Development of effective vaccine against local isolates of foot and mouth disease virus in Bangladesh

PhD Thesis

DEPARTMENT OF MICROBIOLOGY
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Development of effective vaccine against local isolates of foot and mouth disease virus in Bangladesh

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN THE FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Dedicated to…

My Beloved Parents, Who Cherished
My Life with Their Blessings

Quotation…

“If everything is God’s will, then so is the invention of the vaccine, just like the seatbelt.”

Els Borst

Certification

It is hereby certified that student bearing Reg. No. 138, Session 2013-2014 has carried out the research work entitled "**Development of effective vaccine against local isolates of foot and mouth disease virus in Bangladesh**" for the fulfillment of his PhD Degree from University of Dhaka, Bangladesh, under my academic supervision in the Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka.

..……

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Examiner’s Copy

Abstract

Foot and mouth disease (FMD) is a highly contagious picornaviral disease of domestic and wild cloven-hoofed animals throughout the world including Bangladesh. The endemicity of this disease is causing notifiable economic loss and an impediment to the full potential surge of livestock industry in Bangladesh. Vaccination using imported or locally produced vaccines is the existing practice of controlling the disease in Bangladesh, although vaccine failure is common upon vaccination. The causes of these failures are multifold including selection of inappropriate vaccine virus strain(s), inadequate antigenic payload and improper vaccine production process. It was presumed that a quality assured FMD vaccine tailored to the circulating indigenous FMDV strains might efficiently protect the susceptible animals in Bangladesh. Hence, the present investigation was envisaged to carry out an extensive epidemiological study and molecular characterization of the circulatory FMDVs in Bangladesh, selection of appropriate vaccine candidates, optimization of vaccine production processes and effective vaccine development. A total of 319 FMDV infected tissue samples from 47 FMD outbreaks were collected and analyzed over a period of seven years from 2012 to 2018. Polymerase Chain Reaction (PCR) based analysis of the samples revealed that FMDV serotypes O, A and Asia1 are prevalent in Bangladesh with predominance of serotype O (81%) followed by serotype A (13%) and Asia1 (6%). Intra-serotype genetic divergence specifically in serotype O was evident by VP1 protein coding gene based phylogeny studies. Serotype O isolates of Bangladesh clustered in Ind2001d, Ind2001BD1 and Ind2001BD2 sublineages of Ind2001 lineage, although most of the recent isolates belonged to novel and dominating sublineage Ind2001BD1. Phylogeny of FMDV serotype A isolates of Bangladesh clustered all of them in Genotype VII of Asia Topotype. Depending on the prevalence and dominance, FMDV serotype O isolate BAN/TA/Dh-301/2016 from Ind2001BD1 sublineage and serotype A isolate BAN/CH/Sa-304/2016 from Genotype VII were selected as vaccine candidates. Comparative genetic distance analysis confirmed that selected vaccine candidates are significantly ($p=0.001-0.002$) genetically closer to the respective isolates of Bangladesh than the current Indian vaccine strains. Z-test for selection predicted that the vaccines prepared by selected candidates will protect animals against all the circulatory FMDV serotype O and A isolates of Bangladesh. The selected vaccine candidates were adapted and propagated in BHK-21 cell line and subjected to complete genome sequencing. The complete genome of vaccine candidates O/BAN/TA/Dh-301/2016 and A/BAN/CH/Sa-

304/2016 were found to be 8211 nt and 8221 nt in length respectively with an ORF of 6999 nt for each. Deduced amino acid (aa) sequences comparison deciphered that the VP1, VP2 and VP3 regions of O/BAN/TA/Dh-301/2016 had 5%, 3% and 5% structural variability with respective regions of current Indian vaccine strain O/India/R2/75. Three aa substitutions in critical antigenic site 1 in G-H loop of VP1 and 2 aa substitutions in antigenic site 2 of VP2 regions were evident. Similar regions of A/BAN/CH/Sa-304/2016 showed 8%, 6% and 5% variability compared to respective regions of serotype A vaccine strain A/IND40/00 with four aa substitutions in critical antigenic sites of VP1. For the purpose of vaccine production, bulk amount of both the vaccine candidates were successfully inactivated by Binary Ethylenimine within 9-10 hours after the onset of inactivation. Extrapolation of inactivation kinetics of both the vaccine candidates confirmed that there will be less than 1 infectious particle in a 10^4 liter batch after 24 hours inactivation cycle. Complete inactivation of virus was assured by the absence of CPE even at third serial passage of inactivated virus in BHK-21 cell monolayers. Inactivated virus particles in the fluids were significantly (p=0.002-0.003) concentrated by ultrafiltration system using 100 kDa hollow fiber cartridge. After concentration, the final concentrations of inactivated virus particles in the fluids were found to be 16.33±1.15 µg/ml and 14.33±0.57 µg/ml for serotype O and A respectively. Vaccines were formulated with Montanide ISA 201 oil adjuvant and the final concentrations of inactivated FMD viral antigens per 2 ml of vaccine were 14.5 µg and 12.41 µg for serotype O and A respectively. Absence of adverse reactions in the recipient guinea pigs during i*n-vivo* vaccine efficacy testing confirmed the safety of developed vaccines. The minimum inactivated virus particle contents that protected 100% animals from virus challenge and elicited SN_{50} titer significantly higher than the protective level was 3.53 µg and 3.10 µg for FMDV serotype O and A vaccine candidates respectively. In 2D-MNT tests, the antigenic relationship (r) values of the circulating FMD serotype O and A field viruses with respective vaccine strains were found to be >0.3. Strong antigenic relationship between field virus and selected vaccine strains confirmed that vaccine produced using these candidate viruses will protect all genetically divergent FMDV strains of respective serotypes circulating in Bangladesh.

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ABBREVIATIONS

2D-MNT- Two Dimensional Micro Neutralization Test

3D – Three Dimensional

aa – Amino Acid

bp – Base pair

BHK - Baby Hamster Kidney

BLAST- Basic Local Alignment Search Tool

CDS – Coding DNA Sequence

CPE-Cytopathic Effect

CRE - Cis-active Replicative Element

DLS- Department of Livestock Services

DMEM - Dulbecco's Modified Eagles' Medium

DMSO - Dimethyl Sulfoxide

EIF - Eukaryotic Initiation Factor

ELISA - Enzyme Linked Immunosorbent Assay

FEL - Fixed Effects Likelihood

FMD – Foot and Mouth Disease

FMDV – Foot and Mouth Disease Virus

FPLC- Fast Performance Liquid Chromatography

GDP- Gross Domestic Product

IFEL - Internal Fixed Effects Likelihood

IRES - Internal Ribosome Entry Site

MEGA - Molecular Evolutionary Genetics Analysis

NCBI- National Centre for Biotechnology Information

NTP - Nucleotide Triphosphate

OIE - Office Des Epizooties

ORF - Open Reading Frame

PCP-FMD - Progressive Control Pathway for FMD Control

PCR - Polymerase Chain Reaction

PD_{50}- Protective $Dose_{50}$

PDB- Protein Data Bank

PDFMD- Project Directorate on Foot-and Mouth Disease

PVS- Protein Variability Server

SAT - South African Territories

SLAC - Single Likelihood Ancestor Counting

SNT_{50}- Serum Neutralization $Titer_{50}$

$TCID_{50}$- Tissue Culture Infective $Dose_{50}$

UTR – Un-translated Region

VNT - Virus Neutralization Test

VP - Viral Protein

Vpg - Viral Genome-Linked Protein

WRLFMD - World Reference Laboratory for Foot and Mouth Disease Virus

ABBREVIATED NAMES OF AMINO ACIDS

G - Glycine

V - Valine

L - Leucine

I - Isoleucine

F - Phenylalanine

P - Proline

Y - Tyrocine

W - Tryptophan

S - Serine

T - Threonine

A - Alanine

M - Methionine

N - Asparagine

Q - Glutamine

D - Aspartate

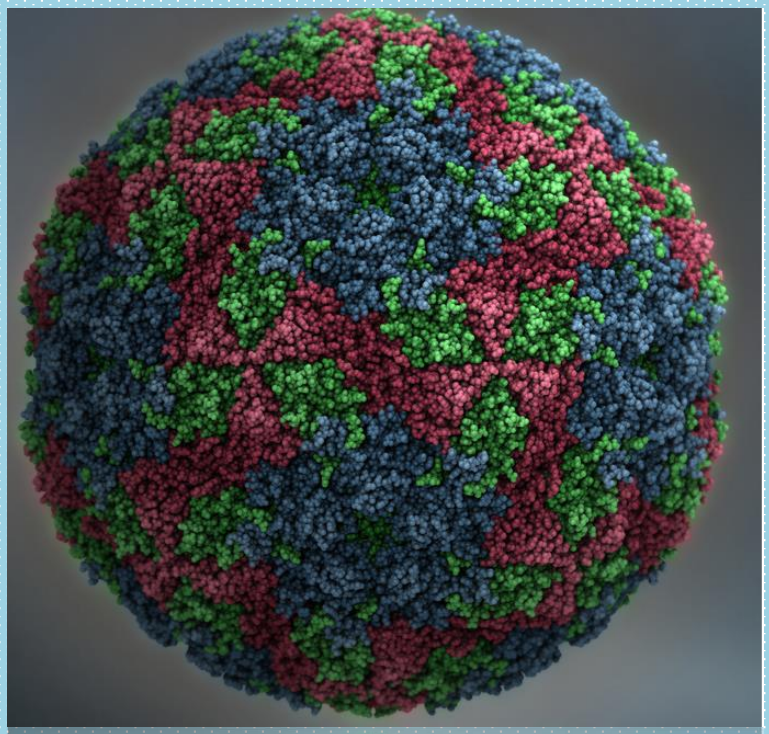
E - Glutamate

K - Lysine

R - Arginine

C - Cysteine

H - Histidine



Chapter 01:

Introduction and Literature Review

1.1 General Introduction

Foot and mouth disease (FMD) is an acute, febrile, and highly contagious vesicular disease affecting more than 70 species of domestic and wild cloven-hoofed animals including cattle, buffalo, sheep, goats, deer, and pigs (Alexandersen *et al.*, 2003). This disease is characterized by high fever, depression, anorexia, excessive salivation and localized vesicles formation in feet, mouth, mammary glands and occasionally at other locations. Subsequently, the vesicles rupture leading to pain, lameness, and reluctance to move or rise. Secondary infections particularly in affected feet, chronic lameness, delayed wound healing and mastitis are the common sequelae of FMD in dairy cattle. Mortality can be high in young animals, where the virus can affect the heart (M. J. Grubman & Baxt, 2004).

The etiological agent of the disease is foot-and-mouth disease virus (FMDV), a virus of the genus *Aphthovirus*, family *Picornaviridae* (Ma *et al.*, 2014; OIE, 2012; Sobrino *et al.*, 2001). The virus is a single-stranded positive-sense RNA virus and the genome is surrounded by an icosahedral capsid composed of 60 copies each of four structural proteins VP1, VP2, VP3 and VP4 (Jackson *et al.*, 2003). The FMDV genome is over 8 kb in length and contains a protein cap 3B, also known as VPg. The genome contains a single large open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). The ORF encodes a single polyprotein that is processed by virus-encoded proteases to yield different polypeptide precursors and mature viral proteins, including four structural proteins (VP1, VP2, VP3 and VP4) and eight non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C and 3D) (Ma *et al.*, 2014; Sobrino *et al.*, 2001). There are seven immunologically distinct serotypes of FMDV namely A, O, C, Asia1, SAT (South African Territories)-1, SAT-2 and SAT-3 (Bachrach, 1968; E. Domingo *et al.*, 2002; Knowles & Samuel, 2003).

FMDV is considered to be the most important animal pathogen worldwide because of its high contagiousness and potential for causing severe economic loss in susceptible animals. The disease has direct and indirect impacts on economy and the direct economic losses are due to reduced milk and meat production, death of animals, weight loss and loss of draught power and reduced fertility in animals. Indirect losses result from treatment and vaccination costs and denied access to both local and international markets. Annual impact of FMD in terms of visible production losses and vaccination expenses in endemic regions alone amount to between US$6.5 and 21 billion (Knight-Jones & Rushton, 2013). The

economic losses due to this disease in Bangladesh was estimated as high as 125 million USD/annum (DLS, 2014).

Livestock sector is an important sub-sector of agriculture of Bangladesh and its overall contribution to the agricultural Gross Domestic Products (GDP) is 13.62% and to national GDP is 1.54%. Country's 20% labour force is associated with rearing of animals and a major portion of the rural population is directly or indirectly linked to the livestock sector which plays a pivotal role in people's day to day economy (DLS, 2014). Total FMDV susceptible domestic animal population in Bangladesh is about 55.13 million of which 24.08 million cattle, 1.48 million buffalo, 26.10 million goats and 3.47 million sheep (DLS, 2014). These animals have great potentials for contributing to the economy of Bangladesh but prevalence of infectious diseases like FMD are the main obstacles in the development of the livestock sector to its optimum capacity. FMD is endemic in Bangladesh with the circulation of FMDV serotype O (80-85%), A (10-15%) and Asia1(up to 5%). Genetic diversity within serotype specifically in O is also present. Along with previous sublineage Ind2001d, two novel sublineages Ind2001BD1 and Ind2001BD2 within Ind2001 lineage of serotype O have emerged in Bangladesh (Siddique *et al.*, 2018). In endemic areas, vaccination is the major tool to control FMD. For the reason various types of imported and locally manufactured FMD vaccines are used in Bangladesh. Vaccine failure is very common in Bangladesh which is attributed to vaccine virus and circulating field virus mismatch, introduction of new mutant viruses or importation of vaccines of heterologous strains from abroad (Ullah *et al.*, 2015; Zinnah *et al.*, 2010). These shortcomings urge the development of an effective FMD vaccine tailored to the circulatory FMDV serotypes of Bangladesh. Furthermore, Bangladesh is implementing OIE/FAO prescribed PCP-FMD road map but the country is yet at early stage or no-control program implemented stage. For implementing effective FMD control program in Bangladesh an effective mono- to tri-valent vaccine will be required. Therefore, the present study emphasized on molecular epidemiology and comparative genome analyses of circulating FMDV serotypes in Bangladesh, prediction of potential vaccine candidates and finally development of effective vaccine using the seed virus of local strains through a number of potency assay and challenge tests.

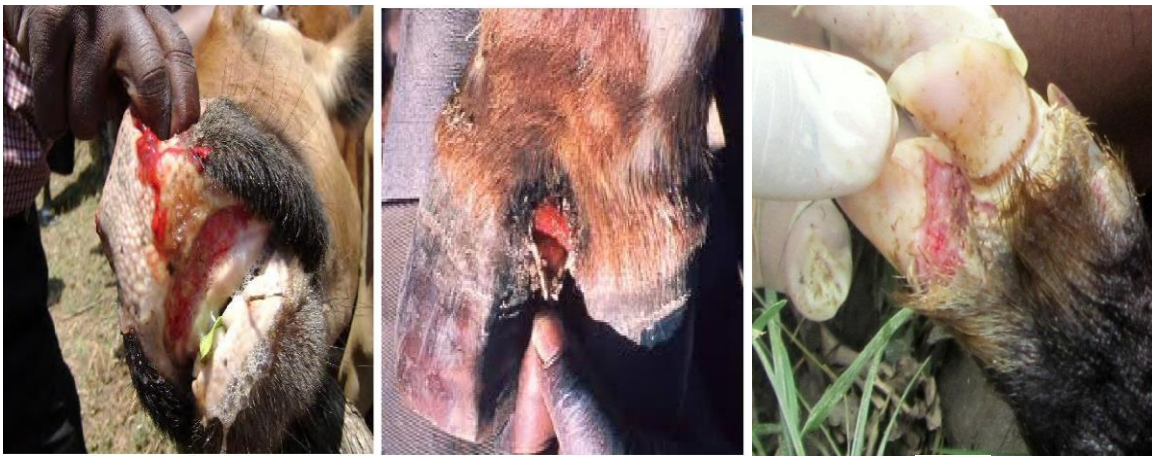
1.2 Review of Literature

1.2.1 Foot and Mouth Disease (FMD)

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating viral disease of cloven-hoofed animals, including cattle, buffaloes, swine, goats, sheep, and other wild ruminants (Alexandersen *et al.*, 2003; F. Brown, 2003).

1.2.1.1 Clinical Signs and Symptoms of FMD

The common clinical signs and symptoms of FMD are high fever, salivation, vesicles (blisters) formation with subsequent rupture and tissue erosion in interdigital space of feet, in and around the buccal cavity, on the mammary gland, and occasionally at other locations. Pain and discomfort from the lesions leads to depression, anorexia, excessive salivation, lameness and reluctance to move or rise. Disease signs usually appear within 2 to 3 days after exposure (**Figure 1.2.1.1**) and can last for 7 to 10 days (M. J. Grubman & Baxt, 2004). The severity of clinical signs may vary with the strain of virus, the exposure dose, the age and breed of animal, the host species and the immunity of the animal. The signs can range from a mild or inapparent infection to severe one. Mortality is low in adult animals but very high in young animals is associated with multifocal myocarditis (OIE, 2012).



(a) **(b)** **(c)**

Figure 1.2.1.1: Lesions due to Foot-and-Mouth Disease in cattle (a,b) and pig (c)

1.2.1.2 Transmission of Foot and Mouth Disease Virus (FMDV)

During acute infection, transmission is facilitated by virus shedding from ruptured vesicles and in bodily excretions and secretions, including breath, milk and semen (Alexandersen *et al.*, 2003) **(Figure 1.2.1.2)**. Susceptible ruminants can be infected by very low doses of

inhaled virus through direct contact with the breath of other acutely infected animals, or indirectly by resuspension of aerosols from contaminated materials. Pigs are relatively resistant to FMDV infection via inhalation routes (Alexandersen *et al.*, 2003). Other routes of infection such as ingestion or through abrasions require a higher dose of virus. Depending on conditions, FMDV can survive for days to months in the environment and in various animal products including meat (Charleston *et al.*, 2011). There is a rapid immune response to infection associated with FMDV clearance, but some ruminant hosts continue to harbor virus, becoming carriers with low and declining levels of FMDV in specific nasopharyngeal epithelial sites and associated lymphoid tissues (Charleston *et al.*, 2011).

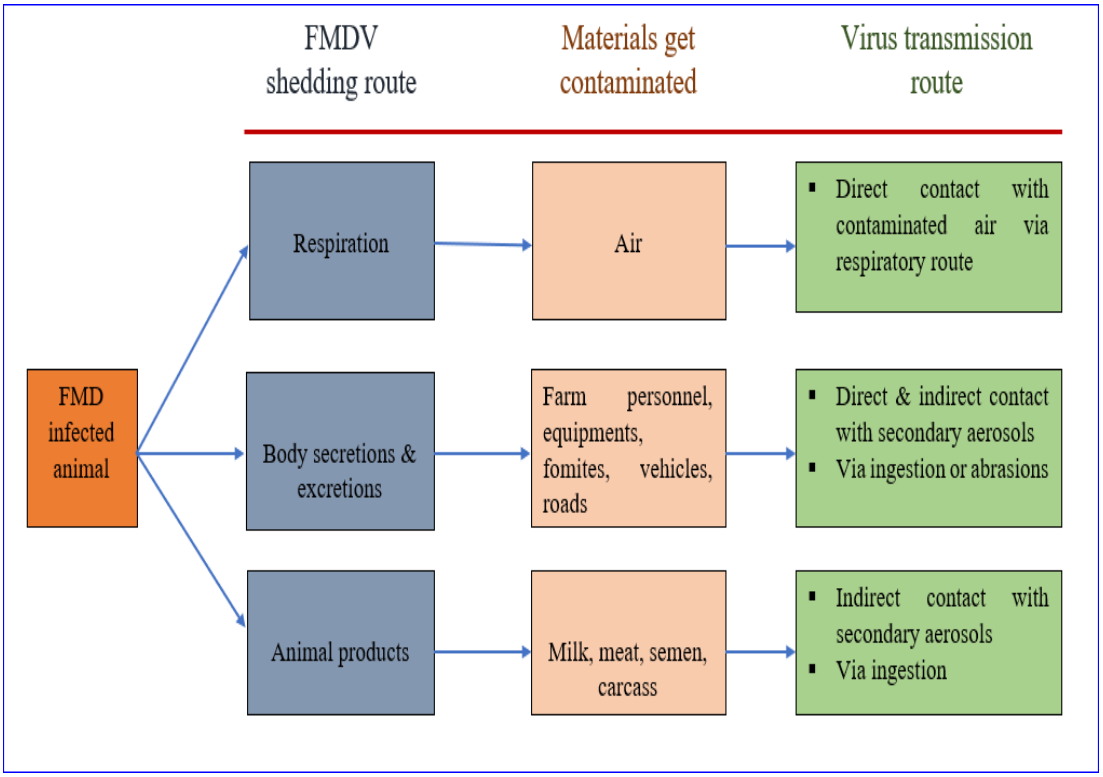


Figure 1.2.1.2: Principal routes of FMD virus spread among susceptible animals

The incubation period of FMD is highly variable, 2-14 days, and depends on the strain and dose of virus, the route of transmission, the animal species and the husbandry conditions (Charleston *et al.*, 2011). Charleston *et al.* (2011) found that period of infectiousness in cattle is only 1.7 days and animals are not infectious until 0.5 days after the appearance of clinical signs (Charleston *et al.*, 2011).

1.2.1.3 Economic Impacts of Foot and Mouth Disease

Although FMD is a disease of low mortality, the global impact of the disease is colossal due to the huge numbers of animals affected. This impact can be segregated into two catagories: (1) direct losses due to reduced production; and (2) indirect losses from costs of FMD control, poor access to markets and limited use of improved production technologies (**Figure 1.2.1.3**). The annual impact of FMD in terms of visible production losses and vaccination in endemic regions alone amount to between US$6.5 and 21 billion. In addition, outbreaks in FMD free countries and zones cause losses of >US$1.5 billion a year. The impacts of FMD are not the similar throughout the world. FMD production losses have a big impact on the world's poorest where more people are directly dependent on livestock. FMD reduces fertility of breeding animals leading to less efficient herd structures and discouraging the use of FMD susceptible, high productivity breeds. Overall the direct losses limit livestock productivity affecting food security. In countries with ongoing control programs, FMD control and management creates large costs. These control programs are often difficult to discontinue due to risks of new FMD incursion. In FMD free countries outbreaks occur periodically and the costs involved in regaining free status have been enormous (Knight-Jones & Rushton, 2013).

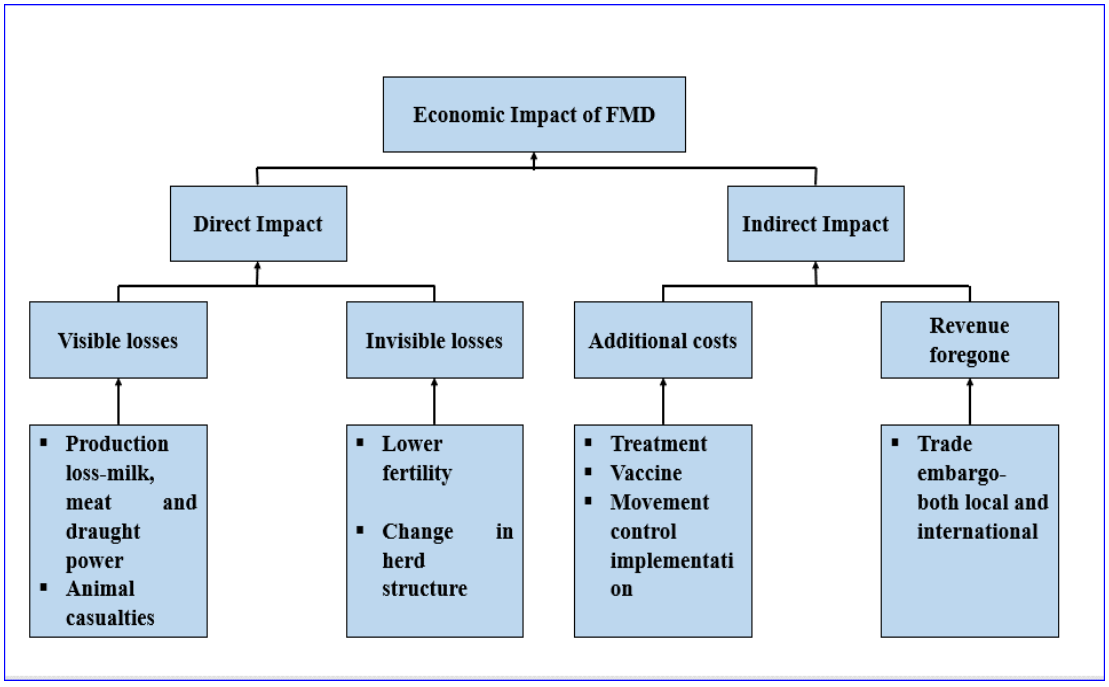


Figure 1.2.1.3: Economic impact of FMD outbreaks

Morzaria (2010) reported the total economic loss due to FMD is 60 million US dollar per year in Bangladesh and in India it is 4.45 billion per year (Morzaria, 2010). Department of

Livestock Services (DLS), Bangladesh and FAO jointly estimated that the annual loss in Bangladesh due to FMD is 125 million USD (DLS, 2014).

1.2.2 Foot-and-Mouth Disease Virus

The causal agent of FMD is foot-and-mouth disease virus (FMDV), a prototype member of the genus *Aphthovirus* under the family *Picornaviridae* (Ma *et al.*, 2014; Sobrino *et al.*, 2001). It is one of the smallest animal viruses. FMDV is the first animal pathogen that was identified as a virus (Loeffler & Frosch, 1898).

1.2.2.1 Physical Properties of Foot-and-Mouth Disease Virus

The length of FMDV genome is over 8 kb in length, which is contained within a icosahedral symmetry capsid (R. Acharya, Fry, E., 1989). The RNA on its own is infectious if inside a cell. The virus is sensitive to acidic conditions (<pH6.0) (Randrup, 1954), temperatures above 50°C and UV light, but persistence in contaminated fodder and the environment for up to 1 month is possible in temperate climates.

1.2.2.2 Virion Structure

FMDV virion is 27-28nm in diameter with spherical shape and icosahedral symmetry. The virion consists of 70% protein, 30% RNA and a small amount of lipid (Putnak & Phillips, 1981). The viral genome has approximately 8000-8200 bases. Sizes of the virion proteins are: VP1(1D) = 29 nm, VP2(1B) = 30 nm, VP3(1C) = 22 nm, and VP4(1A) =8 nm (M. J. Grubman & Baxt, 2004). The sedimentation constant of virions in sucrose gradients is 146S, which is widely used in vaccine manufacturing for the determination of intact virions (Barteling & Vreeswijk, 1991). The capsid proteins of FMDV are composed of 60 copies of the 4 structural proteins VP1, VP2, VP3, and VP4 of FMDV and display an icosahedral structure (Logan *et al.*, 1993). One copy of VP1, VP2, VP3, and VP4 proteins forms a protomer; 5 protomers assemble into a pentamer; and 12 pentamers form a complete viral capsid (Palmenberg, 1990) (**Figure 1.2.2.2**). The most obvious feature of the surface of FMDV particles is a surface-protruding G-H loop formed by the βG and βH chains of residues 140–160 of VP1. The G-H loop contains a highly conserved arginine-glycine-aspartate (RGD) sequence. This sequence is an important neutralizing site of FMDV (Bittle *et al.*, 1982); this sequence is also the primary component of the cell adsorption site and can interact with integrin receptors on the cell membrane to mediate the initiation of virus infection (Logan *et al.*, 1993). Except for VP4, the capsid proteins VP1, VP2, and VP3 are all associated with antigenicity and can interact with the RGD-

directed integrin protein subunits and with heparan sulfate proteoglycans (HSPGs) on the cell surface. The variations of the amino acid residues in FMDV capsid proteins that form viral particles not only can affect the adsorption ability between viruses and cell receptors but also may affect the biological characteristics of viruses (Bittle *et al.*, 1982). Elements of the attachment site for FMDV involving the G-H loop (residues 134-160) and C-terminus (200-213) of VP1 are exposed on the surface. Moreover, this G-H loop, which is a major antigenic site of FMDV, forms a prominent, highly accessible protrusion, a feature not seen in other picornaviruses. It is this loop that is perturbed in the variant viruses. Thus, this virus seems to use a novel escape mechanism whereby an induced conformational change in a major antigenic loop destroys the integrity of the epitope (Parry *et al.*, 1990).

