RT primers (C-Rev and A-Rev) at 55 °C for 2 h. The full-length cDNA corresponding to the complete genome of O IND R2/1975 virus, was PCR amplified as a small fragment (SF) of 5′ UTR of approximately 0.36 kb upstream of the poly (C) tract, a large fragment (LF) of 5′UTR from nucleotides 363 to 1009 and a fragment from the L region of FMDV until the poly A tract (L-Poly A; ~7.2 kb). The schematic representation of these fragments has been shown in Fig. 1 (step I). All the PCRs were carried out using a Phusion® High Fidelity DNA polymerase (NEB, USA), as per the manufacturer's instruction.

The PCR amplicons corresponding to the SF and LF were digested with *Sma*I RE and joined (SF + LF) by *in vitro* ligation method (step II, Fig. 1). This approach allowed the incorporation of homopolymeric poly (C) tract of 44 nucleotides between the SF and LF regions. Later the above joined fragment (SF + LF) was once again amplified using primers A-For and B-Rev. To synthesize the double-stranded complete cDNA of the O IND R2/1975 FMDV, the PCR amplicons SF + LF and L-Poly A were digested with *Dra*I RE and annealed together by *in vitro* ligation method (step III). Finally, the annealed cDNA was amplified by long PCR using the primers A-For and C-Rev and gel purified. Subsequently, the full length PCR amplicon was ligated into pT7 blue blunt vector (Novagen, Germany) through blunt end ligation procedure (step IV). The ligated product was used to transform chemically competent *Escherichia coli* XL1 blue cells (Agilent Technologies, U.K.) and ampicillin-resistant colonies were screened for recombinant plasmid. Positive clones were characterised by restriction endonuclease analysis and subsequent nucleotides sequencing (ABI 3130 Genetic analyzer, Applied Biosystems, CA, USA). The final construct was designated as pT7blue O-IND-R2/1975.

Genetic marker in the form of *Eco*RV restriction site was engineered into the VP4 coding region of cDNA clone to differentiate recombinant rescued virus from wild-type virus. *Eco*RV marker (GATATC) was engineered in place of the wild type sequence

Table 1
List of oligonucleotide primers used in this study.

Primers designation	Nucleotides sequence (5′–3′)	RE site	Nucleotide position	Purpose
A-For	GGACCAGGACA ***ACTAGT*** TTGAAAGGGGGCG	*Spe*I	1–13	PCR of amplicon SF
A-Rev	TATA ***CCCGGG*** GGGGGGGGGGGGGGGGGGGG ATGAAAG	*Sma*I	357–363	PCR of amplicon SF and RT reaction
B-For	GCTA ***CCCGGG*** CCCCCCCCCCCCCCCCCC TAAGTTTTAC	*Sma*I	364–373	PCR of amplicon LF
B-Rev	GCGCGACG ***TTTAAA*** GAAGCCCCAGTCCCCTTCTCAG	*Dra*I	970–1001	PCR of amplicon LF
C-For	CGGC ***TTTAAA*** AGTGCCCAGTTTGAAAAGCTTC	*Dra*I	988–1019	PCR of amplicon L-Poly A
C-Rev	ATTG ***GCGGCCGC*** TTTTTTTTTTTTTTT	*Not*I		PCR of amplicon L-Poly A and RT reaction
*Eco*RV F1	GTGACAATGCGATAAGCGGAG		1794–1814	First round of site-directed mutagenesis to introduce *Eco*RV marker
*Eco*RV R1	CTCCGCTTATCGCATTGTCAC		1794–1814	First round of site-directed mutagenesis to introduce *Eco*RV marker
*Eco*RV F2	CAATGCGATATCCGGAGGCTCC		1798–1819	Second round of site-directed mutagenesis to introduce *Eco*RV marker
*Eco*RV R2	GGAGCCTCCGGATATCGCATTG		1798–1819	Second round of site-directed mutagenesis to introduce *Eco*RV marker
6xHis Ins F	CACCACCACGCGAGAACGCTGCCTACC		3712–3730	For insertion of hexa-histidine tag in VP1 protein
6xHis Ins R	ATGATGATGCGCCTTCTGGGCCAACAC		3695–3711	For insertion of hexa-histidine tag in VP1 protein

Bold and italic nucleotide bases represent the restriction enzyme sites used for ligation and cloning in order to construct the full-length cDNA clone. The underlined bases represent the sequence encoding the His_6-tag.

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