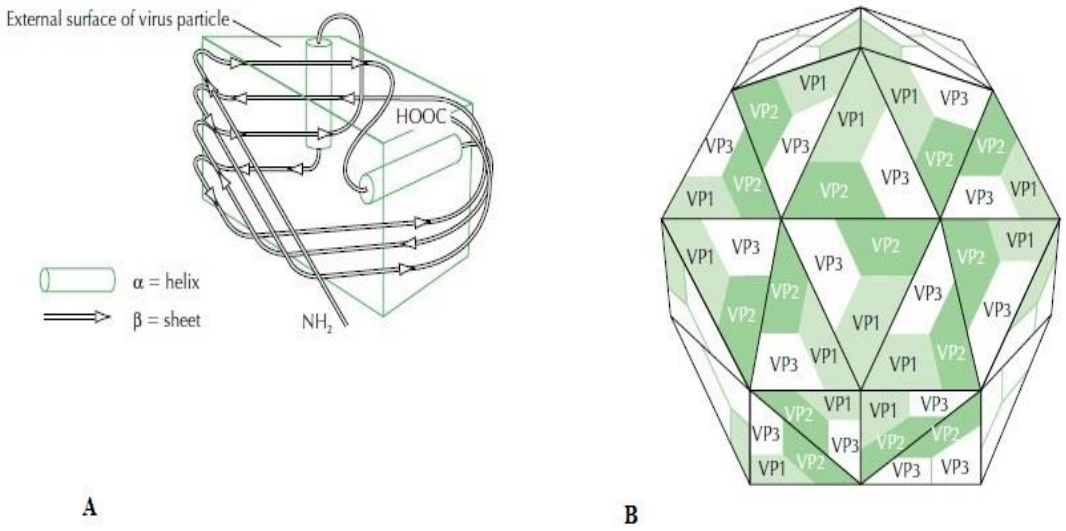


Figure 1.2.2.2: Structural presentation of the Picornavirus capsid proteins. A: The 'eight-strand antiparallel b-barrel' subunit structure found in all T = 3 icosahedral RNA virus capsids. B: Picornavirus particles are icosahedral structures with triangulation number T = 3. Three virus proteins (VP1, 2, and 3) comprise the surface of the particle. A fourth protein, VP4, is not exposed on the surface of the virion but is present in each of the 60 repeated units that make up the capsid (Cann, 2005).

1.2.2.3 FMDV Genome

The genome of picornaviruses functions as a messenger (+) RNA, polyadenylated at its 3′ end and covalently linked to a small protein (VPg) at its 5′ end. The genome organization of FMDV is similar to that of other picornaviruses, including a large single open reading frame (ORF) flanked by highly structured 5′ and 3′ untranslated regions (5′ UTR and 3′ UTR, respectively). FMDV genomes are over 8 kb in size, where most variability in genome size is due to insertions/deletions in the UTRs (Carrillo *et al.*, 2005). Translation

occurs as a single polypeptide precursor (ORF) that is cleaved into functional proteins, mostly by virus encoded proteases (E. Domingo *et al.*, 2002). Primary processing of the FMDV ORF results in three large intermediate polyproteins L/P1, P2 and P3. Protease cleavage by FMDV proteins L, 3C and 2A produces smaller sub products and 12 final mature proteins L, 1A, 1B, 1C, 1D, 2A, 2B, 2C, 3A, 3B, 3C and 3D.

1.2.2.3.1 5′ UTR

The 5′ UTR is approximately 1300 bases in length, which plays important roles in FMDV replication. However, the specific role of S fragment, poly(C) tract, and L fragment pseudoknots to FMDV biology is still unknown.

The first element of the 5′ UTR is known as the S-fragment, consisting of a long stem-loop that is presumably involved in genome replication and stability.

The cardiovirus poly(C) tract has been shown to affect virulence (Duke *et al.*, 1990), while shortening of the FMDV poly(C) tract affected virus growth in vitro but not virulence in a suckling mouse model (Rieder *et al.*, 1993).

Down-stream of a poly(C) tract and a pseudoknots region (Carrillo *et al.*, 2005; P. W. Mason *et al.*, 2003), there is a cis-active replicative element (cre) that consists of a highly conserved stem-loop known to be essential for FMDV genome replication (P. W. Mason *et al.*, 2003).The cre is essential for picornaviral replication and contains a conserved AAACA motif which in poliovirus functions as a template for $3D^{pol}$-mediated uridylylation of 3B (Murray & Barton, 2003). Mutation of the FMDV cre stem region reduced virus RNA replication (P. W. Mason *et al.*, 2002).

A type II internal ribosome entry site (IRES) constitutes the last element of the 5′ UTR and is responsible for initiating the cap-independent translation of the viral RNA. The FMDV IRES contains four structural domains that interact with cellular translation initiation factors and participate in RNA–RNA interactions. Among these domains, the central domain (known as domain 3) is critical for IRES activity (Garcia-Nunez *et al.*, 2014). The approximately 500-nt FMDV IRES, which is responsible for cap-independent polyprotein translation, is predicted to contain four structural domains that interact with cellular factors involved in host translation initiation (Ramos & Martinez-Salas, 1999). Domain 2 binds the polypyrimidine tract-binding protein at a UUUC motif. Domain 3

contains at least three loop structures that are conserved in all picornaviruses, including a GNRA motif essential for the maintenance of the IRES tertiary structure and potentially involved in long-range RNA-RNA interactions. The Y-shaped domain 4 and domain 5 interact with the host translational factors eIF4B and eIF4G (Lopez de Quinto & Martinez-Salas, 2000). The IRES facilitates the initiation of protein synthesis in a CAP-independent pattern, allowing it to mediate ribosome excerpt to an internal site within the viral RNA. This process is facilitated by eukaryotic translation initiation factors (eIFs). Initiation of translation by the IRES begins with specific binding of the central domain initiation factor, eIF4G, to the J-K domains, which is stimulated by eIF4A. Then, these initiation factors induce a restructuration of the region and promote recruitment of ribosomal pre-initiation complexes. PTB and ITAF45 trans-acting factors are also necessary to stabilize the active conformation. Both binding of eIF4G to the IRES and IRES-directed translation are significantly impaired by mutations that impact the integrity of the double-stranded secondary structure. In fact, the primary sequence within the IRES of different FMDV isolates can be up to 50% variable and still retain similar overall secondary structure using compensatory base changes in helical elements.

A 22-nt spacer for ribosomal recognition separates the IRES from the first of two polyprotein start codons, and an additional 84 nt separates the first start codon from the second. Picornavirus utilization of the second start codon seems to be affected by its adjacent sequence and by sequences in the IRES. Unlike poliovirus, FMDV utilizes the second start codon twice as often as the first (Carrillo *et al.*, 2005).

Comparative studies by Carrillo *et al.* (2005) showed average lengths of S fragment of 322 and 373 nt for SAT and Euroasiatic isolates, respectively, and spanning positions 1 to 393 in the 5′ UTR alignment (Carrillo *et al.*, 2005), where L fragment ranged from 604 to 751 nt long. Remarkably, apparently unrelated virus isolates contained identical deletions in the L fragment. Notably, the 22-nt region between the IRES and the first AUG was highly variable, possibly underlying FMDV's preferential use of the second start codon. In summary, the FMDV 5′ UTR, although tolerant for nt substitutions and insertions/deletions, showed significant conservation, especially in the IRES, where novel sequence motifs were identified (Carrillo *et al.*, 2005).

1.2.2.3.2 FMDV Coding Region (ORF)

Protein synthesis starts at one of two functional in-frame AUG codons, separated from each other by an indispensable but highly variable tract of approximately 80 to 84 nucleotides. The long ORF that follows the AUG codon encodes a polyprotein of about 2330 amino acids (aa), although length and composition among natural isolates and even among passaged viruses can be variable.

Leader (L) proteinase: The leader (L) protein, a member of the papain-like cysteine proteinase, is located at 5′ end of the ORF and contains two in-frame initiation codons (84 nt in distance, Lab/Lb), that cleaves itself from the viral polyprotein (Strebel & Beck, 1986) acting as a trans-proteinase and initiation factor eIF4G at G479/R480 resulting the shut-off of host protein synthesis (Glaser *et al.*, 2001). It's also an important determinant of virulence in animals. FMDV L^{pro} is expressed as long (Lab) and short (Lb) isoforms that result from alternative usage of either the first or second start codon, respectively, with the sequence between the two AUGs predicted to form a stable hairpin structure crucial to IRES activity (Hinton *et al.*, 2000). L^{pro} isoforms have indistinguishable activities and specificities and have been proposed to play a role in virulence that may involve the regulation of host interferon responses (Piccone *et al.*, 1995). Carrillo *et al.* (2005) reported that Lb isoform is less conserved and the predicted secondary structure of the terminal regions of L^{pro} remained relatively unaltered. They also predicted that the variability observed at the L^{pro} N and C termini may affect specific virus-host interactions, including ribosomal recognition of alternative start codons (Carrillo *et al.*, 2005).

Structural proteins: The four viral proteins, VP1, VP2, VP3 and VP4 are structural proteins and form the capsid of the virus. These structural proteins are involved in capsid assembly and stability, virus binding to target cells, and antigenic specificity, influencing significant aspects of virus infection and immunity (Jackson *et al.*, 2003). The high level of variability in FMDV external capsid proteins likely reflects the selective pressures on them (Carrillo *et al.*, 2005).

(i) 1A (VP4): Picornaviral 1A is cleaved from the 1AB (VP0) precursor by unknown mechanisms, and this cleavage is required for virion maturation and infectivity (Moscufo *et al.*, 1991). During poliovirus entry into host cells, 1A is involved in receptor mediated capsid conformational changes resulting in membrane ion channel formation and the

release of viral RNA into the cytoplasm. (Carrillo *et al.*, 2005). Carrillo *et al.* (2005) also reported 1A as the most conserved FMDV protein, with 81% of the aa being invariant, including the N-terminal myristylation site and a previously identified heterotypic swine and bovine T-cell epitope (Carrillo *et al.*, 2005).

(ii) 1B (VP2): 1B, a protein of 218 or 219 aa, plays a critical role in virion structural stability and maturation. This protein stretches from position 1917-2570 nucleotides in viral genome containing B-C loop, G-H loop, and E-F loop forms the second antigenic site for the virus. An H-rich region at the 1B/1C interphase is proposed to mediate 1B-1C hydrogen bonding and is likely involved in virion sensitivity to acid, a characteristic with implications for virus stability and transmission (R. Acharya, Fry, E., 1989). Carrillo *et al.* (2005) reported that only the N-terminal half of each VP2 T-cell epitope is invariant, while their C-terminal regions are highly variable (Carrillo *et al.*, 2005).

(iii) 1C (VP3): 1C, a protein of 219 to 221 aa, contains important conformational neutralizing epitopes and makes significant contributions to capsid stability (Logan *et al.*, 1993). It also contains G-H loop playing role in antigenicity. The N termini of five 1C molecules contribute to capsid stability (R. Acharya *et al.*, 1990). Carrillo *et al.* (2005) reported 1C as a highly variable protein, with only 39% of the amino acids being invariant (Carrillo *et al.*, 2005).

(iv) 1D (VP1): 1D is the most studied FMDV protein due to its significance for virus attachment and entry, protective immunity, and serotype specificity (Jackson *et al.*, 2003). There are at least 4 G-H loop in FMD virus playing pivotal role in attachment and antigenicity (Bai *et al.*, 2010). Trypsin sensitive neutralizable antigenic sites 1 are located in the well-known regions 133-160 (the G-H loop) and 200-213 (the C-terminus region) of VP1. In the region 43-60 of VP1, amino acid residues 43, 44, 45 and 48 are involved in antigenic site 3. Carrillo *et al.* (2005) showed that 1D ranges in size from 217 to 221 aa, with only 26% invariant 1D residues (Carrillo *et al.*, 2005). Exposure of the 1D N terminus, a region which includes a 10-aa motif which is conserved in several other picornaviruses, is critical for poliovirus entry into the cell. The FMDV 1D sequences studied by Carrillo *et al.* (2005) lacked this N-terminal motif, suggesting that some aspects of viral entry may differ from those of other picornaviruses (Carrillo *et al.*, 2005).

Nonstructural proteins: In general, regions encoding FMDV nonstructural proteins are highly conserved between isolates, exhibiting higher percentages of invariant residues. Carrillo *et al.* (2005) showed that the most conserved FMDV regions were those encoding 2B and 3C, which exhibited the highest percentage of invariant nucleotides and amino acids in the genome. Notably, the nonstructural proteins L^{pro}, 3A, and 3B exhibited variability comparable to that of the structural proteins 1B, 1C, and 1D, suggesting that these proteins are subjected to selective pressures distinct from those on the other nonstructural proteins (Carrillo *et al.*, 2005).

(i) 2A: FMDV 2A is an 18-aa peptide similar to the C terminus of cardiovirus 2A, a protein which induces a modification of the cellular translation apparatus resulting in 2A release (Donnelly *et al.*, 2001). The high level of 2A conservation likely reflects structural and functional constraints associated with the small size of the protein (Carrillo *et al.*, 2005).

(ii) 2B: FMDV 2B localizes to sites of virus genome replication in ER-derived vesicles (Tesar *et al.*, 1989). The 2B protein in other picornaviruses enhances membrane permeability, blocks protein secretory pathways, suppresses apoptotic responses by affecting intracellular Ca^{2+} homeostasis, and is implicated in virus-induced cytopathic effects (Doedens & Kirkegaard, 1995). Carrillo *et al.* (2005) predicted a previously undescribed, conserved transmembrane domain between positions 120 and 140, suggesting that 2B is an integral membrane protein and consistent with the proposed localization of 2B to ER-derived vesicles (Carrillo *et al.*, 2005).

(iii) 2C: FMDV 2C is homologous to poliovirus 2C, an ATPase affecting the initiation of minus strand RNA synthesis and whose precursor, 2BC, induces vesicle formation in the cytoplasm (Klein *et al.*, 1999). FMDV 2C localizes to membrane-associated virus-replicating complexes (Tesar *et al.*, 1989). The presence of three NTP-binding domains (GXXXXGK, DXXG and NKXO) in the 2C protein turns this region interacting with nucleic acids and is therefore also implicated in RNA synthesis (Dever *et al.*, 1987; Hodgman, 1988). The protein exhibits motifs characteristic of the helicase superfamily (Gorbalenya, Blinov, *et al.*, 1989; Gorbalenya, Koonin, *et al.*, 1989).

(iv) 3A: FMDV 3A has been implicated in virus virulence and host range, similar to the 3A proteins of other picornaviruses (Lama *et al.*, 1998). Deletions in 3A have been associated with FMDV attenuation in cattle and with the porcinophilic phenotype of O

Taiwan/97 (Beard & Mason, 2000). However, evidence suggests that other viral genetic determinants are necessary for these phenotypes (Knowles *et al.*, 2001). FMDV 3A ranges from 143 to 153 aa, with insertions/deletions preferentially occurring at positions 70 to 110 and 130 to 150 (Carrillo *et al.*, 2005). In fact, 3A is one of the most variable proteins encoded by FMDV, with fewer invariant aa than the variable capsid proteins 1B and 1C and the highly variable L^{pro} (Knowles *et al.*, 2001). The variable nature of 3A suggests that it may be highly informative for epidemiologic or forensic purposes. Additionally, the likely role for 3A in virulence and host range suggests that interactions with host factors underlie 3A's variability and the diversifying selection predicted to act upon it (Carrillo *et al.*, 2005). It has been reported that FMDV 3A protein inhibits the RLR-mediated IFN-β induction, by which the FMDV 3A protein evades the host innate immune system (D. Li *et al.*, 2016).

(v) 3B: 3B gene encodes three forms of the VPg protein. FMDV is the only picornavirus to encode multiple 3B proteins (3B1, 23 aa; 3B2, 24 aa; and 3B3, 24 aa), whose homologue in poliovirus primes genomic RNA synthesis during virus replication (Falk *et al.*, 1992). Carrillo *et al.* (2005) reported 3B3 as highly conserved in all examined isolates, supporting previous experimental evidence indicating that only 3B3 isoform is essential for virus viability (Falk *et al.*, 1992; J. M. Pacheco *et al.*, 2003). 3B1 and 3B2 are more variable, and in fact, contain residues to undergo diversifying selection. The deletion of 3B1 and 3B2 may affect FMDV virulence and host range (J. M. Pacheco *et al.*, 2003). Therefore, 3B1 and 3B2 protein variability reflects host range-specific functions (Carrillo *et al.*, 2005).

(vi) 3C: FMDV $3C^{pro}$ is a 213-amino-acid serine proteinase involved in the cleavage of most viral precursor proteins and of host factors associated with translation (eIF4A and eIF4G) and transcription (histone H3). It catalyzes ten of the thirteen cleavages necessary to complete FMDV polyprotein processing. Its protease activity also affects host cell transcription since $3C^{pro}$ is responsible for the cleavage of the cellular histone H3 as well as the elongation factors eIF4G and eIF4A, resulting in cessation of host cell transcription. A previous mutational analysis of $3C^{pro}$ identified the catalytic triad residues C163, H46, and D84 and residues critical for proper protein folding, i.e., D84 and Y136. A high degree of overall $3C^{pro}$ conservation indicates a limited tolerance for mutations, likely due to significant structural/functional constraints (Carrillo *et al.*, 2005).

(vii) 3D: The 469-amino-acid viral RNA-dependent RNA polymerase $3D^{pol}$, responsible for generating minus- and plus-sense genomic RNA, is one of the most conserved FMDV proteins. Analysis by Carrillo *et al.* (2005) indicated that although it is conserved, $3D^{pol}$ is more tolerant of substitutions, as their results extended the proportion of variable residues from 8.6% to 26%. D245, N307, and G295, which are essential for maintaining the functional integrity of the picornaviral polymerases (Koonin, 1991), were invariant in FMDV $3D^{pol}$, along with other residues described as being highly conserved among all RNA-dependent RNA polymerases (George *et al.*, 2001), including the NTP-binding residues G337, D338, and D339. There are three hypervariable, hydrophobic antigenic regions in $3D^{pol}$ (aa 1 to 12, 64 to 76, and 143 to 153) (Carrillo *et al.*, 2005; George *et al.*, 2001).

1.2.2.3.3 3′ UTR

The 3'-UTR, a region of about 90 nt of heterogeneous sequence, is a highly ordered structure, and stimulate the cap-independent translation and likely affect other aspects of viral infection cycle (Bai *et al.*, 2010). The picornavirus 3′ UTR binds several viral and host proteins and is believed to contain structural cis-acting elements required for negative-strand RNA synthesis (Agol *et al.*, 1999). Removal of the terminal poly(A) tract or mutagenesis of structural elements abrogates the infectivity of FMDV infectious clones (M. Saiz *et al.*, 2001). FMDV 3′ UTR sequences stimulate virus-specific, IRES-dependent translation (P. W. Mason *et al.*, 2002) and likely affect other aspects of viral replication, including genome circularization. The FMDV 3′ UTRs are highly variable in length among different, as reported by Carrillo *et al.* (2005). A secondary structure analysis of the FMDV 3′ UTR by them also confirmed the Y shape which is also predicted for other picornaviral 3′ UTRs, suggesting that the structure plays an important role in the 3′ UTR function. (Carrillo *et al.*, 2005).

1.2.3 Classification of Foot-and-Mouth Disease Virus

The FMDV exists as seven immunologically distinct serotypes namely A, O, C, Asia1 and the Southern African Territories (SAT)-1, SAT-2 and SAT-3 based on the nucleotide sequence of the VP1 (1D) structural protein. Each serotype further contains multiple subtypes or topotypes that are usually related to geographical region of the disease occurrence (Bachrach, 1968; E. Domingo *et al.*, 2002; Knowles & Samuel, 2003).

Serotype O: Serotype O is the most prevalent of the seven serotypes of FMDV and (Samuel & Knowles, 2001) and is classically divided into 10 or 11 antigenic subtypes. The genetic diversity of serotype O is much greater, allowing the classification of many distinct lineages (Knowles & Samuel, 2003). Researchers have identified eight distinct genetic lineages, which fall into geographically distinct regions. During the last 10-12 years, 2 prominent lineages have been found circulating in Indian region: PanAsia and Ind2001. A new genetic group named as Ind2011 under serotype O appeared in 2011 in India. Geographically, the Ind2011 lineage was restricted to the southern regions of India in the states of Karnataka, Tamilnadu, Andhra Pradesh, and Kerala (Subramaniam *et al.*, 2015). Moreover, there are 11 topotypes of FMDV serotype O with 15% nucleotide differences with each other have been reported namely Europe -South America (Euro-SA), Middle East-South Asia (ME-SA), South-East Asia (SEA), Cathay, West Africa (WA), East Africa-1 (EA-1), East Africa-2 (EA-2), East Africa-3 (EA-3), East Africa-4 (EA-4), Indonesia-1 (ISA-1) and Indonesia-2 (ISA-2).

Serotype A: Serotype A viruses is extremely diverse, both antigenically and genetically, such that in early 1970s, 32 subtypes had been described (Knowles & Samuel, 2003). Three topotypes-ASIA, AFRICA, and EURO-SA (Europe–South America) and multiple diverse lineages and sublineages have been identified for serotype A. The ASIA topotype is widespread and is found in most countries in Asia with sporadic incursions into North Africa. Although the G-VII lineage (also known as genotype 18) usually circulates in countries containing virus pool 2 (commonly in Bangladesh and India, rarely in Bhutan and Nepal, but until now not in Sri Lanka), this lineage has also been reported in Saudi Arabia in 1995, 2015 and 2016 (Bachanek-Bankowska *et al.*, 2018) and , Albania and the former Yugoslav Republic of Macedonia in 1996, Myanmar in 2010. In India, viruses of the A/ASIA/G-VII lineage were isolated in 1983 and until 2001 were co-circulating with the A/ASIA/G-VI lineage. After 2001, only the G-VII lineage has been reported.

Serotype C: Type C appears to have become confined to the Indian sub-continent and was identified by Waldmann & Trautwein in 1926. Historically, it has been recorded in Europe, South America, EA, North Africa, Angola and southern Asia and was not recorded since 1996, but it was believed that type C still in circulation in India (FMDV conf. India 2012). More recent studies have shown that comparisons of partial VP1 sequences can be used to

classify FMD type C viruses into eight topotypes: EuroSA, Angola, Philippines, ME-SA, Sri Lanka, EA and Tajikistan.

Serotype Asia1: FMD serotype Asia 1 was first identified from a sample originating from Pakistan in 1957 (JoB Brooksby, 1958). This is unique to Asian region where it causes only a small proportion of FMD cases (J. K. Mohapatra *et al.*, 2008). Even though a wide variation were observed in VP1 nucleotide sequences, it was not sufficient to classify Asia 1 viruses into more than one topotype (Knowles & Samuel, 2003). A recent study on viruses belonging to serotype Asia 1 from outbreak during 2003 to 2007 in Asian countries has characterized these viruses into six different groups, I to VI (Valarcher *et al.*, 2009).

Serotype SAT-1, SAT-2 and SAT-3: Several African field strains collected since 1931 were re-examined by Brooks in 1948 who demonstrated a new strain from the South African Territories (SAT 1). Two more strains from Southern Africa (SAT 2 and SAT 3) were also identified (JB Brooksby, 1982). Five different topotypes have been identified for SAT to date, namely: South Africa, Zimbabwe, Zambia, Namibia, Botswana, Malawi, and Uganda. Three of the five topotypes occur within different regions of Zimbabwe, whilst the remaining countries have a single topotype within their borders. (Vosloo *et al.*, 2002).

1.2.4 Distribution of Foot-and-Mouth Disease Virus

1.2.4.1 World Distribution of Foot-and-Mouth Disease Virus

FMD is endemic in India and occurs both in cattle and buffalo. The outbreaks in India are ascribed to serotypes A, O and Asia-1 of which serotype O is predominant (Maroudam *et al.*, 2010). FMD has also been endemic in several parts of the world, particularly in Asia, South Africa, the Middle East, and South America. The sporadically occurrence of some serotypes in various regions of the world is shown in **Figure 1.2.4.1a** (FAO, 2018). The serotype O is the most distributed strain in many countries (Kitching, 1999). In South East Asia (SEA) countries, Brunei, Indonesia, East Malaysia and Singapore are recognized internationally free of the disease without vaccination while China, Philippine, Thailand are very prone to FMD outbreaks. The Republic of Korea had been free from foot and-mouth disease (FMD) since 1934, until outbreaks were reported in 2000. Korea then remained free from FMD until 2002, when a further outbreak in a pig farm occurred (Oem *et al.*, 2008). In the year 2005, FMDV Asia1 reemerged in China. In 2010, outbreaks of

of FMD were recorded in Southern Cambodia, Iran, and Republic of Korea (Rashtibaf *et al.*, 2012; Yoon *et al.*, 2012; Young *et al.*, 2013). Also, during 2010 and 2011, there have been reports of FMD outbreaks that occurred in East Asia affected the People's Republic of China, Republic of Korea, Japan, Mongolia, the Russian Federation, and North Korea (Ma *et al.*, 2014). In India, the most important event was the re-emergence of lineage O/ME-SA/Ind-2001 in 2008. Notably, this lineage, normally prevalent in India, Bangladesh, Nepal and Bhutan, although found in Saudi Arabia and Libya in 2013; and in Tunisia and Algeria in 2014–2015. FMD outbreaks in wild boars were found in Bulgaria in January 2011, where the disease was last recorded in 1996. Subsequently, 12 outbreaks in livestock by FMDV O/ME-SA/PanAsia2 were recorded. Egypt and the Palestinian Autonomous Territories also experienced FMD outbreaks by SAT2 in 2012. Another noticeable event was the emergence of FMDV Asia1 Sindh-08 in the Middle East. In South America, one outbreak of FMDV serotype O, topotype Euro-SA was reported in Paraguay in 2011, which was recognized as FMD-free with vaccination at the time. (Brito *et al.*, 2015).

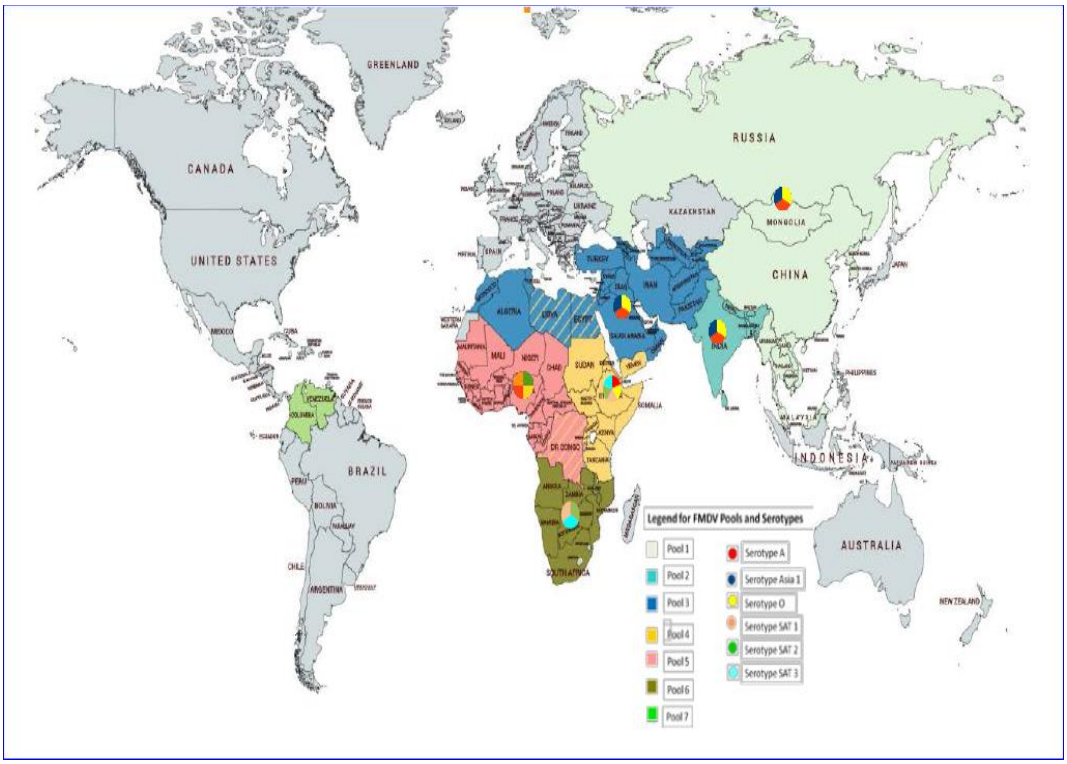


Figure 1.2.4.1a: The Situation of FMD serotypes outbreaks during 2013-2017; Source: FAO (2018).

The FMD viruses circulating all over the world are divided into 7 different pools. The distribution of virus pools is depicted in **Figure 1.2.4.1b** (FAO, 2018).

POOL	REGION/COUNTRIES – colour pools as in Map	SEROTYPES
1	SOUTHEAST ASIA/CENTRAL ASIA/EAST ASIA Cambodia, China, China (Hong Kong, SAR), Taiwan Province of China, Democratic People's Republic of Korea, Republic of Korea, Laos People's Democratic Republic, Malaysia, Mongolia, Myanmar, Russian Federation, Thailand, Viet Nam	A, Asia 1 and O
2	SOUTH ASIA Bangladesh, Bhutan, India, Mauritius, Nepal, Sri Lanka	A, Asia 1 and O
3	WEST EURASIA & MIDDLE EAST Afghanistan, Algeria, Armenia, Azerbaijan, Bahrain, **Egypt**, Georgia, Iran (Islamic Republic of), Iraq, Israel, Jordan, Kazakhstan, Kuwait, Kyrgyzstan, Lebanon, **Libya**, Morocco, Oman, Pakistan, Palestine, Qatar, Saudi Arabia, Syrian Arab Republic, Tajikistan, Tunisia, Turkey, Turkmenistan, United Arab Emirates, Uzbekistan	A, Asia 1 and O
4	EASTERN AFRICA Burundi, Comoros, **Democratic Republic of Congo**, Djibouti, **Egypt**, Eritrea, Ethiopia, Kenya, **Libya**, Rwanda, Somalia, Sudan, South Sudan, United Republic of Tanzania, Uganda, Yemen	O, A, SAT 1, SAT 2 and SAT 3
5	WEST/CENTRAL AFRICA Benin, Burkina Faso, Cameroon, Cabo Verde, Central Afr. Rep., Chad, **Democratic Republic of Congo**, Congo, Côte d'Ivoire, Equatorial Guinea, Gabon, Gambia, Ghana, Guinea-Bissau, Guinea, Liberia, Mali, Mauritania, Niger, Nigeria, Sao Tome Principe, Senegal, Sierra Leone, Togo	O, A, SAT 1 and SAT 2
6	SOUTHERN AFRICA Angola, Botswana, **Congo D. R.,** Malawi, Mozambique, Namibia, South Africa, Zambia*, Zimbabwe	{O, A}*, SAT 1, SAT 2 and SAT 3
7	SOUTH AMERICA Colombia, Venezuela (Bolivarian Republic of)	O and A

Egypt, Libya and **Democratic Republic of Congo** (highlighted in bold) are indicated as being in multiple pools, since they have evidence of FMDV originating from two or more pools. * ONLY IN NORTH ZAMBIA AS SPILL-OVER FROM POOL 4

Figure 1.2.4.1b: List of countries representing each virus pool for the period 2013 – 2017; Source: FAO (2018).

1.2.4.2 Foot-and-Mouth Disease Virus in Bangladesh

FMD was first officially documented in 1958 in Bangladesh (Pirbright Laboratory 2010) (**Figure: 1.2.4.2)** during extensive outbreaks in many parts of the country.

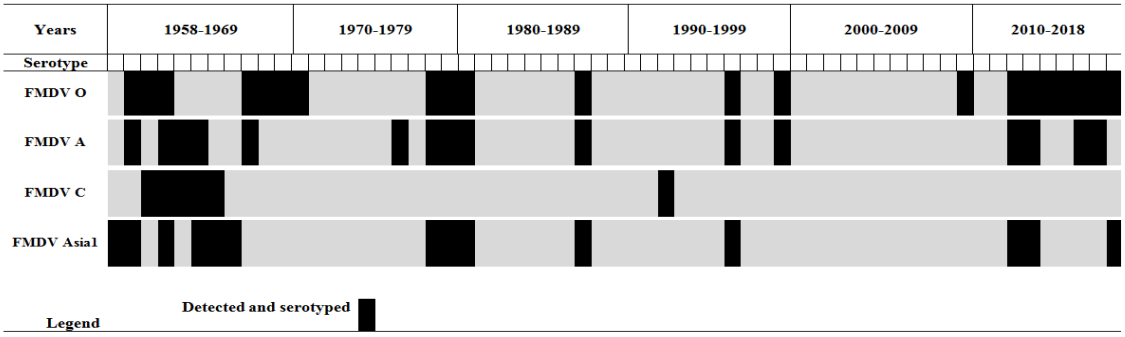


Figure: 1.2.4.2: Occurrence of FMDV serotypes in Bangladesh.

FMD still remains endemic in Bangladesh and the disease occurs throughout the year in all parts of the country with the predominance of FMDV serotype O (80%–85%) followed by serotype A (10%–15%) and Asia1 (up to 5%) (Siddique *et al.*, 2018). Serotype O virus was first isolated between 1987 and 1997 in Bangladesh (Freiberg *et al.*, 1999), again between 1998 and 2000 (Islam *et al.*, 2001), 2008-2008 (Loth *et al.*, 2011) and between 2011 and 2013 (Nandi *et al.*, 2015; Sultana *et al.*, 2014; Ullah *et al.*, 2014). The phylogenetic reconstruction showed that all isolates of 2008 and 2009 belonged to ME-SA topotype, but fell into two distinct sublineages, one named Ind-2001 (the other has not been named). Within both sublineages, the isolates Bangladesh in 2009 were closely related to viruses from Nepal collected during 2008 to 2009. Additionally, both sublineages contained older viruses from India collected in 2000 and 2001(Loth *et al.*, 2011). Sequencing and phylogenetic analysis of VP1 sequences of 2011-2013 isolates revealed that serotype O sequences were closely related to the Ind2001 sublineage of Middle East–South Asia (ME-SA) topotype that was previously circulating in Bangladesh (Nandi *et al.*, 2015). A further study based on VP1 coding region sequences analysis of FMD samples collected during 2012–2016 in Bangladesh revealed that FMDV serotype O was responsible for 82% of the outbreaks in Bangladesh, demostrating its dominance over serotype A and Asia1. During this period two novel sublineages of serotype O, named as Ind2001BD1 and Ind2001BD2 within the Ind2001 lineage also emerged in Bangladesh as well as the circulation of Ind2001d sublineage also continued in the area. The sublineage Ind2001BD1 showed its dominance over other sublineages and majority of the recent isolates of FMDV serotype O were found to be under this sublineage (Siddique *et al.*, 2018).

Serotype A viruses were also reported in Bangladesh simultaneously with serotype O (Freiberg *et al.*, 1999; Islam *et al.*, 2001; Nandi *et al.*, 2015; Siddique *et al.*, 2018). FMDV Serotype A viruses reported during different time period in Bangladesh belonged to the genotype VII of ASIA topotype that was dominant in India during the last decade (Nandi *et al.*, 2015).

Circulation of FMDV Asia-1 serotype in Bangladesh is episodic. The serotype was first isolated in Bangladesh between 1987 and 1996 (Marquardt *et al.*, 2000), between 1996 and 2000 (Islam *et al.*, 2001) and again between 2012 and 2013 (Ullah *et al.*, 2015). The VP1 phylogeny showed that the sequences of all local circulatory serotype Asia1 collected

during the year 2012–2013 clustered within the genetic lineage C which re-emerged in India in 2005 (Ullah *et al.*, 2015).

1.2.5 Identification of Foot-and-Mouth Disease Virus

Complement fixation test (CFT) was the earliest laboratory test used to detect FMDV, but was replaced later by serotype specific antigen detecting Enzyme Linked Immunosorbent Assay (ELISA) due to its better sensitivity and specificity (Ferris *et al.*, 1988). Subsequently molecular methods like conventional RT-PCR assays had been developed and optimized which also could provide serotype-specific results. But one limitation of molecular method was its inability to analyze large number of samples at a time. To overcome this limitation, real time RT-PCR was developed and shown to have high sensitivity and specificity for the detection of FMDV genomes of all seven serotypes (Reid *et al.*, 2002). Real time RT-PCR has been found effective on a large number of different types of samples. More recently, another antigen detection method- "Lateral Flow Device" (LFD) has been evaluated and shown to be pan-reactive to all FMDV serotype except for serotype SAT 2. The technique is easy and rapid, and has the potential to be used for pen-side diagnosis for FMD suspected outbreak (Ferris *et al.*, 2009).

1.2.6 Nucleotide Sequencing

As FMD virus genome consists of RNA, it is usually first transcribed into cDNA prior to performing the nucleotide sequence. Combination of reverse transcription (RT) with PCR technique provides a rapid and powerful tool for studying diverse RNA genomes. Nucleotide sequencing of the VP1 protein coding gene has been used extensively for studying molecular epidemiology as well as to determine the relationships among the field isolates of FMDVs. The technique is also deployed for investigating genetic variation, molecular evolution in carrier animals, and to identify the source of an infection in outbreak conditions (Bastos, 1998; Esteban Domingo *et al.*, 1985; DoPAZO *et al.*, 1988; Sáiz *et al.*, 1993). The first genetic relationships among the FMD virus serotypes A, O, and C as well as their subtyping were also accomplished by VP1 gene sequence analyses (Beck & Strohmaier, 1987). The authors also demonstrated that a single nucleotide change could be detected by analyzing the nucleotide sequences of a virus isolate. Subsequent studies using this gene sequence based molecular approach have provided crucial epidemiological insights (Sáiz *et al.*, 1993), the identification of trans-boundary virus

transmission (Sáiz *et al.*, 1993; Samuel *et al.*, 1999), and evidence of prolonged persistence of a particular virus type in the field (Freiberg *et al.*, 1999; Samuel *et al.*, 1999). Sequence data has also been instrumental in identifying outbreaks resulting from inadequately administered inactivated vaccines (Beck & Strohmaier, 1987; Suryanarayana *et al.*, 1998).

1.2.7 Control of Foot-and-Mouth Disease

FMD control in endemic areas is implemented by diagnostics, surveillance and regular mass vaccination. In FMD-free areas, control strategies include depopulation of infected and in-contact animals, together with restrictions on movement of animals and animal products. There has been also provisions of emergency vaccination when the outbreak is extensive and timely depopulation of large numbers of animals becomes unmanageable (Vannie *et al.*, 2007). However, currently available FMDV vaccines confer protection against clinical FMD although they do not prevent primary infection of the nasopharyngeal mucosa (Cox *et al.*, 2006; Stenfeldt, Diaz-San Segundo, *et al.*, 2016) and persistent FMDV infection occurs at similar prevalence in vaccinated and naïve cattle (Cox *et al.*, 2006; J.M. Pacheco *et al.*, 2015; Stenfeldt, Pacheco, *et al.*, 2016). Thus, vaccination is an efficient measure to prevent clinical FMD and has proven to be a highly useful strategy in preventing dissemination of FMD outbreaks (Parida, 2009), as shown during the 2001 FMD outbreak in Europe when The Netherlands opted to vaccinate all FMDV susceptible species, but vaccinated animals were killed ("vaccination-to-kill policy") to regain FMD-free status and lift trading restrictions more rapidly. However, establishment of 'vaccination-to-live' policies still encounter impediment mainly due to susceptibility of vaccinated cattle to become persistently infected. Nevertheless, there has been recently a growing demand for 'vaccination-to-live' policies as an alternative to large-scale depopulation for the control of disease. As a consequence, new regulations have been approved by the OIE and the FAO, and currently affected countries can regain FMD free status 6 months after the report of the last case following the use of the 'vaccinate- to-live' policy, rather than the one year period previously established (Diaz-San Segundo *et al.*, 2017).

FAO and OIE jointly have initiated a working tool for assisting and facilitating FMD endemic countries or regions to progressively reduce the impact of FMD and the load of FMD virus. The tool is known as 'The Progressive Control Pathway for Foot and Mouth Disease (PCP-FMD)'. The tool designed several stages assigning a definite goal (**Figure**

1.2.7a). FAO also planned a tentative time-frame for each of the FMD endemic country for fulfilling the defined goal. As an FMD endemic country, there is also a time frame for Bangladesh to attain the defined goal. As prescribed by FAO, eventual progression to Stage 2 is the logical goal of Bangladesh by the year 2019 (**Figure 1.2.7b**). The goal of stage 2 is implementation of risk-based control for FMD outbreak and vaccination is to be started in this stage. However, countries have the freedom not to progress further than Stage 2 or 3, both of which provide sustainable management of FMD to a certain level. Eventually vaccination is the core step either for control and management of FMD to a certain level or complete eradication.

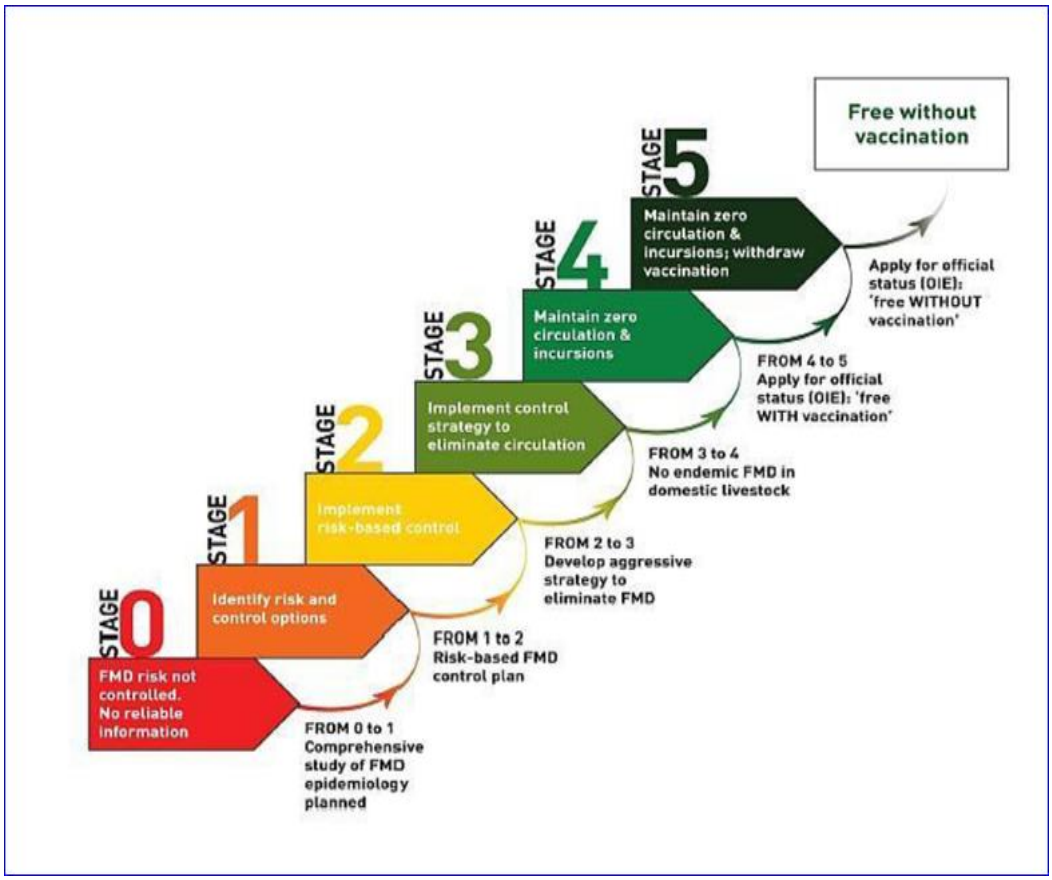


Figure 1.2.7a: The Progressive Control Pathway for FMD control (PCP-FMD) stages (adapted from FAO).

Country	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
Afghanistan*	1	2	2	2	3	3	4	4	4	4
Bangladesh	1	1	1	1	1	2	2	2	2	3
Bhutan	1	1	1	1	1	2	2	2	2	3
India	3	3	3	3	4	4	4	4	4	4
Nepal	1	1	1	1	2	2	2	2	2	3
Pakistan*	1	1	1	2	2	2	2	2	3	3
Sri Lanka	1	1	2	2	3	3	4	4	4	5

Figure 1.2.7b: Time frame for attaining the goal assigned by FAO for Bangladesh (marked area) (adapted from FAO).

1.2.8 Foot and Mouth Disease Vaccines

FMD vaccines were among the first animal vaccines to be developed, with efforts to immunize animals by exposure to infectious virus beginning at the end of the 19th century (M. Lombard *et al.*, 2007). But a practical vaccine was never realized due to the unpredictability of viral virulence and the existence of multiple viral serotypes (Vallée, 1922). The first inactivated vaccine was developed by Waldmann et al. around 1937 using vesicular fluid obtained from tongues of deliberately infected cattle, and subsequently inactivated with formaldehyde (Waldmann & Zimmermann, 1955). But industrial production of inactivated vaccines did not begin until the 1950s after Frenkel described the culture of tongue epithelium from healthy slaughtered animals (M Lombard *et al.*, 2007). Further breakthroughs in inactivated FMD vaccine production included the growth of FMDV in BHK cell suspension cultures in the 1960s (Capstick *et al.*, 1962), the introduction of ethylene imines for FMDV antigen inactivation (H. Bahnemann, 1973; F Brown *et al.*, 1963), and the use of oil-adjuvants in the 1970s (Michelsen, 1961). Besides the FMD inactivated vaccines, novel vaccines that do not use inactivated antigens have been developed but its use is still very limited.

Current inactivated whole virus vaccines: routine vaccination against FMD is usually applied to cattle in countries or zones recognized as “FMD-free with vaccination”, and in countries where the disease is endemic. Current FMD vaccines consist of Binary Ethylenimine (BEI) inactivated purified antigen in formulation with various adjuvants. Vaccines in endemic countries commonly contain more than one serotype of virus, depending upon the epidemiological situation of the particular country. Protective immune

responses require booster vaccination every six months for at least the first two years of age with subsequent annual revaccination (Cox *et al.*, 2006; Parida, 2009).

Novel vaccines: Several novel FMDV vaccines have been introduced by researchers

(i) Recombinant protein and peptide vaccines: by the mid-1970s researchers had developed information concerning the virus capsid structure and determined that VP1 had a prominent surface exposure (Laporte, 1969; Rowlands *et al.*, 1971). Based on this information a number of strategies were utilized to develop protein vaccines as alternatives to the inactivated vaccine. VP1 protein produced by recombinant DNA technology in E. coli protected both swine and cattle from virus challenge (Kleid *et al.*, 1981). Bittle et al. (Bittle *et al.*, 1982) synthesized peptides corresponding to G–H loop and C-terminal regions of VP1 and demonstrated that the peptides could induce high levels of neutralizing antibody in cattle and protected guinea pigs from challenge. Additional studies combining these peptides with peptides that represented FMDV T-cell epitopes showed that the T-cell peptides were recognized by a significant number of cattle and pigs. Large-scale evaluation of peptide vaccines revealed that this type of vaccine is not able to induce significant protection in cattle (Diaz-San Segundo *et al.*, 2017)

(ii) Empty capsid vaccines: an alternate approach to protein or peptide vaccines is the development of immunogens that contain the entire repertoire of immunogenic sites present on the intact virus particle thereby decreasing the possible selection of antigenic variants from the quasispecies. Empty viral capsids are virus particles lacking nucleic acid which are naturally produced in infected cells and are as immunogenic as virions (E. Domingo *et al.*, 1990). Utilizing various expression systems empty capsids were synthesized and assembled in cell culture. Subsequently a number of approaches have been used to deliver the products either as proteins expressed in *E. coli* (M. J. Grubman *et al.*, 1993) or recombinant baculoviruses (J.-C. Saiz *et al.*, 1994) or by direct intramuscular inoculation of recombinant vectors including naked DNA (Chinsangaram *et al.*, 1998), poxviruses (Berinstein *et al.*, 2000; Sanz-Parra *et al.*, 1998), and human adenovirus (Mayr *et al.*, 1999; Mayr *et al.*, 2001). Thus far the most successful strategy has been the delivery of the FMDV capsid sequence with a recombinant, replication-defective human adenovirus type 5 (Ad5). In efficacy studies an Ad5 vector containing the capsid and 3Cpro coding regions of FMDV protected swine (Moraes *et al.*, 2002) and cattle (Juan M Pacheco *et al.*, 2005) from direct inoculation challenge.

(iii) **Live attenuated vaccines:** It has been shown that FMD viruses lacking the leader protease coding region (leaderless) are attenuated in vivo, and use of this virus after chemical inactivation was as effective as the wild-type (WT) inactivated antigen (Chinsangaram *et al.*, 1998; P. Mason *et al.*, 1997). Taking advantage of this knowledge, Uddowla and coworkers further developed leaderless FMDV with mutations in 3B and 3D and leaderless FMDV inactivated vaccine in formulation with adjuvant fully protected cattle against challenge (Uddowla *et al.*, 2012). A newly developed marker vaccine with deletion of some residues in the 3A has been reported to protect swine against challenge with homologous wild type FMDV (P. Li *et al.*, 2014), although this vaccine candidate has never been tested in cattle.

1.2.9 Development of Inactivated FMD Vaccine

As inactivated vaccines evolved, several problems manifested themselves, such as the incomplete viral inactivation of formaldehyde treated antigens. This problem was solved by the introduction of BEI inactivated antigens. In early vaccine production systems concentration of the antigen was achieved through the use of aluminum hydroxide gel adsorption, or polyethylene glycol precipitation. Although these methods are effective and still in use in some parts of the world, they have been largely replaced by industrial ultra-filtration and chromatography in order to remove unwanted cellular protein contaminants and viral non-structural proteins (Rodriguez & Grubman, 2009). At the beginning of the 21st century the protocol for production of inactivated FMD vaccines incorporated the use of serological tests for differentiating infected from vaccinated animals, formulation of vaccines by multiple serotypes and subtypes and a number of adjuvants (Doel, 2003). However, the basic technology for vaccine production has remained the same, still requiring the growth of large volumes of virulent FMDV, subsequent virus inactivation and antigen concentration and purification (Rodriguez & Grubman, 2009).

1.2.9.1 Vaccine Candidates Selection

Foot and mouth disease (FMD) virus exists as seven serotypes within which are numerous variants necessitating careful selection of vaccine strains. Two dimensional Microneutralization test (2D-MNT) based on the use of polyclonal vaccine antisera is widely used for this selection. However, inherent variability in the matching antisera used makes the tests poorly reproducible and creates difficulties in interpretation. Considering

this limitation Mahapatra *et al.* (2008) introduced monoclonal antibodies based neutralization test but prediction of antigenic match based on monoclonal antibodies reactivity did not correlate closely with the results of a polyclonal antibodies based ‘‘gold-standard’’ 2D-MNT method (Mahapatra *et al.*, 2008). Currently, FMDV capsid sequence based molecular approach is being used for the prediction of vaccine strains (Reeve *et al.*, 2010). ID gene sequence based phylogenetic analysis is carried out for the exploration of FMDV clusters within a serotype and vaccine candidate is primarily selected from the most abundant cluster (Mohapatra *et al.*, 2008). Then genetic distances of predicted and current vaccine strains from circulatory isolates are determined using MEGA program (Mahapatra *et al.*, 2017). Z-test for selection (using MEGA) is carried out for the evidence of significant difference among the vaccine strain and circulatory isolates and the absence of same indicates the selected vaccine candidates will protect the all circulating FMDVs in a region (M. Mahapatra *et al.*, 2015). Besides the mentioned analyses, selection analyses, protein variability analyses and comparative FMDV VP1 nt sequence and 3D protein analyses are also carried out to facilitate vaccine candidate selection (M. Mahapatra *et al.*, 2015).

1.2.9.2 Propagation of Vaccine Candidates in Cell Culture

FMD viruses grow well on BHK-21 cell line enabling large-scale production of antigen with good complement fixing properties (Revenson & Segura, 1963). It has also been reported that with increase in the passage number in BHK-21 clone 13 cell line, the titer of FMDV increased significantly (Sellers, 1955). Nair (1987) reported that the susceptibility and infectivity titers of IBRS-2 and MVPK cell lines were less as compared to BHK-21 cells, and thus had no advantage over BHK-21 cell line for vaccine production (Nair, 1987). For commercial FMD vaccine production both BHK-21 clone 13 adherent and suspension cell lines are used. Though adherent culture in roller bottle yields higher virus titer over suspension culture, the later one facilitates spillover production in vaccine industry (Hassan, 2016). The CPE usually develops within 48 hours, but it can be seen as early as 12 hours post infection as cell detachment and destruction with high virus concentration. If no CPE is observed the cells are frozen and thawed followed by 2 blind passages on fresh cell culture and examined for CPE for another 48 hours (Westbury *et al.*, 1988). Cell culture supernatants from post-infected monolayer are collected either by

treating with 1% chloroform for 1 h and centrifugation (Patil *et al.*, 2002), or by centrifugation only (Daoud *et al.*, 2013).

1.2.9.3 Inactivation of Virus

Complete inactivation of Foot and Mouth Disease virus is a critical requirement in the production of FMD vaccine to ensure the safety of the product (Aarthi *et al.*, 2004). Formaline and aziridine such as acetylethylenimine compounds were widely used for inactivating FMDV. However, both of them have serious drawbacks. Waldmann *et al.*, (1941) showed inactivation plots were not linear and often "tailing off", which may cause incomplete inactivation (Soliman *et al.*, 2013). Some of the outbreaks of FMD in Germany and UK have been attributed to the use of such vaccines (Patil *et al.*, 2002). Aziridine compounds have better inactivation capacity though they are toxic to human and may alter immunogenic property of the immunogen by affecting thermal stability (H.G. Bahnemann, 1975). Bahnemann (1975) used 2-bromoethyleneamine (BEA) in alkaline solution to reduce the toxicity of acetylethylenimine (AEI), which is called binary ethyleneimine (BEI). Binary ethylenimine (BEI) is used in particular for FMD vaccine production as this method circumvents the direct handling of the very toxic other aziridine.

For monitoring the rate and linearity of the inactivation process, Aarthi *et al.*, (2004) plotted the $\log_{10}$ of Plaque Forming Unit (PFU) and Ismail *et al.*, (2013) plotted the $\log_{10}$ Tissue Culture Infective $Dose_{50}$ ($TCID_{50}$) against time and built inactivation curves by regression.

1.2.9.4 Virus Particles Concentration and Quantification

The concentration of inactivated FMD virus particles is one of the crucial steps for efficacious vaccine production. Virus particles can be concentrated by polyethylene glycol (PEG) precipitation or polyethylene oxide (PEO) adsorption (Adamowicz *et al.*, 1974; Wagner *et al.*, 1970). Both PEG and PEO were successfully used to concentrate virus particles on a large scale. Ultrafiltration is an alternative concentration system that is being used currently on industrial scale (Adikane *et al.*, 1997).

Sucrose density gradient analysis is the recommended method to quantify FMD virus particles concentration in spite of its technical complexities (Barasa *et al.*, 2008). To overcome the limitation of the conventional method, Spitteler *et al.* (2011) developed size-exclusion chromatographic method to quantify FMDV particles.

1.2.9.5 Adjuvants and Vaccine Formulation

Nonliving vaccine antigens are usually weak immunogenic and adjuvants are added in the vaccine formulation to increase their immunogenicity and to extend the duration of protection (Aguilar & Rodriguez, 2007). Several kinds of adjuvants have been studied for their potency to promote immune responses to FMDV vaccines. Aqueous or oil adjuvants are commonly incorporated in the FMDV inactivated vaccines. The aqueous vaccine is prepared by adsorbing the virus on to aluminium hydroxide gel adjuvant, but this adjuvant is not ideal for use in pigs as its protective efficacy is low in this species (P.V. Barnett *et al.*, 1996). Oil-adjuvanted vaccines are usually formulated as water-in-oil emulsion using mineral oils, such as Marcol and Drakeol. The mineral oil is usually premixed with an emulsifying agent before the addition of a proportion, or all, of the aqueous phase of the vaccine, and emulsified by use of a colloid mill or continuous mechanical or flow ultrasonic emulsifier (OIE, 2012). More complex double emulsions (water/oil/water) are produced by emulsifying once more in an aqueous phase containing a small amount of detergent such as Tween 80 (P.V. Barnett *et al.*, 1996). A double emulsion (water-in-oil-in-water) commercial adjuvant Montanide1 ISA 206 (Seppic Inc, France) is presently being used in many Asian and South American countries (Doel, 2003), and has also been tested in cattle with recombinant empty capsid vaccines produced in baculovirus (Mohana Subramanian *et al.*, 2012). Another mineral oil-based double emulsion commercial adjuvant Montanide ISA-201 has been developed to improve cellular immune response and found superior to ISA-206 and GAHOL oil adjuvants (Dar *et al.*, 2013). Ibrahim and his coworkers, in another comparative study confirmed the superiority of ISA-201 FMD vaccine over Montanide ISA oils (201, 206, 61 and 50) FMD vaccines (Ibrahim *et al.*, 2015). The antigen payload that is used to formulate FMD vaccine may vary between 1 to10 µg or more of 146S particles per dose. The relationship between 146S antigen and vaccine potency is not a linear function and therefore complicates the vaccine formulation (Paul V Barnett *et al.*, 2003).

1.2.9.6 Routes of Vaccine Administration

Currently used FMD vaccines formulated with the water-in-oil-in-water emulsion or alum/saponin adjuvants are administered through intra muscular route. But administration of vaccine through the skin represents an interesting strategy since the skin contains numerous resident antigens presenting cells (APCs) such as, Langerhans cells and dermal

DCs. As such, (Eble *et al.*, 2009) demonstrated that intra dermal vaccination of pigs against FMD with 1/10 dose resulted in comparable vaccine efficacy as intra muscular vaccination with a full dose. More recently, intra dermal vaccination using a needle free system device with 1/16 dose of commercial FMD vaccine resulted in complete protection of cattle (Pandya *et al.*, 2012). Similarly, vaccination using Ad5-vectored vaccine by the subcutaneous route, instead of the conventional intra muscular route, protected animals with a dose 25-fold lower than the protective dose of intra muscular immunization (M.J. Grubman *et al.*, 2012). In guinea pigs FMD vaccine is always administered through subcutaneous route during vaccine efficacy testing (Daoud *et al.*, 2013; Motamedi Sedeh *et al.*, 2007)

1.2.9.7 Vaccine Efficacy Testing

Vaccine efficacy is estimated in vaccinated cattle directly, by evaluating their resistance to live virus challenge. Vaccine efficacy should be established for every strain to be authorized for use in the vaccine (Nesya Goris *et al.*, 2008; N. Goris *et al.*, 2007). Efficacy tests in other target species, such as sheep, goats, pigs or buffalo are either different or not yet standardized. In general, a successful test in cattle is considered to be sufficient evidence of the quality of a vaccine to endorse its use in other species (OIE, 2012).

Among the laboratory animals, guinea pigs are the best to model the pathogenesis of FMDV epithelial vesiculation (di Girolamo *et al.*, 1985). Similar to natural hosts, extensive vesicles develop at the inoculation site within 24 hours, the vesicles rapidly rupture and the epithelium is desquamated. Secondary vesicles develop on the tongue or mouth, leading to salivation, food refusal and weight loss. Within 4 to 5 days, these vesicles begin to heal and desquamation is completed in about 3 weeks (R. C. Knudsen *et al.*, 1979). Like the natural hosts, animals are pyrexic for a short period and viraemia is cleared rapidly, coinciding with a rapid antibody response, with serum neutralizing antibody titers detectable from 3 days post-infection. Moreover, Mortality rates in guinea pigs are low, reported to be of the order of 5% (Knudsen et al., 1979). Due to the reproducibility of the FMDV response, guinea pigs have been used extensively for FMDV vaccine efficacy trials (Cartwright *et al.*, 1982; Daoud *et al.*, 2013; Guo *et al.*, 2005; Yao *et al.*, 2008).

The virus neutralization test (VNT) is the method of choice for indirect assessment of vaccine potency (Mattion *et al.*, 2009). Two dimensional microneutralization test (2D-

MNT) is the most reliable *in-vitro* alternative assay for vaccine matching purpose (Bornali *et al.*, 2015; Kitchin *et al.*, 1988; M. M. Rweyemamu, 1984). The test is used to determine antigenic relation of field viruses with vaccine strain and this antigenic relation is expressed as 'r' value.

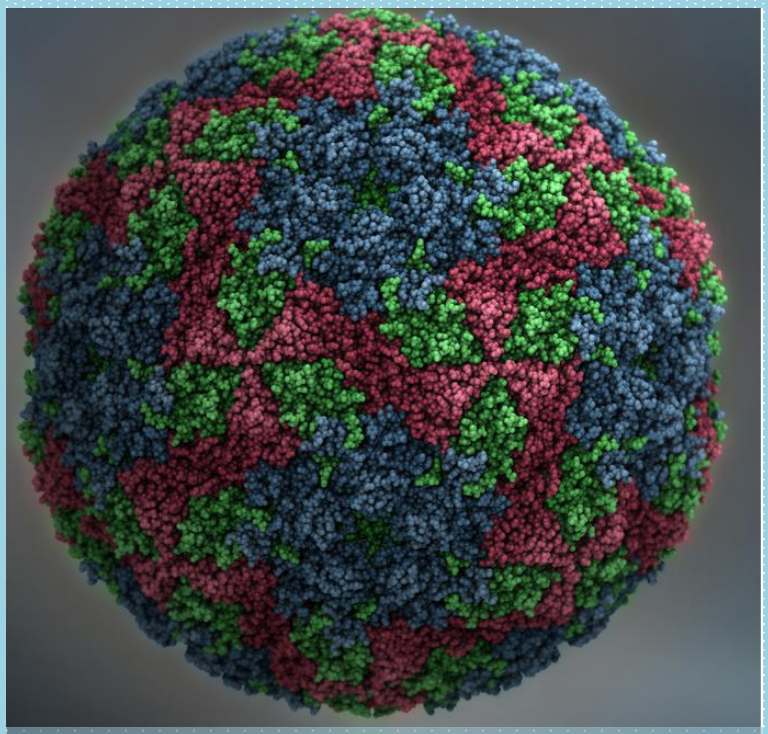
1.3 Hypothesis of the Work

FMD is endemic in Bangladesh and considered as one of the major constraints of livestock development. Vaccination is eventually the only tool to combat FMD in all endemic countries including Bangladesh. Other measures like restriction of livestock movement and quarantine for FMD control are practiced in organized farms only. Vaccination program in Bangladesh is inadequate and inappropriate in terms of selection of vaccine strains. Various types locally manufactured and imported FMD vaccines are used in Bangladesh. Local manufacturers use none-characterized FMDV strains for vaccine manufacturing and vaccine strains are selected without studying detailed molecular epidemiology of the disease in the country. Vaccine strains selection without epidemiological study often results in inaptness of the vaccines. FMD vaccines are imported in the country mainly from India, France and Russia. The thorough molecular epidemiology and genetic analyses of circulatory FMDVs in Bangladesh carried out by Microbial Genetics and Bioinformatics Laboratory (MGBL) research group revealed significant vaccine virus-field virus mismatch. The vaccine strains in the imported vaccines are not in the same topotype/lineage/genotypes circulating in our country. Vaccine failure due to genetic heterogeneity in field virus than vaccine strain was also reported by this research group (Ullah *et al.*, 2015). MGBL research group investigated several FMD outbreaks in some organized dairy farms of the country which were vaccinated by local FMD vaccines or imported one 20-40 days prior to onset of the outbreak. This finding predicts poor in-process quality control or inadequate antigenic pay load or mismatched strain in manufactured vaccines. For effective FMD control program and to achieve goals of PCP-FMD stage 2 in Bangladesh, an effective mono to tri-valent vaccine prepared with indigenous FMDV strain is urgently needed. Therefore, this research work was carried out based on the hypothesis-**a new foot and mouth disease vaccine developed using indigenous circulatory strains of FMDV serotypes with ensuring OIE standard will protect the natural outbreaks of FMDV in susceptible animals of Bangladesh.**

1.4 Working Objectives

On the basis of the hypothesis of the work, the specific objectives are-

i. Genome wide and antigenic properties analyses of the FMDV serotypes O and A circulating in Bangladesh; and selection of vaccine candidate(s);
ii. Analyses of comparative genomics of the selected vaccine candidates with other circulatory FMDVs and with currently used vaccine strains; and
iii. Optimization of vaccine manufacturing processes at laboratory scale, quality assurance of the products and efficacy testing.



Chapter 02:

Materials and Methods

2.1 Research Plan

The following research plan was designed for the development of effective vaccine against local isolates of Foot and Mouth Disease Virus in Bangladesh.

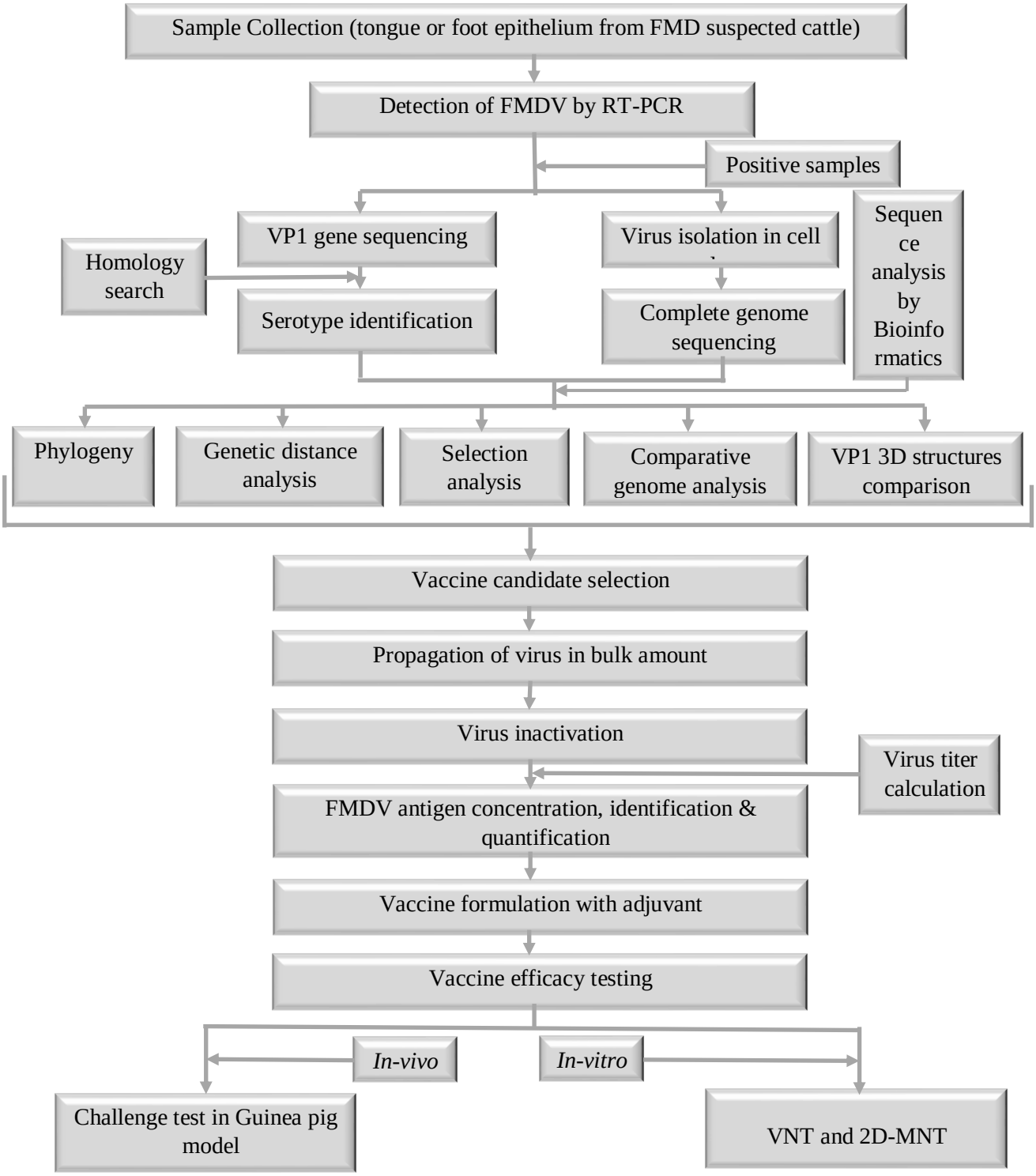


Figure 2.1: Research plan for isolation of FMDV from infected animal tissues with subsequent genome sequencing, bioinformatics analyses, vaccine candidate selection and vaccine development.

2.2 Sample Collection and Transportation to Laboratory

The FMD research group of Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka collects tissue samples regularly from FMD outbreaks all over the country. During the period of 2012 to 2018, a total of 319 tongue or foot epithelium tissue samples were collected from 47 different FMD outbreaks in 27 distinguished districts of Bangladesh **(Appendix-I)**. Ethical approval (Ref. 37/Biol.Scs./2016-2017, Date:23.05.2017) was taken from the "Ethical Clearance Certificate for Animal Involving Committee" of the faculty of Biological science, University of Dhaka **(Appendix-V)**. Moreover, all the samples were collected by registered veterinary doctors with appropriate care to the animals.

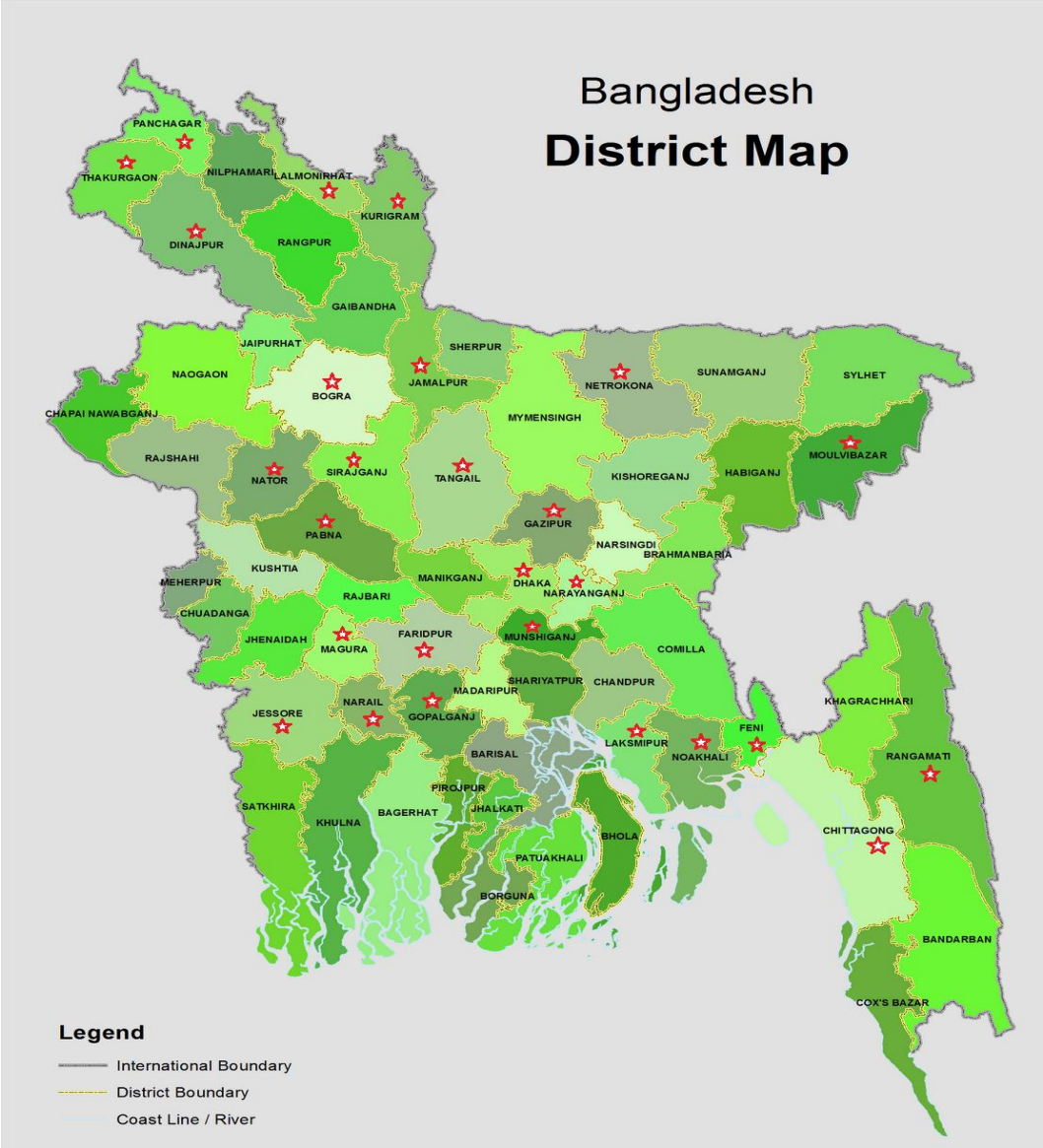


Figure 2.2a: FMD sample collection areas (marked with red stars)

The samples were collected in cryo-vials and transported to the MGBL maintaining cool chain and stored at -80^{0}C until further use. The samples were named according to three letter country code (i.e. BAN for Bangladesh), followed by a two letter district code (e.g. CH for Chittagong), then upazilla and laboratory record number (e.g. Sa-304) and finally by year of outbreak. For example, BAN/CH/Sa-304/2016 representing FMDV from Bangladesh, Chittagong Sadar-laboratory record number 304 and collected in 2016. A pre-set questionnaire **(Appendix-V)** was filled during the collection of samples which describes the history of infected animals.

2.3 Detection of FMDV by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.3.1 Extraction of Viral RNA from Tissue Samples

Approximately 60 mg of tissue from each sample was measured and placed in an elution tube. Four hundred (400) µl lysis buffer (66 µl/10 mg tissue) was added to the samples and homogenized with automated Maxwell® 16 Instrument (Promega, USA) until completely lysed. Homogenized lysate was incubated on ice for 10 minutes to ensure complete sample lysis.

The lysate of each sample was transferred to a 2 ml micro centrifuge tube. Eight hundred and thirty-five (835) µl of blue RNA dilution buffer (provided in the kit, **Appendix-II**) was added to lysates. The mixture was kept on ice as much as possible. Clearing agent (provided in the kit) was re-suspended completely by shaking and 75 µl added to the samples and mixed thoroughly for 30 seconds by vortex. The mixture was then heated in a pre-set 70^o C heat block for 3 minutes. After heating, mixed again for 30 seconds. Samples were incubated at room temperature for 5 minutes for proper cooling. Clearing column was assembled by placing one clearing column into a collection tube. About 700 µl of sample was transferred to clearing column and centrifuged at 12000 x g for 2 minutes. Flow through from the collection tube was transferred to well#1 of the Maxwell® 16 LEV RNA Cartridge without disrupting the pellet of clearing agent. If the volume of sample is more than 700 µl then centrifugation and sample loading to well#1 step was repeated. Plunger was set at well#7 of the Maxwell® 16 LEV RNA Cartridge. The Maxwell® 16 RNA Cartridge contains an array of chamber facilities RNA extraction. Three hundred (300) µl of Nuclease free water was loaded in the elution tube. After that The Maxwell® 16 RNA Cartridge and elution tube were placed in the specified chamber of the instrument,

the RNA extraction program was set in the instrument and after about 34 minutes RNA yield was collected from the elution tube. The extracted RNA was stored at -80^0 C until reverse transcribed for cDNA synthesis.

2.3.2 Preparation of Complementary DNA (cDNA) from Extracted RNA

The extracted RNA was reverse transcribed into cDNA using GoScript™ Reverse Transcription System (Promega, USA) as per the manufacture's instruction. The detailed method of cDNA preparation is deciphered below:

2.3.2.1 Target RNA and Primer Combination and Denaturation

Sterile thin-walled dilution tubes (ExtraGene, USA) and reaction tubes (Eppendorf, USA) were placed on ice. The extracted RNA and the 1.2kb Kanamycin Positive Control RNA were thawed on ice. On ice, the experimental RNA was mixed with Oligo(dT)$_{15}$ Primer and Nuclease-free water was added to make a final volume of 10µl per RT reaction. Three reactions, one for the target RNA/Primer combination, one for positive control and another for negative control were set as outlined in **Table 2.3.2.1.**

Table 2.3.2.1: RNA/Primer Mixture for cDNA Preparation

Positive Control	
1.2kb Kanamycin Positive Control RNA (1µg)	5 µl
Oligo(dT)$_{15}$ Primer (0.5µg/reaction)	2 µl
Nuclease-Free Water	3 µl
Final Volume	**10 µl**

Negative (No RNA Template) Control	
Random (Hexameric Primer)	2 µl
Oligo(dT)$_{15}$ Primer (0.5µg/reaction)	2 µl
Nuclease-Free Water	6 µl
Final Volume	**10 µl**

Experimental Reaction	
Experimental RNA	5 µl

Random (Hexameric Primer)	2 µl
Oligo(dT)$_{15}$ Primer (0.5µg/reaction)	2 µl
Nuclease-Free Water	1 µl
Final Volume	**10 µl**

Each reaction tube was closed tightly and placed at 70° C for 5 minutes in a preheated heat block. Afterwards, the primer/RNA mixture was quickly chilled on ice for 5 minutes. Then the tubes were spun for 10 seconds and kept closed on ice until the reverse transcription reaction mix was added.

2.3.2.2 Reverse Transcription

The reverse transcription reaction mix was prepared by combining the following components of the GoScript™ Reverse Transcription System in a sterile 1.5ml micro centrifuge tube (Eppendorf, USA) on ice. Thirty (30) µl of reaction mix was prepared for each cDNA synthesis reaction to be performed. The volumes needed for each component is described in **Table 2.3.2.2**.

Table 2.3.2.2: Reaction Mixture for cDNA Preparation

Positive Control	
RNase Free H_2O	14 µl
GoScript™ 5X Reaction Buffer	8.0 µl
GoScript™ Reverse Transcriptase	2.0 µl
MgCl2 (25mM)	6.0 µl
Final Volume	**30 µl**

Negative Control	
RNase Free H_2O	12 µl
GoScript™ 5X Reaction Buffer	8.0 µl
GoScript™ Reverse Transcriptase	2.0 µl
MgCl2 (25mM)	6.0 µl
dNTP Mix (final concentration 1.0 mM each dNTP)	2.0 µl
Final Volume	**30 µl**

Experimental Reaction	
RNase Free H_2O	11.0 µl
GoScript™ 5X Reaction Buffer	8.0 µl
GoScript™ Reverse Transcriptase	2.0 µl
MgCl2 (25mM)	6.0 µl

dNTP Mix (final concentration 1.0 mM each dNTP)	2.0 µl
Recombinant RNasin® Ribonuclease Inhibitor	1.0 µl
Final Volume	**30 µl**

The reaction mix was vortexed gently to mix, and kept on ice. Reverse transcription reaction mix (30 µl aliquots) was then added to each reaction tube on ice. Ten (10) µl of RNA and primer mix was added to each reaction for a final reaction volume of 40 µl per tube. The tubes were subsequently incubated at 25° C for 5 minutes to anneal the primers. First-strand synthesis was carried out by incubating the reaction at 42° C for 60 minutes. Reverse transcriptase was inactivated by incubating the reaction mix at 70° C for 15 minutes.

2.3.3 Amplification of VP1 Coding Gene by RT-PCR and Screening for FMDVs

The cDNA was directly amplified by adding the products of the heat inactivated reverse transcription reaction to the PCR mixture and proceeding with thermal cycling. GoTaq® Hot Start Colorless Master Mix (Promega, USA) was used for PCR amplification. Composition of the GoTaq® Hot Start Colorless Master Mix is given in **Table 2.3.3a**.

Table 2.3.3a: Composition of GoTaq® Hot Start Colorless Master Mix

GoTaq® Hot Start Colorless Master Mix (2X)
GoTaq® Hot Start Polymerase
dNTPs (400µM each)
2X Colorless GoTaq® Reaction Buffer (pH 8.5)
$MgCl_2$ (4 mM)

PCR mix was prepared with the addition of 2X GoTaq® Hot Start Colorless Master Mix, 400nM of each of the primers and Nuclease free water. Although GoTaq® Hot Start Polymerase, component part of the master mix was bound to a proprietary antibody that blocks polymerase activity at temperature below 70° C, PCR mix was prepared maintain ice-cold condition. After mixing the mixture by vortex, short centrifugation was done. After that, reaction mix was dispensed into sterile, thin walled tubes. Both Positive and Negative control reaction was performed to authenticate the PCR. PCR preparation was done like the outline in **Table 2.3.3b**.

Table 2.3.3b: PCR Preparation for the screening of FMDV

PCR Mixture Components	Positive Control (50 µl reaction)	Negative Control (50 µl reaction)	Experimental Reaction (50 µl reaction)
Nuclease Free Water	Up to 50 µl	Up to 50 µl	Up to 50 µl
GoTaq® Hot Start Colorless Master Mix (2X)	25	25	25
Upstream Primer (10 µM)	2 µl (400nM)	2 µl (400nM)	2 µl (400nM)
Downstream Primer (10 µM)	2 µl (400nM)	2 µl (400nM)	2 µl (400nM)
Template	Variable (< 500 ng)	No Template	Variable (< 500 ng)

The RT product of each sample was tested with primer pairs to screen the presence of FMDV. Initial screening was done with VP1UF (Ullah *et al.*, 2014)-NK61 (Reid *et al.*, 2000; Samuel & Knowles, 2001) and 16F (Nandi *et al.*, 2013)-NK61 primers pair combinations. All the PCR tubes containing the appropriate mixtures were heated at 94°C for 5 minutes in the thermal cycler (Applied Biosystem, USA) to ensure Denaturation of all DNA templates. Thirty five (35) cycles of these segments were repeated with a final extension of 10 min at 72°C. After this, PCR tubes were stored at -20°C until further analysis. The cycling profile for each primer: target combination was optimized accordingly (**Table 2.3.3c**).

Table 2.3.3c: Primers used for FMDV screening

FMDV Serotypes	Primers	Sequence (5`-3`)	Annealing Temp. (^{0}C)	Location	Reference
All	16F	GAGAACTACGGWGGWGAGAC	55	VP1	Nandi *et al.*, 2015
All	16R	GCACCGWAGTTGAAGGAGGT	55		
All	VP1UF	GCRCAGTACTACRCSCAGTAC	55	VP1	Ullah *et al.*, 2014

All	NK61	GACATGTCCTCCTGCATCTG	55	2B	Samuel and Knowles, 2001

2.3.4 Agarose Gel Electrophoresis

The amplified products were run on 1.0 % agarose gel with a 1kb-DNA ladder (Promega, USA) for the visualization of the amplified products. Sixty (60) ml of 1.0% agarose was prepared in 1x TAE buffer. The mixture was heated in microwave oven for ~5 minutes until melted. Then the boiled mixture was allowed to cool to about 45°C and added 4 µl ethidium bromide (stock 10 mg/ml). The gel was poured off onto gel casing, and comb was inserted. The casing was then allowed to set on a flat surface for about 15 min. Buffer 1x TAE was then poured into tank and comb was removed from gel. Samples were prepared on parafilm (1µl 6x loading dye and 5µl PCR product). Molecular weight marker was prepared with 5µl of molecular weight marker and 1µl of 6x loading dye. Samples and markers were loaded into the appropriate wells formed in the gel. Electrophoresis was started at 100 volts for 35 min. The gel was viewed on AlphaImager HP Gel-documentation system (Cell Bioscience, USA).

2.4 Virus Isolation in Cell Culture

Representative positive samples were inoculated into BHK-21 (Baby Hamster Kidney) cell monolayers for the isolation of Foot and Mouth Disease Virus. The isolates were subsequently subjected to complete genome sequencing.

2.4.1 Preparation of Cell Culture Media

Dulbecco's Modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA) with stable glutamine was used for the culture of the BHK-21 cell line and virus isolation. Commercial formulation of the DMEM was according to the study of Dulbecco and Freeman (Dulbecco & Freeman, 1959). DMEM was supplemented with antibiotics [penicillin and streptomycin (10000 µg/ml), Gentamycin (10 mg/ml) and Fungizone (Gibco, USA)] to prevent bacterial, mycoplasma and fungal contamination. There was slight difference from original formulation where only 0.05 mg/l riboflavin was used to avoid negative photo oxidative effects. Two different types of formulations of DMEM were prepared for cell culture and virus isolation purposes. Those were growth media with 10% FBS (Fetal Bovine Serum) and maintenance media with 2% FBS respectively.

2.4.2 Preparation of Cell Line

BHK-21 (Baby Hamster Kidney) cell line was used for infection and isolation of FMDV. Before infection, BHK-21 cell monolayers were prepared and maintained. The cell line preserved at -80^{0}C with cryo-protectant DMSO (Dimethyl Sulfoxide) was revived. The cell containing vial was thawed at 37^{0}C in water bath and the cell suspension was added with 3.5 ml of growth medium in a 15 ml sterile falcon tube. DMSO was removed by centrifugation of the falcon tube at 1000 rpm for 2.30 minutes and decanting the supernatant. After decanting the supernatant containing DMSO, pellet was re-suspended in 5 ml fresh growth medium. Re-suspended cell suspension was placed in a 25 cm^2 cell culture flask by a pipette and was spread gently over the entire inner surface of the flask. The flask was capped and incubated at 37^{0}C in an incubator (Nuaire, USA) with humidified atmosphere of 5% CO_2. Cell growth was monitored for next 48 to 72 hours until complete monolayer development.

2.4.3 Sub-culturing the Cells

The growth of the cells was observed under inverted microscope (Leica, Germany). The cells were sub-cultured after the development of complete monolayer to multiply the number of the monolayers. For this, spent media were decanted from cell culture flask and the monolayer of the cell was washed with 5 ml PBS (Phosphate Buffered Saline) to remove unwanted residuals. Then 800 µl of cold 0.25% trypsin-EDTA solution (caisson, USA) was added on the washed monolayer in the flask followed by incubation at 37 ºC for 5 minutes. Finally, 3.2 ml of fresh 10% DMEM (growth medium, Appendix I) was added into the flask and the cells were pipetted up and down several times to break up the clumps of cells. Using the same pipette, the cell suspension was drawn up and quickly distributed in new flasks in required volume (0.5 to 1.0 ml). Fresh growth medium was added to make the final volume 5 ml. After capping, the flasks were incubated at 37 ºC in a CO_2 incubator until monolayer development.

2.4.4 Inoculum Preparation

Tissue samples found positive to FMDV screening (described in Section **2.3**) were selected to infect BHK-21 cell monolayers. Tissues were ground using sterile mortar and pestle. Sterile sands were added to the pestle to facilitate tissue grinding. The ground tissues were suspended by adding 5 ml of DMEM. A blank control was also prepared by the same procedure but without tissue samples. Both the tissue and blank suspensions were taken

into two different 15 ml falcon tubes and centrifuged at 4^0 C (Tomy MX 307, Japan) with a speed of 1500 rpm for 5 minutes. The supernatant of both the tubes were taken and filtrated by 0.22 µm Millipore syringe filter assembly (Millipore Sterivex - GS 0.22 µm disposable filter units). The tissue filtrate was used as positive inoculum and the rest as negative inoculum to infect BHK-21 cell monolayers.

2.4.5 Sample Inoculation and Virus Culture

Cell culture flasks having 80-90% confluent monolayers (log phase) were selected to be infected by virus. The spent media from the flasks were decanted and the monolayers were washed by PBS. Two hundred (200) µl filtrate of sample was inoculated in the cell monolayer and in negative control, same volume of filtrate of blank media were used instead of sample filtrate. The flasks were labelled properly and incubated at 37^0C for 60 minutes for adsorption of the virus in the cells. Then fresh growth medium (2% DMEM) was added to the flasks to make the final volume of 5 ml. After that, cell culture flasks were incubated at 37^0C for 24-48 hours. Periodically, the cell culture flasks were monitored for the presence of cytopathic effect (CPE).

2.4.6 Harvesting and Preservation of Virus

The samples developed significant CPE in contrast to negative control were selected and the fluid of each flask was harvested separately. A small scaled scrapper was used to dissociate adherent cell from the inner flat bottom surface of the flasks. The total content of the flask was collected into a sterile 15 ml falcon tube and centrifuged at 4^0C with1000 rpm for 2 minutes to collect cell culture supernatant. Major portion of the supernatant was poured into another sterile 15ml falcon tube leaving cell pellet with 0.5ml supernatant. Remaining cell pellet with supernatant was subjected to freeze-thaw cycles for 2-3 times to disrupt the cells and release of intracellular virus. After this step, previously collected cell culture supernatant was mixed with disrupted cells in falcon tube. Finally, the viral suspension was clarified from the cell debris by centrifugation at 7000 rpm for 20 minutes to collect clear cell culture supernatant (Daoud *et al.*, 2013). Same procedure was followed for negative control.

The collected cell culture supernatant of each falcon tube was further subdivided into aliquots of 1 ml in sterile 2ml cryo-vials and stored at -80° C freezer (Nuaire, USA) until further use.

2.5 VP1 Protein Coding Gene Sequencing

VP1 protein coding genes of all the positive samples were sequenced for serotype identification and bioinformatics analyses.

2.5.1 Extraction of Viral RNA and cDNA preparation

RNA was extracted either from tissue samples or cell culture supernatant following the procedure described in **sub-section 2.3.1** except in case of extraction from cell culture supernatant, 450 µl of cell soup was mixed with 50 µl of lysis buffer in a 2 ml micro centrifuge tube. The mixture was vortexed to lyse the cells. The lysate was kept on ice as much as possible to facilitate complete lysing. Complementary DNA (cDNA) was prepared from extracted RNA like the procedure described in **sub-section 2.3.2**.

2.5.2 PCR Amplification of VP1 Region

VP1 protein coding region of FMDV was amplified by primer pairs VP1UF and NK61 from prepared cDNA following the procedure described in **sub-section 2.3.3**. cDNA from a sample previously identified as FMDV positive was used as positive control and PCR negative control was also included to validate the reaction. Cell culture negative control was subjected to PCR reaction as like as the positive control. PCR was set at 94^0 C for denaturation followed by annealing at 55^0C and extension at 72^0C.

PCR product was visualized by Agarose gel electrophoresis according to protocol mentioned in section **2.3.4**.

2.5.3 Purification of Amplicons

After Agarose gel electrophoresis, PCR positives samples were purified using The Wizard® SV Gel and PCR Clean-Up System (Promega, USA; Appendix I) according to the manufacturer's instructions. Centrifugation based methodology was followed to purify the PCR products. The Wizard® SV Gel and PCR Clean-Up System is based on ability of DNA to bind to silica membranes in the presence of chaotropic salts (guanidine isothiocyanate). After amplification, an aliquot of the PCR product is added to the equal volume of guanidine isothiocyanate containing Membrane Binding Solution (MBS) and directly purified. In case of gel purification, an equal amount of gel cut containing the intended band was mixed with equal volume of MBS in a micr centrifuge tube and incubated at 65ºC in a heat block until completely dissolved. In both the cases, the mixture was transferred to the mini column pre-set with a collection tube (SV mini column

assembly). After short incubation (2 minutes) at room temperature, SV mini column was centrifuged at 16,000×g (14,000 rpm) for 1 minute. Then the flow-through was discarded and SV mini column was washed for two times with Membrane Wash Solution (Supplied in the kit, ethanol added). After washing the SV mini column, DNA was eluted in Nuclease Free Water (Supplied in the kit). The purified PCR product was stored at -20^{o}C until further processing.

2.5.4 Measurement of the Concentration of Amplicons

The concentration of amplicons were measured using a NanoDrop^{TM} spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). PCR product was measured as ng/ µl. The reading of the ratio was between at 260 nm and 280 nm (OD 260 /0D 280). This OD 260/280 ratio provides an estimate of the purity of the nucleic acid (DNA) which is a value of 1.8.

2.5.5 Sequencing of PCR products

After purification of the PCR products, purified products and respective primers were sent to First Base Laboratories, Malaysia for single-pass DNA sequencing by Sanger method. The sequencing reaction was done both for forward and reverse primers (**Table 2.3.3.1c**). The sequences (tracer files) were viewed using sequence viewer software like chromas. Both forward and reverse sequences were assembled into a single contig using SeqMan version 7.0.0 (Lasergene, DNASTAR, USA).

2.5.6 Homology Search and Serotype Identification

Serotypes of the sequences were identified by homology search in GenBank of National Biotechnology Information Centre (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool BLAST (Morgulis *et al.*, 2008; Zhang *et al.*, 2000).

2.6 Sequence Analyses and Vaccine Candidates Prediction

A Sequence-based molecular approach was used for the prediction of potential vaccine candidates from circulatory Foot and Mouth Disease Virus serotypes in Bangladesh. The VP1gene sequences generated in MGBL along with other relevant sequences of this geographical location were retrieved from NCBI database to be used in this study.

2.6.1 Phylogeny and Determination of Topotype, Lineage and Sub-lineage of FMDV Isolates

For Phylogeny, two different datasets were prepared for FMDV serotype O and A. The dataset for serotype O included a total of 68 VP1 gene sequences among which 39 sequenced in our lab and 29 sequences were retrieved from NCBI database. For serotype A, the dataset was prepared by a total of 41 VP1 gene sequences (14 our sequences and 27 retrieved) **[Appendix-III]**.

The sequences of both the datasets were first aligned using built in ClustalW program of MEGA7 (Tamura *et al.*, 2011), then phylogenetic trees were constructed by the maximum-likelihood method (Kumar *et al.*, 2016) based on the Tamura-Nei model (Tamura & Nei, 1993). Gamma distribution, and bootstrap values were set at 1 and 1000 respectively. Topotypes, Lineages and Sublineages of the FMDV isolates were determined from the constructed phylogenetic trees comparing with references sequences.

2.6.2 Genetic Distance Analyses

Genetic distances of current Indian vaccine strains and proposed vaccine candidates from FMDV isolates circulating in Bangladesh were calculated based on the genetic variations among the VP1 protein coding gene sequences. Analyses were conducted in MEGA7 (Kumar *et al.*, 2016) using the Kimura 2-parameter model (Kimura, 1980). The rate variation among sites was modeled with a gamma distribution (shape parameter = 2). The analysis involved 69 nucleotide sequences for serotype O and 21 for serotype A. Codon positions included were 1st+2nd+3rd. All ambiguous positions were removed for each sequence pair. There was a total of 639 positions in the final dataset. Finally, the mean genetic distances of FMDV isolates from current and proposed vaccine candidates were calculated and compared by SPSS Program Version 20. For calculation and comparison of mean, 2-tailed T-Test with 95% confidence interval and 1000 bootstrap value was used.

2.6.3 Selection Analyses

The synonymous (dS) and non-synonymous (dN) changes at every codon of VP1 coding sequences of FMDV isolates circulating in Bangladesh were estimated using four different selective pressure analyses implemented in DataMonkey (Pond & Frost, 2005). The methods used were Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), Internal Fixed Effects Likelihood (IFEL) and Mixed Effects Model of

Episodic Diversifying Selection (MEME). These methods were conducted using the GTR model of nucleotide substitution and neighbor-joining method to determine the rate for dN and dS. The synonymous rate exceeding the non-synonymous rate was considered as negative selection (dS>dN), while the positive selection was defined as when the non-synonymous rate exceeds the synonymous rate (dN>dS). Neutral selection was defined when the non-synonymous rate equals to the synonymous rate (dN = dS). Z-test for selection analyses were conducted in MEGA7 (Kumar *et al.*, 2016) using the Kumar method (Nei & Kumar, 2000). The selection analyses included 68 nucleotide sequences of FMDV serotype O and 20 of serotype A circulating in Bangladesh (**Appendix-III**). All ambiguous positions were removed for each sequence pair. There was a total of 213 codon positions in the final dataset. Values of P less than 0.05 were considered significant at the 5% level in case of all methods.

2.7 Complete Genome Sequencing

The proposed vaccine candidates selected through bioinformatics analyses were subjected to complete genome sequencing to understand the molecular and structural organizations of the virus.

2.7.1 PCR Amplification of the Entire Genome

PCR amplifications were done with internal primer pairs to generate overlapping fragments spanning the entire genome of selected isolates (Abdul-Hamid *et al.*, 2011; Ma *et al.*, 2014; Reid *et al.*, 2000; Samuel & Knowles, 2001; Sanyal *et al.*, 2004; Sultana *et al.*, 2014) (**Table 2.7.1a and Table 2.7.1b**).

Table 2.7.1a: Primer pairs used to amplify the complete genome of FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016

Forward/ Reverse Primer Pair	5'-3' Sequence	Position	Location	Amplicon size (bp)	References
P1F	TTGAAAGGGGGCGCTAGGGT	1-20	5′ UTR	392	Designed in MGBL
M1R	CGGTAAAACTTGGGGGGGGGGGGGGGGTGAAAGGC	360-392	5′ UTR		Ma *et al.*, 2014
20F	TTGAAAGGGGGCRCTAGGGT	1-20	5′ UTR	392	Sultana *et al.*, 2014
M1R	CGGTAAAACTTGGGGGGGGGGGGGGGGTGAAAGGC	360-392	5′ UTR		Ma *et al.*, 2014
LF1F[a]	CCCCCCTAAGTTTTACCGTCGTTCCCG	378-405	5′ UTR	484	Sanyal *et al.*, 2004

P2R	ACATGATGGGTCCGTTAGGA	843-862	5′ UTR		Designed in MGBL
LF1F[a]	ACATGATGGGTCCGTTAGGA	378-405	5′ UTR	587	Sanyal *et al.*, 2004
1R	CCAGTCCCCTTCTCAGATC	948-965	5′ UTR		Reid *et al.*, 2000
1F	GCCTGGTCTTTCCAGGTCT	640– 658	5′ UTR	640	
1OEXR	CCCTCGTGYAGYTCAAGACC	1329–1348	VP4		Sultana *et al.*, 2014
2OF	CCMTTCYTCGAMTGGGTCTA	1254– 1273	VP4	1006	
2OR	TGGTTWCCCACTGCRGTGAC	2241– 2260	VP2		
2OEXF	GGTCTTGARCTRCACGAGGG	1329 – 1348	VP4	862	
2OEXR	CCGTAGACRCCTTTGTGGTC	2172 – 2191	VP2		
3OF	ARGACTTYGTGAGYGGGCC	2035– 2053	VP2	861	
3OR	AAGTGCAGGTTRATGGTGCC	2877– 2896	VP3		
3OEXF	GACCACAAAGGYGTCTACGG	2172 – 2191	VP2	724	
3OR	AAGTGCAGGTTRATGGTGCC	2877– 2896	VP3		
4OF	CAAGGTSTATGCCAACATCG	2507– 2526	VP3	890	
4OR	RTYTGCATCAGGTCCAACAC	3378– 3397	VP1		
5OEXF	GAGAACTACGGTGGTGAGAC	3276– 3295	VP1	718	
NK61	GACATGTCCTCCTGCATCTG	3971 –3994	2B		Samuel & Knowles, 2001
NSP 1F	GAGACGTYGAGTCCAACCC	3939-3958	2B	1051	Abdul-Hamid *et al.*, 2011
NSP 2R	GCCATRGGCGGGATRAA	4972-4989	2C		
NSP 2F	CAGCTCARAGCACGTGACAT	4423-4443	2C	1047	
NSP 3R	ACCATCCCCTCRAAGAAYTC	5449-5469	3A		
NSP 3F	TGACCACTTYGACGGTTA	4860-4878	2C	746	
NSP 4R	CATRATCACTATGTTTGCCA	5585-5605	3A		
NSP 4F	CGRAGGTTYCACTTTGAC	5098-5116	3A	928	
NSP 5R	CACTTTCAAAGCGACAGG	6007-6025	3C		
NSP 5F	GAATTCTTTGAGGGGATGGT	5449-5469	3A	947	
NSP 6R	GGGGGTKCCYTTCTTCAT	6377-6395	3C		
NSP 6F	CRAGCTGAAGGACCCTAC	5831-5849	3B	1106	
NSP 7R	GGACAGGACATGCTCTCAG	6922-6939	3D		
NSP 7F	GGACAGGACATGCTCTCAG	6283-6302	3C	1042	
NSP 8R	AATTTGCGGTCCGTTGT	7307-7324	3D		
NSP 8F	ATGCGCAAAACCAAGCT	6736-6753	3D	942	
NSP 9R	GTCCAGCTCRACTCCCTC	7660-7678	3D		
NSP 10F	AACGTGTGGGATGTGGA	7393-7410	3D	832	
T21R	CAGGAAACAGCTATGAC TTTTTTTTTTTTTTTTTTTTR	8186-8225	3'UTR		
NSP 10F	AACGTGTGGGATGTGGA	7393-7410	3D	832	
T21A	CAGGAAACAGCTATGAC TTTTTTTTTTTTTTTTTTTTA	8186-8225	3'UTR		
NSP 10F	AACGTGTGGGATGTGGA	7393-7410	3D	832	
T21G	CAGGAAACAGCTATGAC TTTTTTTTTTTTTTTTTTTTG	8186-8225	3'UTR		

Table 2.7.1b: Primer pairs used to amplify the complete genome of FMDV serotype A vaccine candidate BAN/CH/Sa-304/2016

Forward/Reverse Primers	5'-3' Sequence	Position	Location	Amplicon size (bp)	Reference
A1F	TTGAAAGGGGGCGCTAGGG	1-19	5′ UTR	374	Abdul-Hamid *et al.*, 2011
A1R	GGGTGAAAGGCGGRCTTCG	355-374	5′ UTR		
A2F3	CCCCCCTAAGTTTTACCGTCR	374-394	5′ UTR	622	
A2R	CCTTCTCAGATCCCGAGTGTCG	974-995	5′ UTR		
682F	CCAGGTCTAGAGGRGTGA	687-704	5′ UTR	974	
5' UTR-4R	GCCTGAATAGGYGACCGGAG	1640-1660	5′ UTR		
5' UTR-4F	CCGTTGAGYGGTTCTTGATCG	1040-1059	L^{Pro}	758	
A4R	CCATGGAGTTCTGGTACTGRTGC	1775-1797	VP4		
A4F	GAGCCTTTCTTCGACTGGGTC	1284-1304	L^{Pro}	994	
A5R	WCACCTCCACGTCCCAGC	2260-2277	VP2		
A6F	AYTCGAGTGTGGGAGTCACS	2029-2048	VP2	640	
A6R	GCTGTTTTRGGGTCTGTTGTCACC	2645-2668	VP3		
A7F	CTGGACYCTGGTRGTGATGG	2477-2496	VP2	502	
A7R	GTACGCCACCATGTASCGG	2960-2978	VP3		
A8F2	GGYYTGGTGACAACAGACCC	2640-2659	VP3	806	
A8R2	SCGTGYTGGTGKGTTTGC	3428-3445	VP1		
A9F	GTACAGGGYTGGGTCTGC	3144-3161	VP3	630	
A9R	CGTGGCRRGAATTGCACC	3756-3773	VP1		
A10F	GGTYCCCAATGGAGCACC	3530-3547	VP1	1006	
NSP 1R2	CTTCTGAGGCGATCCATG	4517-4535	2C		
NSP 1F	GAGACGTYGAGTCCAACCC	3939-3958	2B	1051	
NSP 2R	GCCATRGGCGGGATRAA	4972-4989	2C		
NSP 2F	CAGCTCARAGCACGT GAC AT	4423-4443	2C	1047	
NSP 3R	ACCATCCCCTCRAAGAAY TC	5449-5469	3A		
NSP 3F	TGACCACTTYGACGGTTA	4860-4878	2C	746	
NSP 4R	CATRATCACTATGTTTGCCA	5585-5605	3A		
NSP 4F	CGRAGGTTYCACTTTGAC	5098-5116	3A	928	
NSP 5R	CACTTTCAAAGCGACAGG	6007-6025	3C		
NSP 5F	GAATTCTTTGAGGGGATGGT	5449-5469	3A	947	
NSP 6R	GGGGGTKCCYTTCTTCAT	6377-6395	3C		
NSP 6F	CRAGCTGAAGGACCCTAC	5831-5849	3B	1106	
NSP 7R	GACGCGTAGTCRGCAGC	6922-6939	3D		
NSP 7F	GGACAGGACATGCTCTCAG	6283-6302	3C	657	
NSP 7R	GACGCGTAGTCRGCAGC	6922-6939	3D		
NSP 8F	ATGCGCAAAACCAAGCT	6736-6753	3D	589	
NSP 8R	AATTTGCGGTCCGTTGT	7307-7324	3D		
NSP 8F	ATGCGCAAAACCAAGCT	6736-6753	3D	943	
NSP 9R	GTCCAGCTCRACTCCCTC	7660-7678	3D		
NSP 10F	AACGTGTGGGATGTGGA	7393-7410	3D	833	
T21R	CAGGAAACAGCTATGAC TTTTTTTTTTTTTTTTTTTTR	8186-8225	3′UTR		

PCR reaction mix was prepared with the addition of 2X GoTaq® Hot Start Colorless Master Mix (Promega, USA), 400nM of each of the primer pairs and sufficient Nuclease free water. Positive control and no template negative controls were added in parallel to authenticate the PCR. The cDNA prepared from extracted RNA of the proposed vaccine candidates was used as template. The positioning of the overlapping primer pairs is presented in the schematic representation in **Figure. 2.7.1**

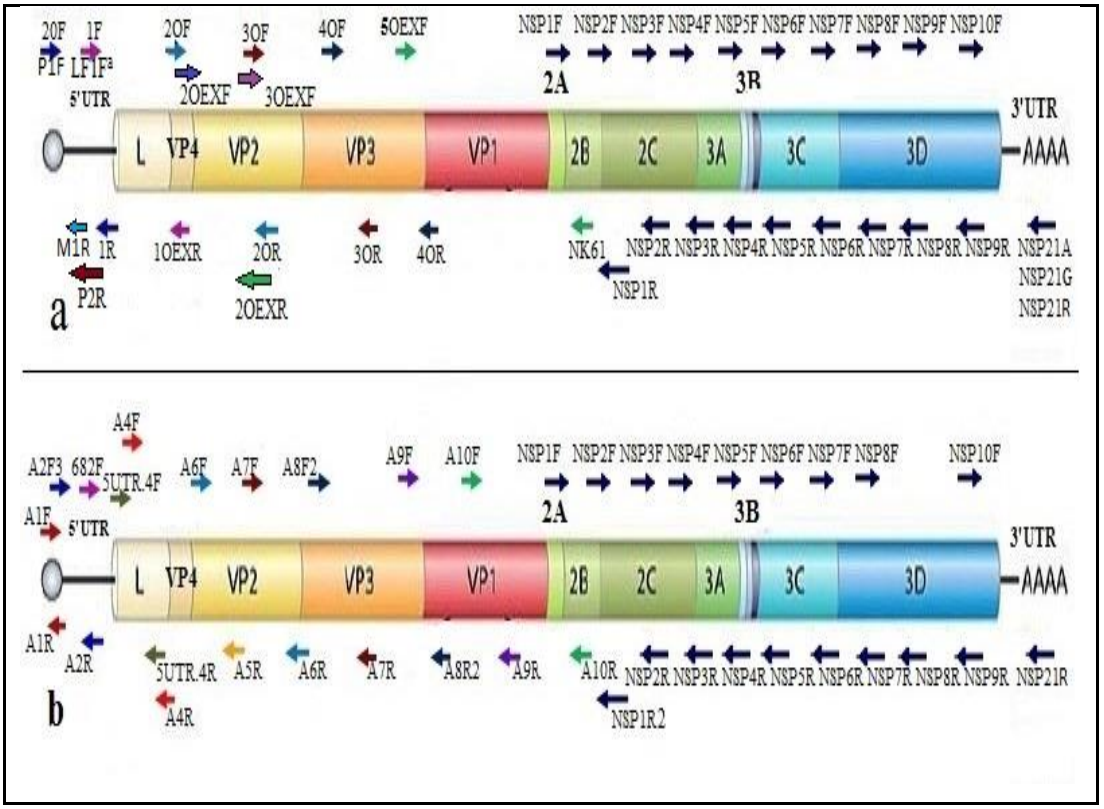


Figure 2.7.1: Primer pairs used to amplify the entire genome of (a) FMDV serotype O and (b) serotype A

The PCR reactions were prepared as the protocol described in **Table 2.3.3.1b**. Reaction mixtures added with template cDNA were heated at 94º C for 5 minutes followed by 94º C for 1 minute, respective annealing temperature for 1 minutes and 72º C for 1 minute 30 seconds. Final Extension was set at 72º C for 7 minutes.

Amplified PCR products were visualized, purified and concentration measured according to the protocols described in section **2.3.4**, **2.5.3** and **2.5.4** respectively. In case of any non-specific amplification, gel purification of the desired amplicon was done according to the method described in section **2.5.3** with an exception that gel was prepared by low melting point Agarose (Invitrogen, USA) in this case. Total PCR product was loaded in more than one well of the gel and run. After completion of the run the gel was placed on UV-

Illuminator (Biometra, USA), desired band was excised and collected in a 1.5 ml microcentrifuge tube, and then purified by Wizard® SV Gel and PCR Clean-Up System (Promega, USA).

2.7.2 Sequencing of the Entire Genome

Sequencing was performed according to protocol described in section **2.5.5**.

2.7.3 Assembling of Raw Sequences

Overlapping sequences spanning the entire genome was assembled in to contigs using SeqMan version 7.0.0 (Lasergene, DNASTAR, USA) and finally all the contigs were assembled into consensus sequences. All the parameters of the assembly project was set as default. Degenerate traces shown in the consensus was fixed by subsequent BLAST search. The assembly project was imported as both SeqMan and FASTA file format.

2.7.4 Complete Genome Annotation

Annotation of the complete genome was done according to National Centre for Biotechnology Information (NCBI) Ref Seq (Reference sequence for Foot-and-Mouth Disease Virus). Pairwise and Multiple alignment of Ref Seq and complete genomes in built-in ClustalW of MEGA7 was performed to annotate the complete genome. Open Reading Frame (ORF) of FMDV was annotated by pairwise and multiple alignment by the built-in ClustalW Codon of MEGA7.

2.7.5 Comparative Sequence Analysis

For inactivated FMD vaccine only three surface capsid proteins VP1, VP2 and VP3 are important for eliciting protective immunity in vaccinated animals whereas VP4 is the internal protein and has no role in producing neutralizing antibody (Reeve *et al.*, 2010). Nucleotide and deduced amino acid sequences of VP1, VP2 and VP3 regions of proposed FMDV serotype O and A vaccine candidates were compared with those of respective current vaccine strains using Needleman-Wunsch algorithm. VP4 and nonstructural protein coding regions were not considered in this study.

2.7.6 Prediction of the 3-D Structure of VP1 Region

The amino acid sequence of VP1 region of both current and proposed FMDV vaccine strains were uploaded one by one to SWISS-MODEL protein structure homology

modeling server (Bertoni *et al.*, 2017) to generate PDB files. Afterwards, the PDB file was visualized via PyMOL.

2.8 Adaptation and Propagation of Vaccine Candidates in Cell Culture

Once the vaccine candidates were selected through sequence based molecular approach, they were adapted to grow rapidly in BHK-21 cell line. Subsequently, bulk amount of cell culture fluid for each serotype was propagated following the procedure described in **sub-section 2.4.1**, **2.4.2**, **2.4.3** and **2.4.5**. But instead of using 25 cm^2 cell culture flasks, 175 cm^2 flasks were used in this case. Two (02) ml of 0.25% trypsin EDTA solution was used to detach the cells during sub-culturing and cells were supplemented with 100 ml of growth medium. Cells monolayers were infected with 1 ml of virus fluid and 100 ml of maintenance medium was added to the flask. Flasks were observed for next 18 to 20 hours for CPE and virus was harvested following the protocol described in **sub-section 2.4.6**.

2.9 Virus Inactivation

2.9.1 Preparation of Binary Ethylenimine (BEI)

Foot and Mouth Disease virus was inactivated by adding Binary Ethylenimine (BEI) to the virus suspension. BEI was prepared from 0.1M 2-bromoethylamine hydrobromide (BEA) by cyclizing in presence of 175mM sodium hydroxide (NaOH) solution at 37^0 C for 1hour (H. G. Bahnemann, 1990; Patil *et al.*, 2002). To prepare 100 ml of BEI, 700 mg of NaOH (Sigma-Aldrich, Germany) was weighed and taken in an autoclaved 250 ml glass bottle (Duran, Germany). Then Nano pure water was added in the bottle up to 100 ml and mixed thoroughly to make 175mM sodium hydroxide (NaOH) solution. Afterwards, 2.05 gm of 2-bromoethylamine hydrobromide (Merck KGaA, Germany) was weighed and added to the already prepared 100 ml of 175mM sodium hydroxide (NaOH) solution and mixed. The bottle was incubated at 37^0 C for 1hour to form BEI. pH of the solution was recorded before and after incubation by a pH meter (HANNA, USA). Finally, the BEI solution was filtered through 0.22 µm syringe filter assembly.

2.9.2 Addition of Binary Ethylenimine (BEI) to the Virus Suspension

The inactivation of FMDV was carried out using 3mM BEI. The prepared BEI was added at 3% (v/v) to the virus suspension to a final BEI concentration of 3mM and the mixture was incubated at 37^0 C for 24 hours (H. G. Bahnemann, 1990; Patil *et al.*, 2002). For inactivating 1.5L of FMD virus suspension in a 2L capacity bottle, 46.4 ml of BEI was

added and incubated. The bottle was shaken at every 30 minutes interval. During inactivation process 900 µl virus samples were taken from the bottle at every hour interval starting from 0 hour up to 24th hour. Each hourly sample was mixed with 100 µl of 20% sodium thiosulfate in a 1.5 ml sterile micro centrifuge tube and preserved at -80^{0} C until further use.

After completion of 24 hours virus inactivation cycle excess BEI in the virus suspension was neutralized by adding 170 ml of 20% sodium thiosulfate solution **(Appendix-II)** at 10% (v/v) to make a final concentration of 2% (Martín-Acebes *et al.*, 2011).

2.9.3 Inactivation Monitoring

Virus inactivation was monitored following the procedures of Aarthi *et al.*, 2004 and Ismail *et al.*, 2013. Virus titer was assayed in terms of Tissue Culture Infective Dose$_{50}$ ($TCID_{50}$).

2.9.3.1 Virus Titration in Hourly Samples

Virus titer in each of the hourly samples collected during inactivation process was assayed in terms of $TCID_{50}$ (Tissue Culture Infective Dose$_{50}$).

2.9.3.1.1 Preparation of 0.5log$_{10}$ Virus Dilution Series

A 0.5log$_{10}$ virus dilution series was prepared in sterile 1.5 ml micro centrifuge tubes (Eppendorf) according to the **Table 2.9.3.1.1**

Table 2.9.3.1.1: Preparation of 0.5log$_{10}$ dilution series

Sl no.	Dilution step	Log$_{10}$ dilution step	Vol. of DMEM (µl)	Vol. of virus to be transferred (µl)	Total volume (µl)
1	$10^{-0.5}$	-0.5	684	316 (neat virus)	1000
2	10^{-1}	-1.0	684	316	1000
3	$10^{-1.5}$	-1.5	684	316	1000
4	$10^{-2.0}$	-2.0	684	316	1000
5	$10^{-2.5}$	-2.5	684	316	1000
6	$10^{-3.0}$	-3.0	684	316	1000
7	$10^{-3.5}$	-3.5	684	316	1000
8	$10^{-4.0}$	-4.0	684	316	1000
9	$10^{-4.5}$	-4.5	684	316	1000
10	$10^{-5.0}$	-5.0	684	316	1000
11	$10^{-5.5}$	-5.5	684	316	1000
12	$10^{-6.0}$	-6.0	684	316	1000

13	$10^{-6.5}$	-6.5	684	316	1000
14	$10^{-7.0}$	-7.0	684	316	1000
15	$10^{-7.5}$	-7.5	684	316	1000

Fifteen sterile 1.5 ml micro centrifuge tubes were taken in a rack. In all tubes 684 µl of DMEM was taken. Three hundred and sixteen (316) µl neat virus was added in the first tube, mixed well and transferred 316 µl in the second tube. The procedure was repeated up to the last tube. The samples were diluted from $10^{-0.5}$ to $10^{-7.5}$ ($0.5\log_{10}$ to$7.5\log_{10}$) dilutions.

2.9.3.1.2 Addition of Virus Dilutions to the Cell Culture Plate

The diluted virus was added to appropriate wells of a 96-well cell culture plate (Nunc) shown in the **Figure 2.9.3.1.4**. Two virus samples were accommodated in a 96-well cell culture plate. Fifty (50) µl of DMEM was added to all the selected wells in the plate except the bottom most row (12^{th}) where 100 µl of DMEM was added. The last row was used as the uninfected cell control. To four wells of each row of H, G, F and E columns, 50 µl of diluted virus starting from the highest dilution ($10^{-7.5}$ in row 11) to the lowest dilution ($10^{-2.5}$ in row 1) was added. Similarly, the dilutions of second virus sample were added to the wells of D, C, B and A columns. The plate was then incubated at 37° C for 1 hour.

2.9.3.1.3 Addition of BHK-21 Cells to the Plate

BHk-21 cell monolayer was detached and cell suspension was prepared following the procedure described in **section 2.4.3**. The concentration of the cells/ml of medium was counted by Countess II (Life Technologies, USA) cell counter following the manufacturer's guidelines. Cell concentration was then adjusted to 10^6/ml and 50 µl of BHK-21 cell suspension was added to each of the selected wells. The plate was covered with lid and incubated at 37° C in a CO_2 incubator for 48-72 hours.

2.9.3.1.4 Staining the Plate and Scoring

After 48-72 hours of incubation the plate was stained with 0.1% crystal violet in 10% formalin solution **(Appendix-I)**. Wells which stained (>50% of surface) were scored - and which did not stain were scored +. The scores were recorded in the result sheet (**Figure 2.9.4.1.4**) and $TCID_{50}$/ml was calculated using Karber's method from the following formula (Kärber, 1931)-

Log_{10} $TCID_{50}$ = L-d(s-0.5)

Where, L= log_{10} of the most concentrated virus dilution tested

d= log dilution factor

s= sum of proportions

(1.3 was added with the calculated value as the test is done in 50 µl volume)

Virus Titration Result Sheet

Date of run: 01/12/2016 Date of staining: 04/12/2016

Virus 1: Serotype: A ID No: FMDV-304 (0 hour) Log_{10}virus tire/50 µL:

Virus 2: Serotype: A ID No: FMDV-304 (1 hour) Log_{10}virus tire/50 µL:

Log_{10} virus dilution series - -2.5 to -7.5 for virus 1 and -2.0 to -7.0 for virus 2

0.5 Log_{10} virus dilution

(50 µL diluted virus + 50 µL infection medium + 50 µL cell suspension)

	Virus 1				Virus 2				
	H	G	F	E	D	C	B	A	
$10^{-2.5}$	+	+	+	+	+	+	+	+	1
$10^{-3.0}$	+	+	+	+	+	+	+	+	2
$10^{-3.5}$	+	+	+	+	+	+	+	+	3
$10^{-4.0}$	+	+	+	+	+	+	+	+	4
$10^{-4.5}$	+	+	+	+	+	+	+	+	5
$10^{-5.0}$	+	+	-	+	+	+	+	+	6
$10^{-5.5}$	+	+	+	-	-	+	+	-	7
$10^{-6.0}$	-	-	-	+	-	-	+	-	8
$10^{-6.5}$	-	+	-	-	-	-	-	-	9
$10^{-7.0}$	-	-	-	-	-	-	-	-	10
$10^{-7.5}$	-	-	-	-	-	-	-	-	11
Cell control (100 µL infection medium + 50 µL cell suspension)									12

Figure 2.9.3.1.4: Virus titration result sheet. '+' indicates presence of CPE and '-' indicates absence of CPE. Four rows were used for a sample and two samples were accommodated in a plate.

2.9.3.2 Drawing of Inactivation Kinetics

Based on sampling hour and the virus infectivity titers of the hourly samples, the inactivation kinetics were calculated statistically using the slope and regression. The log_{10} infectivities of each timed sample was plotted against time of sampling and regression line

was built in Microsoft Excel 2016 work sheet. The regression line was extrapolated to determine the expected time required to inactivate a 10000 L batch and how much virus could be inactivated in a period of 24 hours.

2.9.3.3 Innocuity Testing

The absence of residual virus in the inactivated virus suspension was confirmed by innocuity testing in BHK-21 cell sheet. The test was carried out by inoculating 200 µl of inactivated virus in a 25 cm^2 cell sheet. The flask was observed for 2-3 days for cytopathic effects (CPE). Afterwards the cell sheet was freeze-thawed and passaged into a second cell sheet. The procedure was continued up to passage into a third cell sheet (OIE, 2012)

2.10 Inactivated Virus Particles Concentration

Inactivated FMD virus suspension was concentrated to its $1/10^{th}$ of original volume by ultrafiltration technique using 100 kDa hollow fiber cartridge (Model: UFP-100-C-4MA; GE Healthcare, USA) in AKTA Flux 6 (GE Healthcare, USA) protein concentrator (Adikane *et al.*, 1997). The cartridge was first assembled in AKTA Flux 6 protein concentration system and decontaminated by 1L 20% ethanol solution (v/v) followed by washing the system with adequate autoclaved Nano pure water. Then 1.5L of inactivated virus fluid was loaded in the Feed Tank and the machine was started to concentrate the fluid. Feed pump and permeate pump in the machine was set at 250 and 100 rpm respectively. The feed pressure (Pf) was maintained always below 1 bar. The virus fluid of each serotype was concentrated up to $1/10^{th}$ of its original volume.

2.11 Virus Particles Quantification

Virus particles concentrations in inactivated virus suspension, concentrate and permeate were quantified by size exclusion chromatography in AKTA Pure 25 (GE Healthcare, USA) Fast Performance Liquid Chromatography (FPLC) system. AKTA Pure 25 is fitted with a U9-D UV monitor with 2 mm optical path flow cell with selectable 254/280 nm filters, and a laptop computer running Unicorn® 7.0 control and analysis software. HiPrep Capto Core 700 (GE Healthcare, USA) size exclusion resin column of 1 ml capacity was assembled in AKTA Pure 25 FPLC system. Pumps of AKTA Pure 25 were washed with 20% ethanol and the whole flow path including the column was first decontaminated by 20% ethanol followed by Nano pure water wash. During chromatographic runs the flow rate was set at 1.3 ml/min, mobile phase was 30 mM Tris buffer with 400 mM NaCl, pH

8 (**Appendix-II**). Five hundred (500) µl of sample was injected through injection loop and detection was set at 254 nm. All peak fractions were collected in 15 falcon tubes through outlet1. The virus particles concentration was calculated directly from Unicorn® 7.0 software by integrating the area under the peak and using extinction coefficient 72 for the FMDV at 254 nm wave length (Hosseini *et al.*, 2016; Spitteler *et al.*, 2011).

2.12 Detection of FMDV Particles

The FMDV antigen present in the inactivated virus suspension, concentrate, permeate and their respective peak fractions were detected by FMDV serotype specific antigen detecting sandwich ELISA (Arsh Biotech, India). Manufacturer's prescribed protocol was used to perform the test. Appropriate wells of 96-well micro titer plate (Nunc, Denmark) were coated with 50 µl of serotype specific rabbit anti-FMDV antibodies (coating sera). Then the plate was incubated for 1 hour at 37^0 C and washed three times with wash buffer (PBS+0.1% Tween20). In the second step, 50 µl of reference antigens and test samples were added to appropriate wells, plate was incubated and washed. The third step was the addition of 50 µl of serotype specific guinea pig anti-FMDV antibodies (tracing sera) followed by incubation and washing. In the fourth step, rabbit anti-guinea pig anti-antibodes tagged with HRP (Horse Radish Peroxidase) conjugate was added at 50 µl/well, then the plate was incubated and washed. Finally, OPD (Orthophenyl Diamine di hydrochloride) substrate at 50 µl/well was added and the plate was incubated at 37^0 C for 15 minutes for color development. The color reaction was stopped by addition of 0.1M H_2SO_4 solution and OD (Optical Density) value was read at 492 nm in GlowMax (Promega, USA) micro plate reader.

2.13 Vaccine Formulation

Commercial oil adjuvant Montanide™ ISA 201VG (Seppic, France) was used to formulate monovalent vaccine against FMDV serotype O and A. Equal amount of antigen and adjuvant (w/w) was mixed with homogenizer at 300 rpm at 30^0 C temperature to form water in-oil-in water double emulsions. Thirty (30) ml of monovalent vaccine against serotype O and A was prepared separately.

2.14 Vaccine Sterility and Safety Testing

For sterility testing, 100 µl of formulated vaccine was streaked on nutrient agar plate and incubated overnight at 37^0 C. The plate was then observed for bacterial growth. The safety

test of the vaccine was performed during efficacy testing in guinea pigs. Any undue local and systemic reactions attributable to the vaccines were recorded (OIE, 2012).

2.15 Vaccine Efficacy Testing

Both *In-vivo* and *In-vitro* tests were performed to determine the efficacy of experimentally developed FMD vaccines.

2.15.1 *In-Vivo* Efficacy Testing in Guinea pig Model

In-vivo vaccine efficacy tests were performed in the Animal Laboratory, Toxicology and Biomedical section, BCSIR, Dhaka, Bangladesh. Prior ethical approval (Ref. 37/Biol.Scs/2016-2017, Date: 23.05.2017) was taken from the appropriate authority in University of Dhaka for conducting the test **(Appendix-V)**.

Female guinea pigs of Dunkin Hartley breed having body weights 360-440 g were used in the experiment. A total of 74 guineapigs-24 for virus adaptation and 50 for challenge tests were used in this study. The guinea pigs were purchased from the icddr,b animal lab facilities, Mohakhali, Dhaka. The guinea pigs were reared in the animal laboratory with ad-libitum feeding and watering and vitamin C supplements to gain the expected body weight. Commercial pellets and carrots were given as feed and autoclaved tap water was supplied as drinking water.

2.15.1.1 Adaptation of FMDV Vaccine Candidates in Guinea Pigs

Foot and Mouth Disease virus do not naturally infect guinea pigs. For preparation of challenge virus FMDV serotype O isolate BAN/TA/Dh-301/2016 and A isolate BAN/CH/Sa-304/2016 were adapted in guinea pigs. Twelve female guinea pigs were used for every virus strain where 2 animals were used for each virus passage. One hundred (100) µl of viral suspension was injected intra-dermally in the metatarsal pad of the left hind foot. The animals were then observed daily up to 4-5 days for primary and secondary lesions. Edema and vesicle formation only in the injected foot pad was considered as primary lesions and those in injected as well as other foot pads were considered as secondary lesions (Núñez *et al.*, 2001). After edema and vesicle formation the animals were euthanized and vesicular fluid and epithelia around the vesicles were collected, homogenized, inoculum prepared in sterile PBS and used for further inoculations in the second passage. The procedure was repeated up to 6^{th} passage until complete adaptation

(El-Sayed *et al.*, 2012). The capability of FMDV to produce secondary lesions is considered evidence of adaptation to the new host (Núñez *et al.*, 2001).

(a) **(b)**

Figure 2.15.1.1: Adaptation of FMDV in guinea pigs. Guinea pigs used for virus adaptation (a), injection of virus in foot pad of guinea pigs at first passage (b).

2.15.1.2 Challenge Test in Guinea pigs

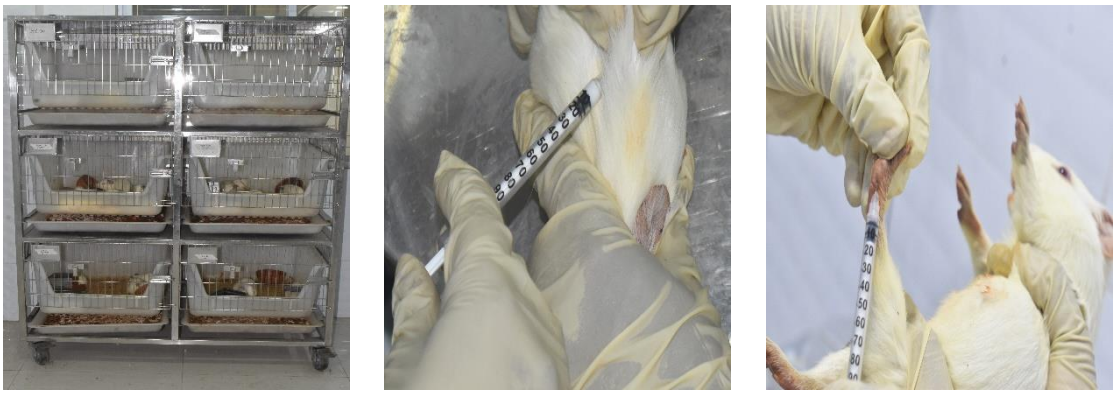
Twenty five healthy guinea pigs were used in the challenge test for each monovalent vaccine. The guinea pigs were grouped into five groups each containing 5 animals(Choudary *et al.*, 2008). The animals of first four groups were vaccinated with different dilutions of vaccine and the 5th group was kept as non-vaccinated control group (**Table 2.15.1.2a**).

Table 2.15.1.2a: Grouping and vaccination of guinea pigs

Group No.	No. of guinea pigs	Dose of vaccine	Route of vaccination	Vaccine dilution factor (log_{10})
1	5	2 ml (Full dose)	Subcutaneous	0.6 (4-fold dilution)
2	5	500 µl	Subcutaneous	
3	5	125 µl	Subcutaneous	
4	5	31.25 µl	Subcutaneous	
5	5	Placebo (2 ml) **(Appendix-I)**	Subcutaneous	-

All the guinea pigs were vaccinated at the same time on the same day. Feeding and watering were kept as usual. The presence of local swelling at injection site or rise in body temperature were observed and recorded (**Table 2.15.1.2b**). On the 21st day of vaccination

all the animals were challenged with 100 $TCID_{50}$ of guinea pig adapted homologous virus in 100 µl (Choudary *et al.*, 2008) intradermally in foot pad of hind left foot.



(a) **(b)** **(c)**

Figure 2.15.1.2: Groups of guinea pigs used in the experiment (a), vaccination in guinea pigs (b), injection of challenge virus (c).

The guinea pigs were observed up to 8th day post challenge for swelling or vesicle formation in any foot pad. Swelling or vesicle formation in all four or in any one of foot pads after challenge was considered as 'un protected'. The data was recorded (**Table 2.15.1.2b**) and Protective $Dose_{50}$ (PD_{50}) of vaccine was calculated by Karber's method (**Section 2.9.3.1.4**).

Table 2.15.1.2b: Post challenge data recording

Group No.	No. of guinea pigs	Swelling at injection site	Rise in body temperature (^{0} C)	Lesion development	No of protected animals	No of un-protected animals
2 ml (Full dose)	5					
500 µl	5					
125 µl	5					
31.25 µl	5					
Placebo (2 ml)	5					

2.15.1.3 Blood Collection

Pre vaccinal blood was collected prior to vaccination on the first day and post vaccinal blood was collected prior to challenge on the 21st day from all the guinea pigs used in the

experiment. One to 2 ml of blood was collected from lateral tarsal vein of hind limbs (Birck *et al.*, 2014). The blood was gently placed in 15 ml falcon tube and kept upright overnight at 4^0 C for clotting of blood. After clotting the falcon tubes were centrifuged at 600xg for 15 minutes and serum was collected by micropipette and preserved in -20^0 C until use (Motamedi Sedeh *et al.*, 2007).

2.15.2 *In-Vitro* Vaccine Efficacy Testing

In-vitro efficacy of the experimental vaccines were assayed by virus neutralization test (VNT) and two dimensional micro neutralization test (2D-MNT).

2.15.2.1 Virus Neutralization Test

The quantitative virus neutralization tests for FMD antibody were performed with BHK-21 cells in 96 well flat-bottomed tissue culture grade micro titer plates (Nunc, Denmark). The preserved sera were thawed and inactivated at 56° C for 30 minutes before testing. Twofold dilution of the sera starting from 1/4 to 1/1024 were prepared in 50 µl of DMEM per well. Two rows of wells for each serum were used and 4 samples were accommodated in a plate (**Figure 2.15.2.1**). Four wells for serum and cell control were maintained separately. Previously titrated homologous virus containing 100 $TCID_{50}$/50 µl was added to each well except in wells of cell control. The plates were then incubated at 37° C for 1. A cell suspension at 10^6 cells/ml was prepared and added to the plates as described in **section 2.4.3 and 2.9.3.1.3**. Plates were covered with loosely fitting lids and incubated in an atmosphere of 5% CO_2 at 37° C for 2–3 days. The plates were then stained and scored according the protocols described in **section 2.9.3.1.4**. Serum Neutralization $Titer_{50}$ (SN_{50}) were calculated by Karber's method (Kärber, 1931).

Virus Neutralization Test (VNT) Result Sheet

Virus ID	:	Isolate: BAN/TA/Dh-301/2016		Serum ID	:	S1	Group1/S1
		Serotype: O				S2	Group1/S2
		$TCID_{50}$:				S3	Group1/S3
		Virus Dose:100 $TCID_{50}$/50 µl/well				S4	Group1/S4
Serum Dilution Series: 1:4 to 1:1024							
Log_{10} SN_{50} Titer	:	S1: 2.55	S2: 2.55	S3: 2.1	S4: 2.25	Log_{10} Virus $TCID_{50}$ Titer	: 2.1

Plate Layout:

		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024		Virus Control	
S1	A	-	-	-	-	-	-	-	+	+	Cell Control	1:4 (+)	1:4(+)
	B	-	-	-	-	-	-	-	+	+		1:8(+)	1:8(+)
S2	C	-	-	-	-	-	-	-	+	+		1:16(+)	1:16(+)
	D	-	-	-	-	-	-	-	+	+		1:32(+)	1:32(+)
S3	E	-	-	-	-	-	+	+	+	+	Serum Cont.	1:64(+)	1:64(+)
	F	-	-	-	-	-	-	+	+	+		1:128(+)	1:128(-)
S4	G	-	-	-	-	-	-	+	+	+		1:256(-)	1:256(-)
	H	-	-	-	-	-	-	+	+	+		1:512(-)	1:512(-)
		1	2	3	4	5	6	7	8	9	10	11	12

"+" =Virus growth (CPE present), "-" = Cell growth (No CPE)

Figure 2.15.2.1: Representative virus neutralization test (VNT) result Sheet. '+' indicates presence of CPE or unprotected wells and '-' indicates absence of CPE or protected wells.

2.15.2.2 Two Dimensional Micro Neutralization Test (2D-MNT)

Two Dimensional Micro Neutralization Test (2D-MNT) was used to determine serological relationship 'r' value between field isolate and vaccine virus of the same serotype (M. Rweyemamu *et al.*, 1978). The guinea pig sera obtained from first group of animals (full dose vaccine group) were pooled and used in the experiment. In case of serotype O, r values were calculated for 7 field isolates of different lineage and year and in case of serotype A, r values were calculated for 4 field isolates (**Table 2.15.2.2)**.

Table 2.15.2.2: List of isolates selected for two dimensional micro neutralization test

Serotype O			Serotype A		
Isolate ID	**District**	**Sublineage**	**Isolate ID**	**District**	**Genotype**
BAN/TA/Dh-301/2016	Tangail	Ind2001BD1	BAN/CH/Sa-304/2016	Chittagong	VII
BAN/MA/Ku-269/2015	Moulivibazar	Ind2001BD1	BAN/DH/Sa-307/2017	Dhaka	VII
BAN/Go/Ka-236/2015 (Pig)	Gopalganj	Ind2001BD1	BAN/DH/Sa-310/2017	Dhaka	VII
BAN/GA/Ka-212/2014	Gazipur	Ind2001d	BAN/GA/Sa-197/2013	Gazipur	VII
BAN/JA/Me-180/2013	Jamalpur	Ind2001d			
BAN/BO/Na-161/2013	Bogra	Ind2001BD2			
BAN/NA/Ha-156/2013	Noakhali	Ind2001d			

2.15.2.2.1 Test Procedure

An aliquot of pooled serum was thawed, heat inactivated at 56° C for 30 minutes. A series of 0.3$\log_{10}$ (2-fold) dilution staring from 1:32 to 1:1024 was prepared according to the T**able 2.15.2.2.1**.

Table 2.15.2.2.1: Preparation of 0.3$\log_{10}$ dilution series

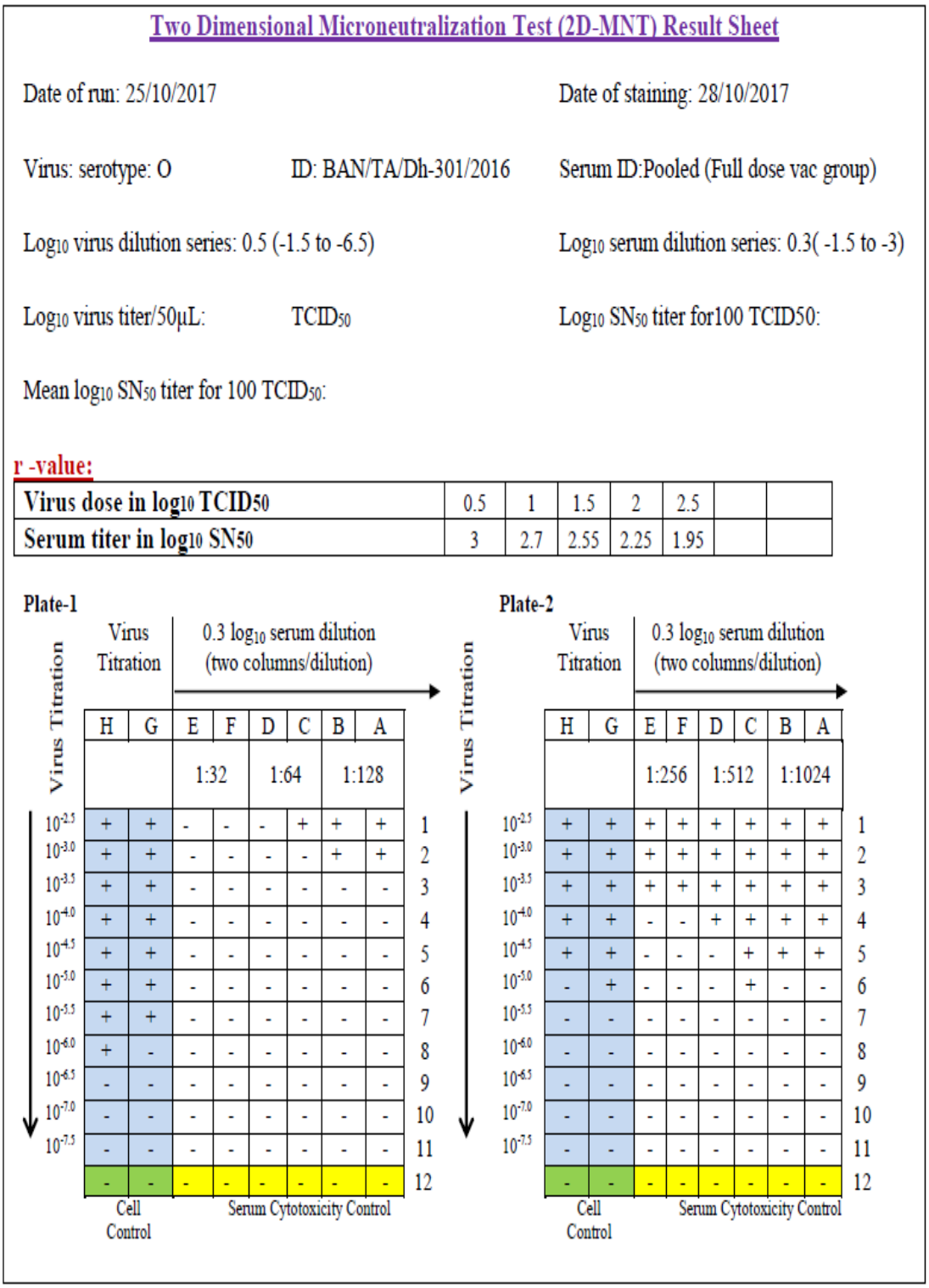
Sl no.	Dilution step	Log_{10} dilution step	Volume of DMEM (ml)	Volume to be transferred	Total volume (ml)
1	1:32	1.5	7.75	250 µl neat pooled serum	8
2	1:64	1.8	4	4 ml	8
3	1:128	2.1	4	4 ml	8
4	1:256	2.4	4	4 ml	8
5	1:512	2.7	4	4 ml	8
6	1:1024	3.0	4	4 ml	8

Four (4) ml volume for each dilution of guinea pig pooled serum was sufficient to test one heterologous and virus along with homologous virus.

The virus was diluted according to the **Table 2.9.3.1.1** except dilution was prepared in 2000 µl here. Two tissue culture 96-well plates for each virus-serum combination was labelled for name of virus, serum, date and plate number according to the **Figure 2.15.2.2.1**. DMEM at 50 µl/well was added in virus titration columns (H1 to H11 and G1 to G11) and serum cytotoxicity control wells (F12 to A12), while 100 µl/ well was added to cell control wells (H12 and G12) in all the plates.

Diluted serum at 50 µl/well was added as designed in the plates. Diluted virus at 50 µl/well was also added in all wells except in cell control wells according to plate design. The plates

were so designed that each serum dilution was treated with each virus dilution. The plates were then gently mixed, covered with lids and incubated at 37^0 C for 1 hour.



Two Dimensional Microneutralization Test (2D-MNT) Result Sheet

Date of run: 25/10/2017 Date of staining: 28/10/2017

Virus: serotype: O ID: BAN/TA/Dh-301/2016 Serum ID:Pooled (Full dose vac group)

Log_{10} virus dilution series: 0.5 (-1.5 to -6.5) Log_{10} serum dilution series: 0.3(-1.5 to -3)

Log_{10} virus titer/50µL: $TCID_{50}$ Log_{10} SN_{50} titer for100 TCID50:

Mean log_{10} SN_{50} titer for 100 $TCID_{50}$:

r -value:

Virus dose in log_{10} $TCID_{50}$	0.5	1	1.5	2	2.5		
Serum titer in log_{10} SN_{50}	3	2.7	2.55	2.25	1.95		

Plate-1

Virus Titration	H	G	E	F	D	C	B	A	
			1:32		1:64		1:128		
$10^{-2.5}$	+	+	-	-	-	+	+	+	1
$10^{-3.0}$	+	+	-	-	-	-	+	+	2
$10^{-3.5}$	+	+	-	-	-	-	-	-	3
$10^{-4.0}$	+	+	-	-	-	-	-	-	4
$10^{-4.5}$	+	+	-	-	-	-	-	-	5
$10^{-5.0}$	+	+	-	-	-	-	-	-	6
$10^{-5.5}$	+	+	-	-	-	-	-	-	7
$10^{-6.0}$	+	-	-	-	-	-	-	-	8
$10^{-6.5}$	-	-	-	-	-	-	-	-	9
$10^{-7.0}$	-	-	-	-	-	-	-	-	10
$10^{-7.5}$	-	-	-	-	-	-	-	-	11
	-	-	-	-	-	-	-	-	12
	Cell Control		Serum Cytotoxicity Control						

Plate-2

Virus Titration	H	G	E	F	D	C	B	A	
			1:256		1:512		1:1024		
$10^{-2.5}$	+	+	+	+	+	+	+	+	1
$10^{-3.0}$	+	+	+	+	+	+	+	+	2
$10^{-3.5}$	+	+	+	+	+	+	+	+	3
$10^{-4.0}$	+	+	-	-	+	+	+	+	4
$10^{-4.5}$	+	+	-	-	-	+	+	+	5
$10^{-5.0}$	-	+	-	-	-	+	-	-	6
$10^{-5.5}$	-	-	-	-	-	-	-	-	7
$10^{-6.0}$	-	-	-	-	-	-	-	-	8
$10^{-6.5}$	-	-	-	-	-	-	-	-	9
$10^{-7.0}$	-	-	-	-	-	-	-	-	10
$10^{-7.5}$	-	-	-	-	-	-	-	-	11
	-	-	-	-	-	-	-	-	12
	Cell Control		Serum Cytotoxicity Control						

Figure 2.15.2.2.1: 2D-MNT result sheet. '+' indicates presence of CPE and '-' indicates absence of CPE. The log_{10} SN_{50} titers have been plotted against their respective virus dose.

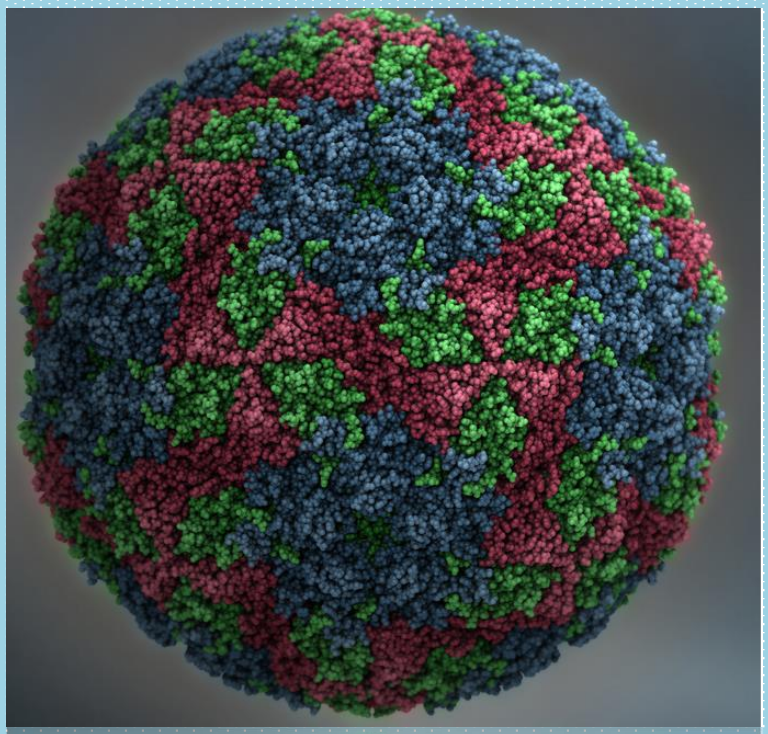
Sufficient cell suspension was prepared from BHK-21 monolayers. Cell concentration was adjusted to 10^6 cells/ml according to the **sections 2.4.3 and 2.9.3.1.3**, then added to the plates at 50 µl/well after completion of 1-hour incubation. After the addition of cells, the plates were incubated for 48-72 hours at 37^0 C with 5% CO_2 in an incubator. Upon completion of incubation the plates were stained, washed and scored according the protocols described in **section 2.9.3.1.4**.

2.15.2.2.2 Determination of Serum Neutralization$_{50}$ (SN_{50}) titer and r value

From the first two columns of both plates (H and G) for homologous and heterologous virus respective virus titer in terms of Tissue Culture Infective Dose$_{50}$ ($TCID_{50}$) was estimated using Karber's method (Kärber, 1931). The $\log_{10}$ dose of virus at any virus dilution was estimated by deducting $\log_{10}$ values, that is, just deducting $\log_{10}$ of a dilution from $\log_{10}$ of virus titer. Specific SN_{50} titer for any virus $\log_{10}$ dose was calculated by Karber's method. The SN_{50} titers were plotted against their respective $\log_{10}$ dose of virus and a linear regression line was constructed. From the regression equation the $\log_{10}$ SN_{50} titer for exactly 2 $\log_{10}$ $TCID_{50}$ was estimated for both homologous and heterologous viruses and r value was calculated from the following formula (M. M. Rweyemamu, 1984)-

$$r = \frac{\text{Arithmetic titer of vaccinal serum against the heterologous field virus}}{\text{Arithmetic titer of vaccinal serum against the homologous vaccine virus}}$$

r values greater than 0.3 indicate that the field isolates are sufficiently similar to the vaccine strain and use of a vaccine based on this strain is likely to confer protection against challenge with the field isolates. Conversely, values less than 0.3 suggest that the field isolate is sufficiently different from the vaccine strains and a vaccine based on these strains is less likely to protect (OIE, 2012).



Chapter 03:

Results

3.0 Results

Foot and mouth disease (FMD) is the most contagious disease of mammals and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. Vaccination and restriction of animal movement are the main tools to prevent this devastating disease. Though inactivated vaccines are used to control the disease worldwide, vaccine failure reports are also very common. This study focused on the molecular epidemiology of circulatory FMDV serotypes in Bangladesh, appropriate vaccine candidate selection and development of a tailored quality assured FMD vaccine suitable to control the disease in the region. The findings of this study can be highlighted like-

- Molecular epidemiology of FMD during 2012-2018;
- FMDV VP1 protein coding sequence-based bioinformatics analyses and selection of appropriate vaccine candidates;
- Complete genome sequencing of selected vaccine candidates and genome wide comparative analysis;
- Development of quality assured inactivated FMD vaccines;
- Efficacy testing of the developed vaccines

All these findings are deciphered in the upcoming text.

3.1 Epidemiology and Characterization of Circulatory FMDVs

During the period of 2012 to 2018, a total of 319 samples were collected from 47 different FMD outbreaks in 27 different districts of Bangladesh by our research group. The time period of my research was included in the mentioned time. Among the total samples, 121 representative samples were tested for serotype identification and molecular characterization of FMDVs circulating in Bangladesh. Analysis of the test results revealed that, 86 samples from 38 outbreaks were identified as FMDV serotype O, whereas, 26 samples from 6 outbreaks and 9 samples from 3 outbreaks were identified as FMDV serotype A and Asia1 respectively. During this 6-year study period, it is observed that FMDV serotype O was responsible for the occurrence of 38 (81%) outbreaks, serotype A was responsible for 6 (13%) and serotype Asia1 was responsible for 3 (6%) outbreaks **(Figure 3.1a)**.

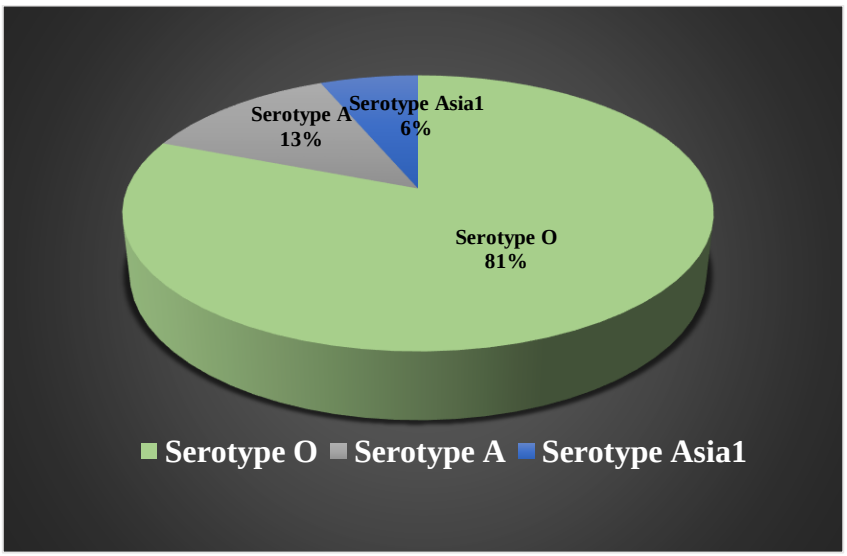


Figure 3.1a: Percentage of FMDV serotypes circulating in Bangladesh.

During 2012 to 2018, FMDV serotype O outbreaks were recorded in 27 districts. Animals of Dhaka and Gazipur districts were affected by all three circulating FMDV serotypes O, A and Asia1. Jashore district was affected solely by serotype Asia1. In addition to Dhaka and Gazipur, outbreaks of serotype A virus were also recorded in Chittagong district (**Figure 3.1b)**.

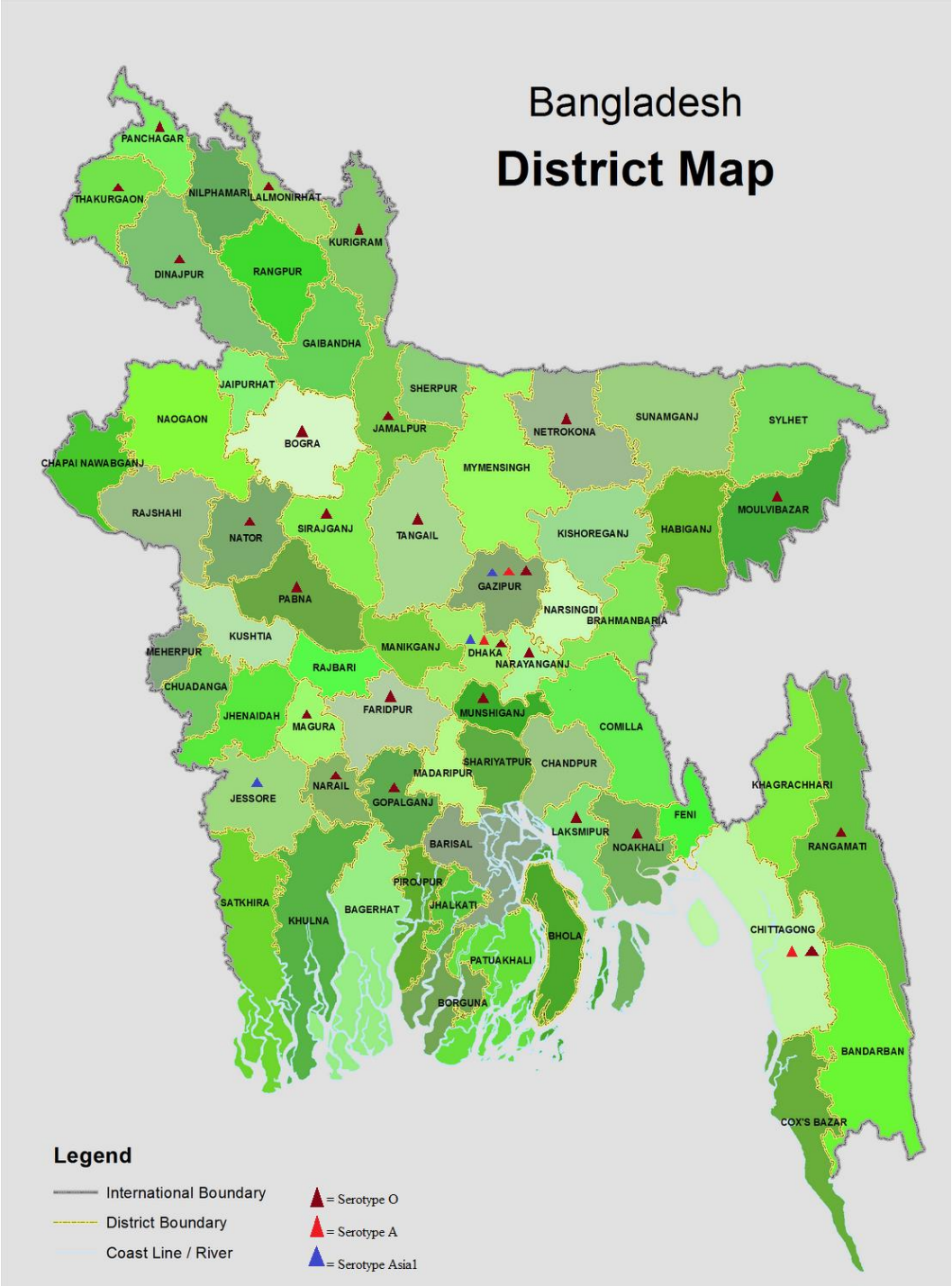


Figure 3.1b: Map showing distribution FMDV serotypes in Bangladesh.

3.2 Sequence Analyses and Vaccine Candidates Prediction

3.2.1 Phylogeny and Identification of Lineage, Sub-lineage or Types of FMDVs

Two different phylogenetic trees using respective datasets **(Appendix-III)** were constructed by the maximum-likelihood method for the determination of lineages,

sublineages or types of circulating FMDV isolates in Bangladesh **(Figure 3.2.1a and 3.2.1b).**

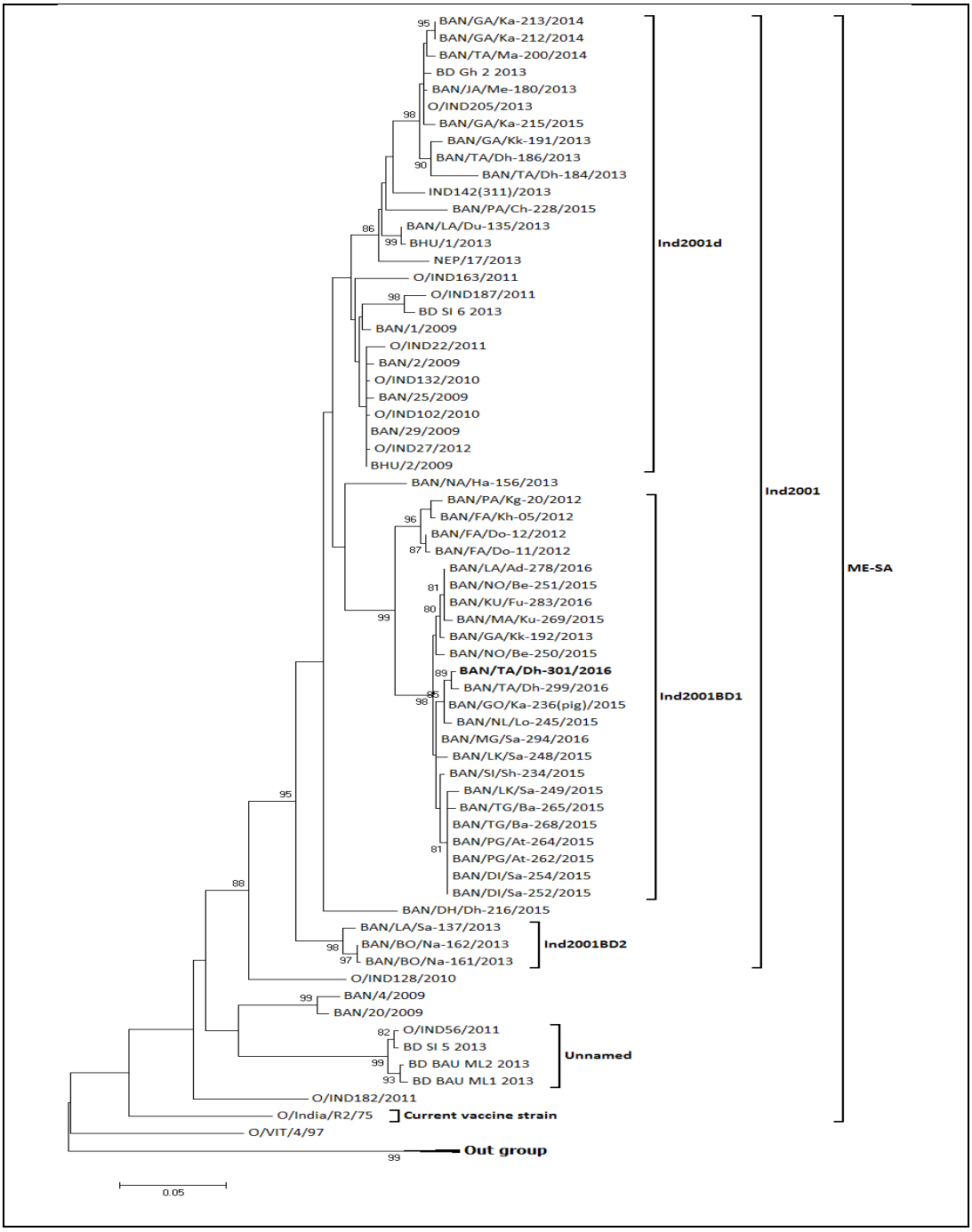


Figure 3.2.1a: FMDV serotype O VP1 coding region based phylogenetic tree constructed by maximum-likelihood method in MEGA7 software. The evolutionary distances were computed using the Tamura-Nei method (Tamura & Nei, 1993) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1).

The phylogenetic tree revealed that all the FMDV serotype O isolates of Bangladesh clustered in Ind2001d, Ind2001BD1 and Ind2001BD2 sublineages of Ind2001 lineage with exception to 3 isolates which are clustered in an unnamed group, but all are under Middle East-South Asia (ME-SA) topotype. Most of the recent isolates are under Ind2001BD1sublineages and this sublineage appeared as dominant sublineage over others in recent years.

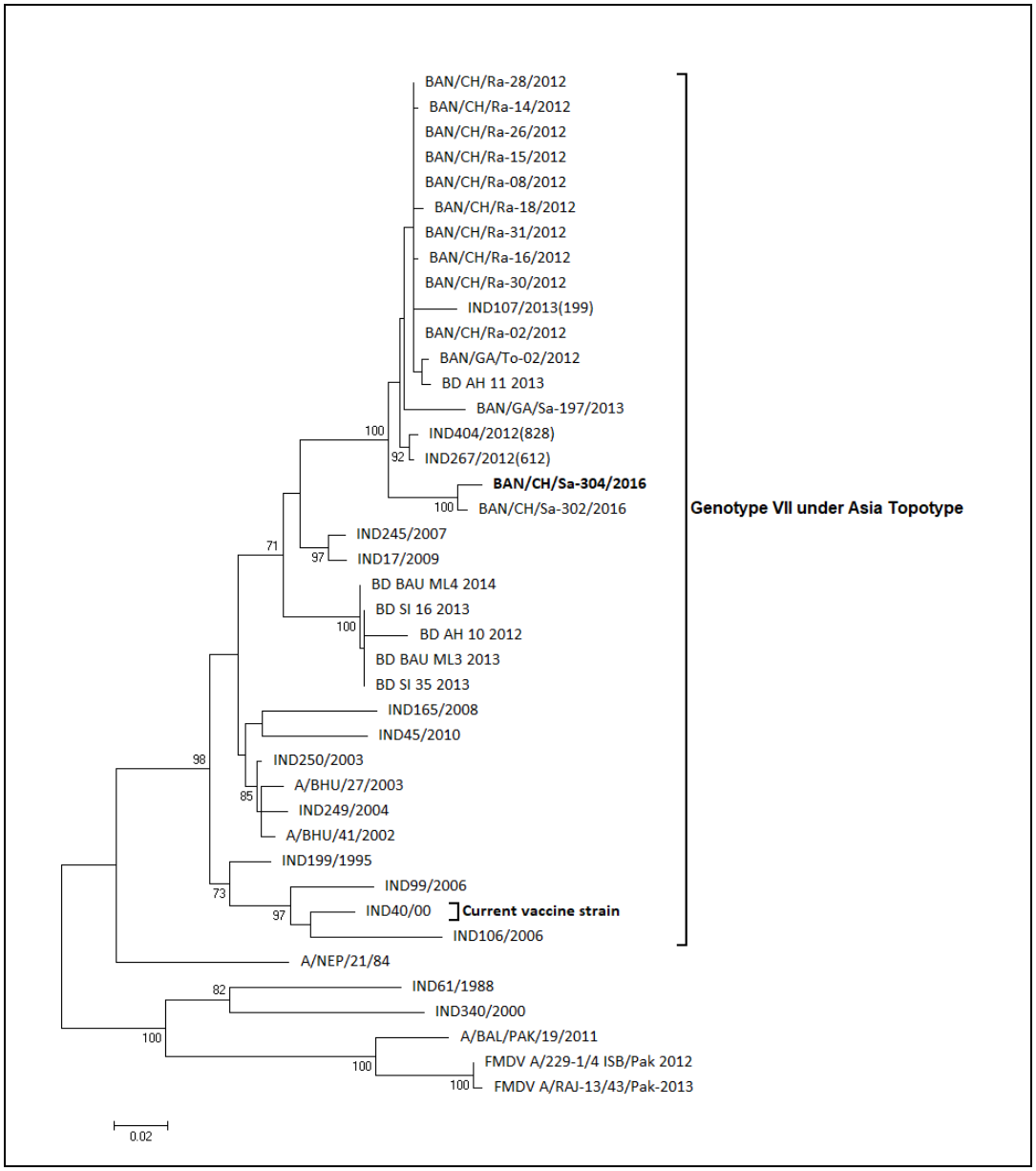


Figure 3.2.1b: FMDV serotype A VP1 coding region based phylogenetic tree constructed by maximum-likelihood method in MEGA7 software. The evolutionary distances were computed using the Tamura-Nei method (Tamura & Nei, 1993) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1).

The phylogenetic tree of FMDV serotype A showed that all respective isolates of Bangladesh are in Genotype VII under Asia Topotype.

For vaccine candidate selection the abundance of the circulation of sublineages in recent years were considered. Under serotype O, Ind2001BD1 was found to be the most abundant circulatory sublineage in Bangladesh. The most recent (at the time of analysis) isolate BAN/TA/Dh-301/2016 from this sublineage was preliminarily selected as vaccine candidate because of its high abundance and dominance. Similarly, BAN/CH/Sa-301/2016 isolate from the only circulating Genotype VII under Asia Topotype of serotype A was selected as vaccine candidate.

3.2.2 Genetic Distance Analyses

Significant genetic divergence in immunologically important site of vaccine strain with circulatory respective pathogen hinders vaccine efficacy. Immunologically important VP1 gene based genetic distance analyses were carried out to estimate degree of genetic divergence or closeness of present and proposed vaccine strains from FMDV isolates circulating in Bangladesh. The results are presented in **Table 3.2.2a**, **3.2.2b**, **3.2.2c** and **3.2.2d**.

Table 3.2.2a: Genetic Distance of Vaccine Strain O/India/R2/75 from FMDV Serotype O Isolates of Bangladesh

Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance
BAN/TA/Dh-301/2016	0.16	BAN/9/2009	0.15	BAN/TG/Ba-268/2015	0.15
BAN/NA/Ha-156/2013	0.17	BAN/5/2009	0.13	BAN/TG/Ba-265/2015	0.16
BAN/GO/Ka-236(Pig)/2015	0.16	BAN/4/2009	0.13	BAN/PG/At-264/2015	0.16
BD_SI_6_2013	0.17	BAN/3/2009	0.13	BAN/PG/At-262/2015	0.16
BD_Gh_2_2013	0.16	BAN/2/2009	0.16	BAN/DI/Sa-254/2015	0.16
BD_SI_5_2013	0.15	BAN/1/2009	0.16	BAN/DI/Sa-252/2015	0.16
BD_BAU_ML1_2013	0.15	BD_AH_21_2011	0.17	BAN/NO/Be-251/2015	0.15
BAN_PA_Kg-16_2012	0.19	BD_AH_20_2011	0.17	BAN/NO/Be-250/2015	0.16
BAN_PA_Sa-12_2012	0.19	AH_19_2013	0.17	BAN/LK/Sa-249/2015	0.16
BAN_PA_Ra-05_2012	0.19	BD_AH_18_2012	0.18	BAN/LK/Sa-248/2015	0.15
BAN_FA_Ka-01_2012	0.20	BD_AH_17_2012	0.18	BAN/NL/Lo-245/2015	0.16
BAN_FA_Kh-05_2012	0.17	BD_AH_16_2013	0.17	BAN/SI/Sh-234/2015	0.16

Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance
BAN/31/2009	0.15	BD_AH_15_2012	0.17	BAN/PA/Ch-228/2015	0.16
BAN/30/2009	0.15	BD_AH_14_2011	0.19	BAN/DH/Dh-216/2015	0.15
BAN/29/2009	0.15	BD_AH_13_2012	0.17	BAN/GA/Ka-215/2015	0.16
BAN/28/2009	0.15	BD_AH_12_2011	0.17	BAN/GA/Ka-213/2014	0.16
BAN/27/2009	0.16	BD_BAU_ML2_2013	0.15	BAN/GA/Ka-212/2014	0.16
BAN/26/2009	0.16	BAN/TA/Dh-299/2016	0.17	BAN/TA/Ma-200/2014	0.16
BAN/25/2009	0.16	BAN/MG/Sa-294/2016	0.15	BAN/GA/Kk-192/2013	0.16
BAN/24/2009	0.13	BAN/MG/Sa-287/2016	0.16	BAN/GA/Kk-191/2013	0.16
BAN/23/2009	0.16	BAN/KU/Fu-283/2016	0.15	BAN/BO/Na-162/2013	0.15
BAN/20/2009	0.12	BAN/LA/Ad-278/2016	0.15	BAN/BO/Na-161/2013	0.15
BAN/11/2009	0.16	BAN/MA/Ku-269/2015	0.16		

Table 3.2.2b: Genetic Distance of Proposed Vaccine Candidate BAN/TA/Dh-301/2016 from FMDV Serotype O Isolates of Bangladesh

Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance
BAN/TA/Dh-301/2016	-	BAN/9/2009	0.07	BAN/TG/Ba-268/2015	0.01
BAN/NA/Ha-156/2013	0.08	BAN/5/2009	0.14	BAN/TG/Ba-265/2015	0.02
BAN/GO/Ka-236(Pig)/2015	0.00	BAN/4/2009	0.14	BAN/PG/At-264/2015	0.01
BD_SI_6_2013	0.08	BAN/3/2009	0.14	BAN/PG/At-262/2015	0.02
BD_Gh_2_2013	0.09	BAN/2/2009	0.07	BAN/DI/Sa-254/2015	0.01
BD_SI_5_2013	0.15	BAN/1/2009	0.07	BAN/DI/Sa-252/2015	0.01
BD_BAU_ML1_2013	0.16	BD_AH_21_2011	0.07	BAN/NO/Be-251/2015	0.02
BAN_PA_Kg-16_2012	0.04	BD_AH_20_2011	0.08	BAN/NO/Be-250/2015	0.01
BAN_PA_Sa-12_2012	0.05	AH_19_2013	0.08	BAN/LK/Sa-249/2015	0.02
BAN_PA_Ra-05_2012	0.04	BD_AH_18_2012	0.08	BAN/LK/Sa-248/2015	0.01
BAN_FA_Ka-01_2012	0.04	BD_AH_17_2012	0.08	BAN/NL/Lo-245/2015	0.01
BAN_FA_Kh-05_2012	0.04	BD_AH_16_2013	0.07	BAN/SI/Sh-234/2015	0.01
BAN/31/2009	0.07	BD_AH_15_2012	0.07	BAN/PA/Ch-228/2015	0.10
BAN/30/2009	0.07	BD_AH_14_2011	0.09	BAN/DH/Dh-216/2015	0.08
BAN/29/2009	0.07	BD_AH_13_2012	0.08	BAN/GA/Ka-215/2015	0.08
BAN/28/2009	0.07	BD_AH_12_2011	0.07	BAN/GA/Ka-213/2014	0.09

Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance
BAN/27/2009	0.07	BD_BAU_ML2_2013	0.16	BAN/GA/Ka-212/2014	0.09
BAN/26/2009	0.07	BAN/TA/Dh-299/2016	0.01	BAN/TA/Ma-200/2014	0.09
BAN/25/2009	0.07	BAN/MG/Sa-294/2016	0.01	BAN/GA/Kk-192/2013	0.02
BAN/24/2009	0.15	BAN/MG/Sa-287/2016	0.01	BAN/GA/Kk-191/2013	0.09
BAN/23/2009	0.07	BAN/KU/Fu-283/2016	0.02	BAN/BO/Na-162/2013	0.08
BAN/20/2009	0.13	BAN/LA/Ad-278/2016	0.02	BAN/BO/Na-161/2013	0.08
BAN/11/2009	0.07	BAN/MA/Ku-269/2015	0.02		

Table 3.2.2c: Genetic Distance of Vaccine Strain IND40/00 from FMDV Serotype A Isolates of Bangladesh

Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance
BAN/CH/Sa-304/2016	0.13	BAN_CH_Ra-18_2012	0.11	BD_AH_10_2012_VP1	0.12
BD_SI_16_2013	0.11	BAN_CH_Ra-16_2012	0.11	BD_BAU_ML4_2014	0.10
BAN_CH_Ra-31_2012	0.10	BAN_CH_Ra-15_2012	0.11	BD_BAU_ML3_2013	0.10
BAN_CH_Ra-30_2012	0.11	BAN_CH_Ra-14_2012	0.11	BD_SI_35_2013	0.11
BAN_CH_Ra-02_2012	0.10	BAN_CH_Ra-08_2012	0.11	BAN/CH/Sa-302/2016	0.13
BAN_CH_Ra-28_2012	0.11	BAN_GA_To-02_2012	0.09	BAN_GA_Sa-197_2013	0.12
BAN_CH_Ra-26_2012	0.11	BD_AH_11_2013	0.11		

Table 3.2.2d: Genetic Distance of Proposed Vaccine Candidate BAN/CH/Sa-304/2016 from FMDV Serotype A Isolates of Bangladesh

Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance	Name of Isolate	Genetic Distance
BAN/CH/Sa-304/2016	-	BAN_CH_Ra-18_2012	0.04	BD_AH_10_2012_VP1	0.11
BD_SI_16_2013	0.10	BAN_CH_Ra-16_2012	0.05	BD_BAU_ML4_2014	0.10
BAN_CH_Ra-31_2012	0.04	BAN_CH_Ra-15_2012	0.05	BD_BAU_ML3_2013	0.10
BAN_CH_Ra-30_2012	0.05	BAN_CH_Ra-14_2012	0.04	BD_SI_35_2013	0.10
BAN_CH_Ra-02_2012	0.04	BAN_CH_Ra-08_2012	0.04	BAN/CH/Sa-302/2016	0.01
BAN_CH_Ra-28_2012	0.04	BAN_GA_To-02_2012	0.04	BAN_GA_Sa-197_2013	0.06
BAN_CH_Ra-26_2012	0.04	BD_AH_11_2013	0.05		

The mean genetic distances of current and proposed FMDV serotype O and A vaccine candidates from their respective isolates of Bangladesh were estimated in SPSS version 20 software and delineated in **Table 2.5.2e**.

Table 3.2.2e: VP1 coding region based genetic distance

Serotype	Vaccine Strain	Genetic Distance from FMDV Isolates of Bangladesh		95% Confidence Interval	
		Mean Distance	Standard Error (SE)	Lower	Upper
Serotype O	O/India/R2/75 (Current vaccine strain)	0.1599	0.001	0.1566	0.1634
	BAN/TA/Dh-301/2016 (Proposed vaccine candidate)	0.0640	0.005	0.0537	0.0739
Serotype A	IND40/00 (Current vaccine strain)	0.1095	0.002	0.1053	0.1142
	BAN/CH/Sa-304/2016 (Proposed vaccine candidate)	0.0579	0.006	0.0458	0.0721

The mean genetic distances of current serotype O vaccine strain O/India/R2/75 and serotype A vaccine strain IND40/00 from their respective isolates of Bangladesh were found to be 0.1599±0.001 and 0.1095±0.002 respectively. Whereas, the same for proposed vaccine candidates BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 were 0.0640±0.005 and 0.0579±0.006. On the basis of the 2-tailed T-test, the mean genetic distance of the current FMDV serotype O vaccine strain O/India/R2/75 from the respective FMDV isolates of Bangladesh is significantly higher (p=0.001) than that of the proposed vaccine candidate BAN/TA/Dh-301/2016. The finding is similar in case of serotype A with a p value of 0.002.

3.2.3 Selection Analyses

Selection analysis was carried out to observe the codon bias to mutation in VP1 protein coding sequences of FMDV isolates of Bangladesh. In selection analyses by FEL, IFEL, REL and MEME methods, significant positive selection ($p<0.05$) was found at codon position 19, 43, 45, 140 and 198 of VP1 protein of FMDV serotype O isolates of Bangladesh. Among the positively selected codons, codon position 45 was identified by

all methods. No positively selected codons were identified in case of serotype A. As high as 82 codon positions in serotype O and 12 codon positions in serotype A were found under negative selection pressure. Z-test in both serotype O and A datasets (**Appendix-III**) for the evidence of evolutionary selection did not exhibit significant ($p>0.2$) difference among the isolates. The results of selection analyses are summarized in **Table 3.2.3**.

Table 3.2.3: Results of VP1 based selection analyses

Method of analysis	Serotype O		Serotype A	
	Codon position for positive selection	No. of negatively selected positions	Codon position for positive selection	No. of negatively selected positions
SLAC	None	20 positions	None	5 positions
FEL	45 ($p<0.05$)	81 positions	None	12 positions
IFEL	45 ($p<0.05$)	82 positions	None	None
REL	45,140,198 ($p<0.05$)	20 positions	None	None
MEME	19,43,45 ($p<0.05$)	n/a	None	None
Z-test for selection	None ($p>0.2$)	-	None ($p>0.2$)	-

3.3 Complete Genome Sequencing

The proposed serotype O and A vaccine candidates BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 respectively were subjected to complete genome sequencing to understand the molecular and structural organizations of the virus. These studies will be helpful of monitoring the virus at the field level and to predict why vaccine failure case occurs if will there be during the use of these strains.

3.3.1 PCR Amplification of the Entire Genome

cDNA prepared from BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 genomic RNA as described earlier were used for entire genome amplification and sequencing. Total of 22 overlapping amplified products for BAN/TA/Dh-301/2016 and 20 for BAN/CH/Sa-304/2016 were generated. Optimized primer combinations were used to amplify 5′ UTR, structural and non-structural regions; and 3′ UTR of the viral genome. The amplification profiles of the entire genomes of both the viruses are delineated in the gel autoradiograph (**Figure 3.3.1a and 3.3.1b)**.

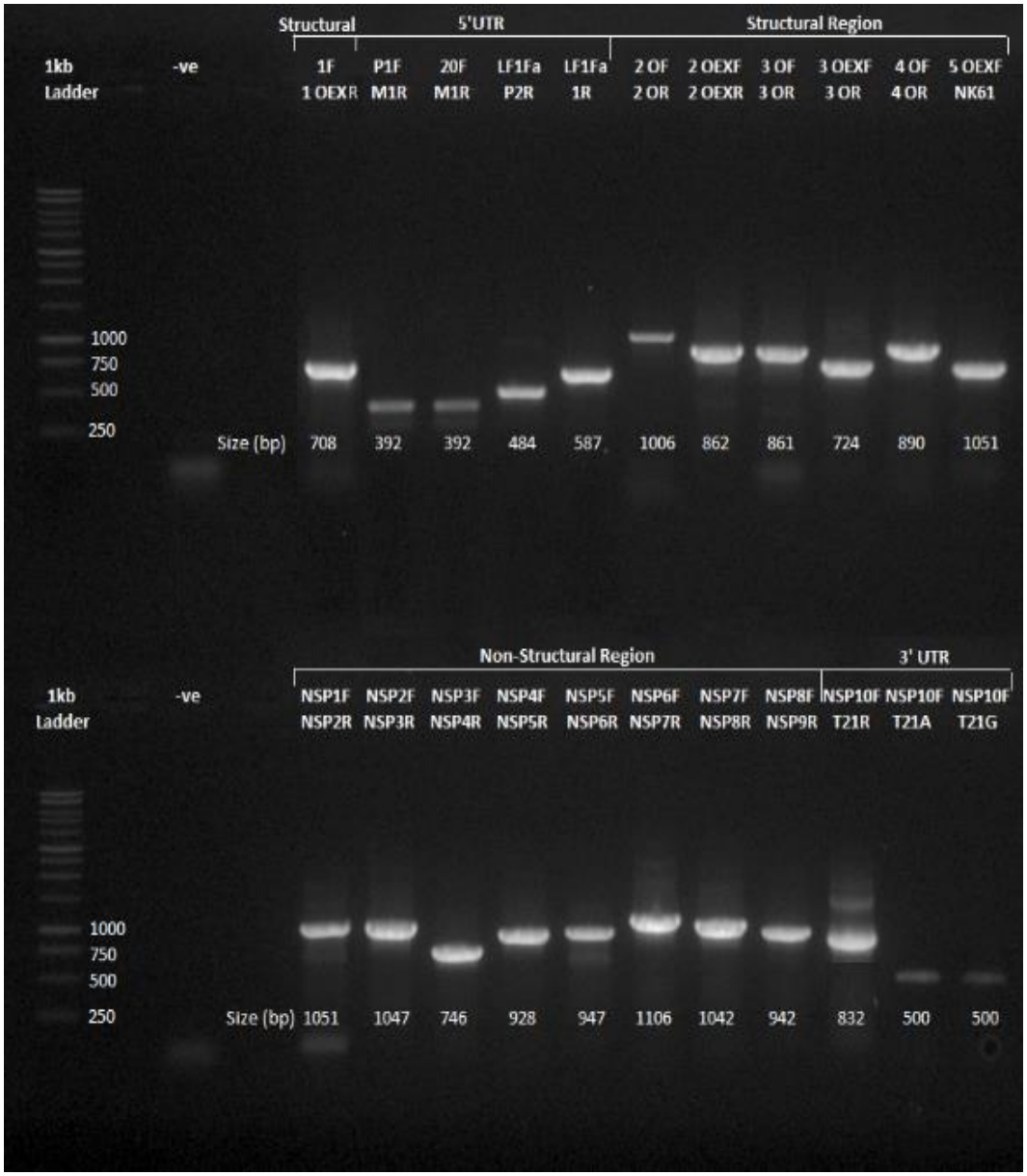


Figure 3.3.1a: PCR amplification of the entire genome of O/BAN/TA/Dh-301/2016. Twenty two overlapping amplicons were amplified using previously optimized primer pairs, which covered the entire genome. 1 kb marker (Promega, USA) was used in 1% gel.

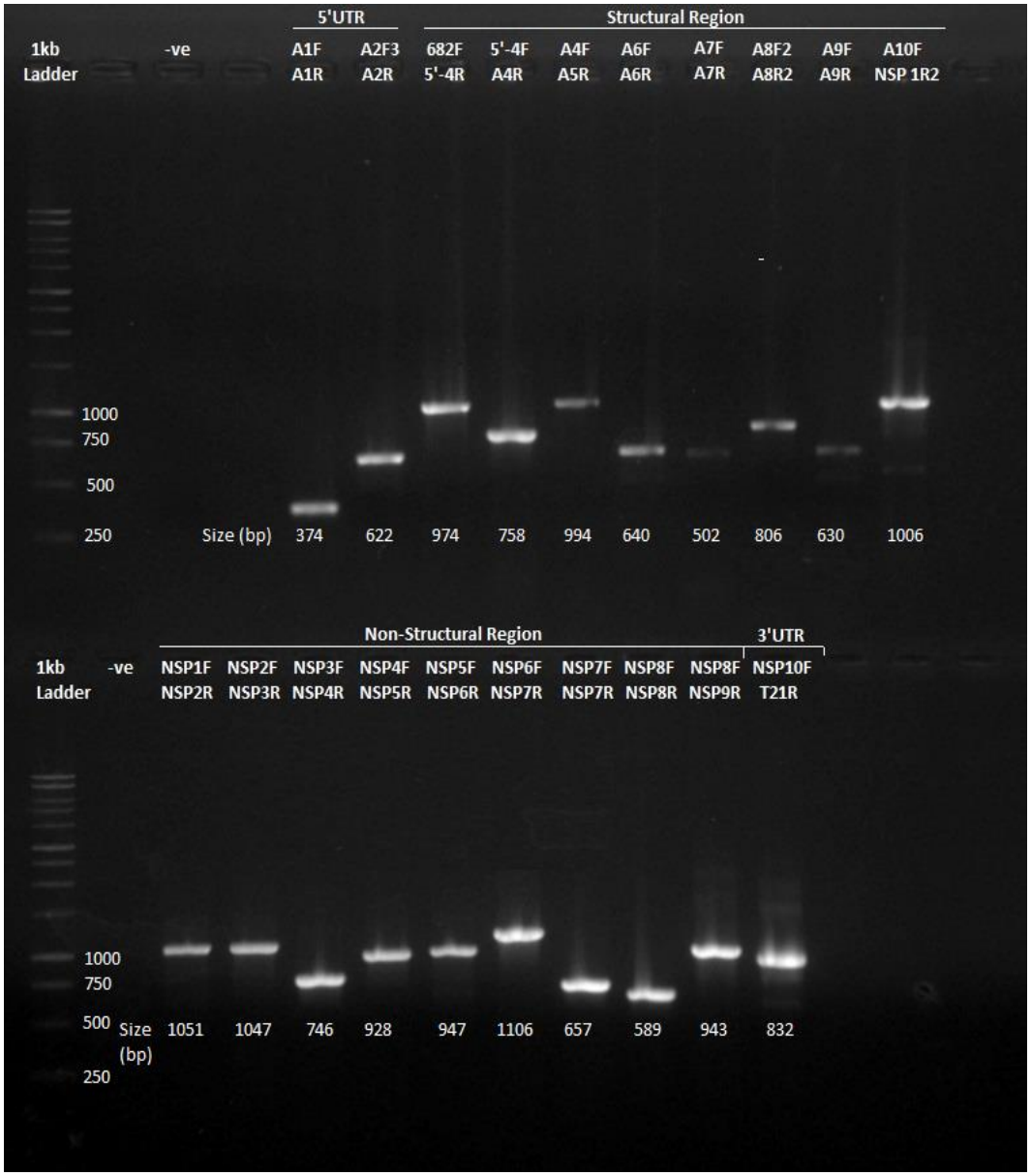


Figure 3.3.1b: PCR amplification of the entire genome of A/BAN/CH/Sa-304/2016. Twenty overlapping amplicons were amplified using previously optimized primer pairs, which covered the entire genome. 1 kb marker (Promega, USA) was used in 1% gel.

3.3.2 Sequencing of the Entire Genome

Sanger sequencing reactions (cycle sequencing) were performed to sequence all of the overlapping amplicons spanning the entire genome of BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 using the same primer pairs applied in RT-PCR amplification of the genome (**Table 2.7.1a and Table 2.7.1b**). The consensus complete genome sequence of both the isolates are presented in **Appendix-IV**.

The complete genomes of BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 FMDV isolates are 8211 and 8221 nucleotides (nt) in length respectively. Both the isolates have an Open Reading Frame (ORF) of 6999 nucleotides. The details of the complete genome annotation of both the isolates are presented in **Table 3.3.2a** and **3.3.2b**.

Table 3.3.2a: Complete genome annotation of FMDV isolates BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016

Region	BAN/TA/Dh-301/2016 (Serotype O)		BAN/CH/Sa-304/2016 (Serotype A)	
	Extent	Length (nt)	Extent	Length (nt)
5´ UTR	1-1098	1098	1-1101	1101
Coding DNA Sequence (CDS)	1099-8097	6999	1102-8100	6999
3´ UTR	8098-8211	114	8101-8221	121

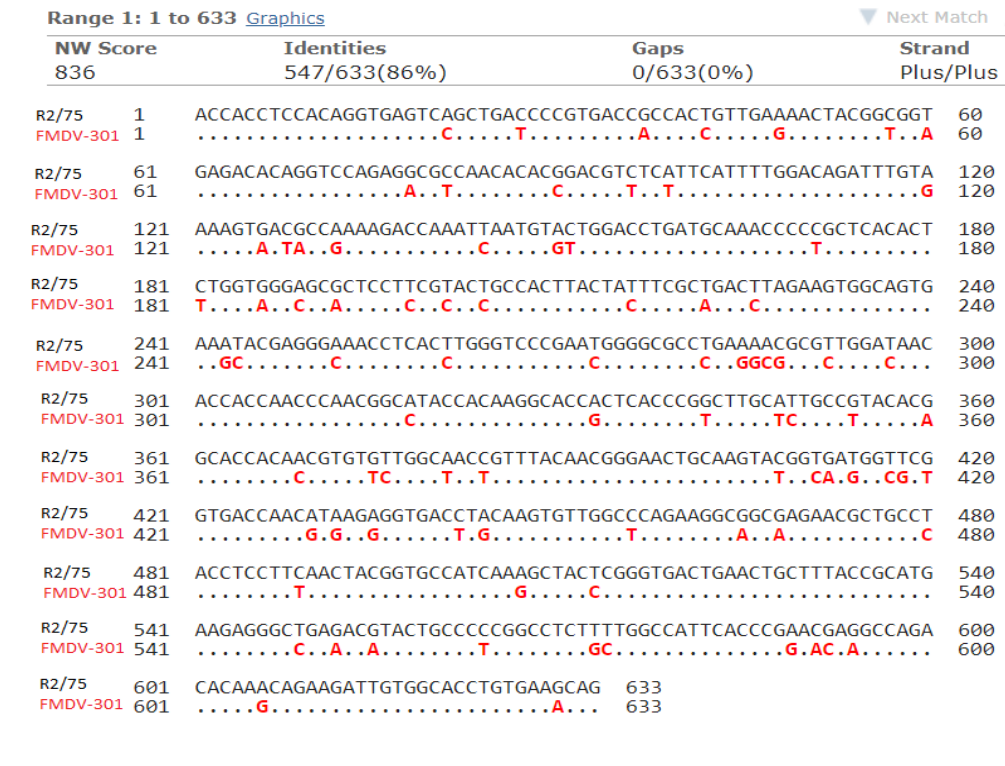
Table 3.3.2b: Length of genes in Coding DNA Sequences (CDS) of FMDV isolates BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016

Genes in CDS	BAN/TA/Dh-301/2016 (Serotype O)		BAN/CH/Sa-304/2016 (Serotype A)	
	Extent	Length (nt)	Extent	Length (nt)
L^{Pro}	1099-1701	603	1102-1704	603
VP4	1702-1956	255	1705-1959	255
VP2	1957-2610	654	1960-2613	654
VP3	2611-3270	660	2614-3273	660
VP1	3271-3903	633	3274-3906	633
2A	3904-3957	54	3907-3960	54
2B	3958-4419	462	3961-4422	462
2C	4420-5373	954	4423-5376	954
3A	5374-5832	459	5377-5835	459
3B	5833-6045	213	5836-6048	213
3C	6046-6684	639	6049-6687	639
3D	6685-8097	1413	6688-8100	1413

3.4 Comparative Genomics and Proteomics Analysis of Surface Proteins

Nucleotide and deduced amino acid sequences of VP1, VP2 and VP3 regions of the proposed FMDV serotype O and A vaccine candidates were compared with those of respective current Indian vaccine strains. Two representative comparative sequence analyses using Needleman-Wunsch algorithm are delineated in **Figure 3.4a** and **3.4b**. The results of comparative sequence analyses of all three structural protein coding regions are summarized in **Table 3.4**.

Nucleotide sequence of VP1



Protein sequence of VP1

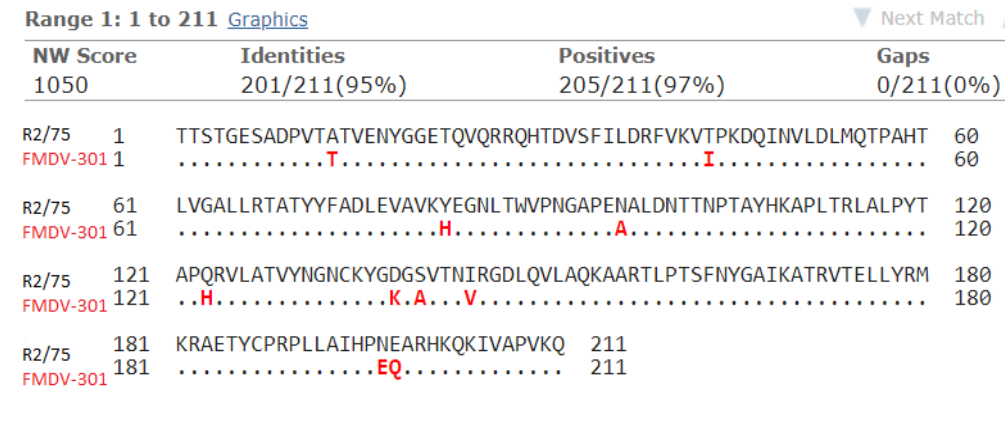


Figure 3.4a: Needleman-Wunsch pairwise alignment of nucleotide and deduced amino acid sequences of VP1 of current and proposed FMDV serotype O vaccine candidates R2/75 and FMDV-301(BAN/TA/Dh-301/2016) respectively. It shows 86 nucleotides and 10 deduced amino acids mismatches between the isolates.

Nucleotide sequence of VP1

Range 1: 1 to 633 Graphics Next Match

NW Score	Identities	Gaps	Strand
915	565/634(89%)	2/634(0%)	Plus/Plus

```
IND40/00   1    ACCACCACGGCCGGGGAATCTGCAGACCCAGTCACCACCACTGTTGAGAACTACGGCGGT   60
FMDV-304   1    ......T.TA.......G.........................C..C..............   60

IND40/00   61   GAGACACAAGTCCAGCGGCGTCACCACACTGACGTCAGCTTCATAATGGACAGATTTGTG   120
FMDV-304   61   ................A..C..............TG.A..T...................   120

IND40/00   121  AAGATTG-GAACCACTAACCCCACACATGTCATTGACCTCATGCAAACCCACCAACACGG   179
FMDV-304   121  ..A....T...TGT..-.GT...........T............................T..   179

IND40/00   180  GCTGGTGGGTGCCCTGTTGCGTGCTGCCACGTACTACTTCTCCGACTTGGAGATCGTGGT   239
FMDV-304   180  AT....T................GC..A............................T.....   239

IND40/00   240  TCGTCACAGCGGCAACCTGACATGGGTACCCAATGGAGCACCTGAGGCAGCCCTGTCCAA   299
FMDV-304   240  C......GAA...........G.....................C.................   299

IND40/00   300  CACAGGAAACCCTACCGCCTACAACAAAGCGCCGTTCACGAGACTTGCGCTCCCCTACAC   359
FMDV-304   300  ...T...........A.............T...A.................T........   359

IND40/00   360  TGCGCCACACCGCGTGTTGGCAACAGTGTACAACGGGACGAACAAGTACTCCGCGGCCAG   419
FMDV-304   360  ...A........T...C.........................................T..   419

IND40/00   420  TGGGCGCACACGGGGTGACCTGGGGCAGCTCGCAGCGCGAATTGCCGCCCAACTTCCTGC   479
FMDV-304   420  ......TG................A..A.....G..A...G.C..T..T.....C.....   479

IND40/00   480  CTCTTTCAATTTTGGTGCAATCAAGGCTGACGCCATCCACGAGCTTCTCGTGCGCATGAA   539
FMDV-304   480  ...C.....C..C..........G...CACTA.............G..............   539

IND40/00   540  GCGTGCCGAACTCTACTGCCCCAGACCACTGTTGGCGGTGGAGGTCTCGTCTCAAGACAG   599
FMDV-304   540  ............................A.......A..............G........   599

IND40/00   600  ACACAAACAGAAGATCATTGCACCAGCAAAACAG   633
FMDV-304   600  ......................G.............   633
```

Protein sequence of VP1

Range 1: 1 to 211 Graphics Next Match

NW Score	Identities	Positives	Gaps
1021	195/211(92%)	200/211(94%)	0/211(0%)

```
IND40/00 1    TTTAGESADPVTTTVENYGGETQVQRRHHTDVSFIMDRFVKIGTTNPTHVIDLMQTHQHG   60
FMDV-304 1    ..ST............................G.........VNVS..............   60

IND40/00 61   LVGALLRAATYYFSDLEIVVRHSGNLTWVPNGAPEAALSNTGNPTAYNKAPFTRLALPYT   120
FMDV-304 61   .......G..............E.........................V..........   120

IND40/00 121  APHRVLATVYNGTNKYSAASGRTRGDLGQLAARIAAQLPASFNFGAIKADAIHELLVRMK   180
FMDV-304 121  ......................A..........V.............R.TT.........   180

IND40/00 181  RAELYCPRPLLAVEVSSQDRHKQKIIAPAKQ   211
FMDV-304 181  .........M.....................   211
```

Figure 3.4b: Needleman-Wunsch pairwise alignment of nucleotide and deduced amino acid sequences of VP1 of current and proposed FMDV serotype A vaccine candidates IND40/00 and FMDV-304 (BAN/CH/Sa-304/2016) respectively. It shows 68 nucleotides and 16 deduced amino acids mismatches between the isolates.

Table 3.4: Nucleotide and amino acid mismatches in VP1, VP2 and VP3 regions of selected vaccine candidate BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 with respective current vaccine strains O/India/R2/75 and IND40/00

Region	No. of mismatches in O/BAN/TA/Dh-301/2016 compared to O/India/R2/75		No. of mismatches in A/BAN/CH/Sa-304/2016 compared to A/IND40/00	
	Nucleotide	Amino acid	Nucleotide	Amino acid
VP1	86	10 (position 13, 43, 82, 96, 123, 138, 140, 144, 197, 198)	68	16 (position 3, 4, 33, 43, 44, 45, 46, 68, 83, 110, 143, 154, 168, 170, 171, 190)
VP2	73	07 (positions 23, 70, 74, 93, 134, 154, 191)	82	12 (position 56, 65, 71, 74, 77, 98, 134, 154, 189, 190, 191, 195)
VP3	73	10 (positions 25,56, 60, 86, 96, 131, 174, 195, 219, 220)	65	10 (position 54, 59(deletion), 67, 68, 69, 92, 94, 139, 204, 220)

The nucleotide sequence of VP1 protein coding region of FMDV vaccine candidate O/BAN/TA/Dh-301/2016 has 14% variability with 86 mismatched nt compared to respective region of current Indian vaccine strain O/India/R2/75. Both the VP2 and VP3 protein coding regions have 11% nucleotide sequence variability with 73 numbers of nucleotides substitutions for each. For serotype A vaccine candidate BAN/CH/Sa-304/2016; VP1, VP2 and VP3 protein coding regions showed 11%, 12%, and 11% nucleotide sequence variability with 68, 82 and 65 number of nucleotide substitutions compared to respective regions of current Indian vaccine strain IND40/00.

The deduced amino acid sequences of selected vaccine candidates had also variability with their respective current vaccine strains. The VP1, VP2 and VP3 amino acid sequences of O/BAN/TA/Dh-301/2016 had 5%, 3% and 5% variability with 10, 7 and 10 number of aa substitutions with respective regions of vaccine strain O/India/R2/75. Three aa (D138K, S140A and N197E) substitutions in antigenically crucial site 1 in G-H loop of VP1 and 2 aa (N134K, T191N) substitutions in site 2 of VP2 regions have been found. In addition, an aa substitution (T43I) near to antigenic site 3 in B-C loop and another (E198Q) near to site 1 in VP1 region are evident.

The same of vaccine candidate A/BAN/CH/Sa-304/2016 had 8%, 6% and 5% variability with 16, 12 and 10 aa substitutions compared to respective regions of A/IND40/00. In VP1

region 16 aa substitutions were evident, of which, only 4 substitutions (S83E, T143A, I154V and D170T) were in antigenically critical sites. No aa substitutions were evident at antigenically critical sites of VP2 and VP3 proteins.

The protein variability between the selected and current vaccine candidates of both serotypes were also confirmed by Wu-Kabat protein variability algorithm (**Figure 3.4c** and **3.4d)**.

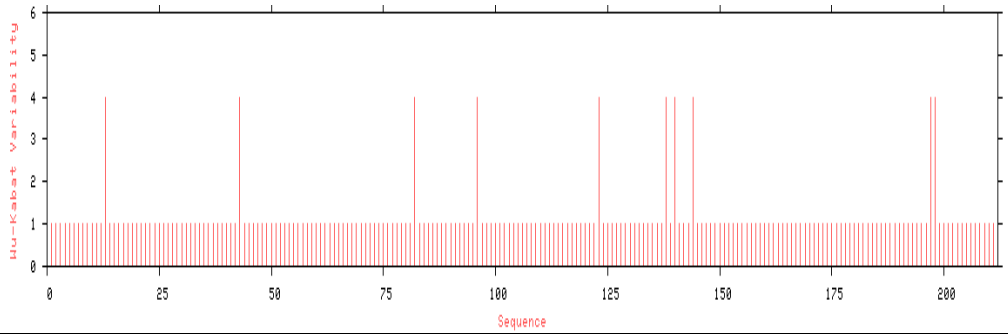
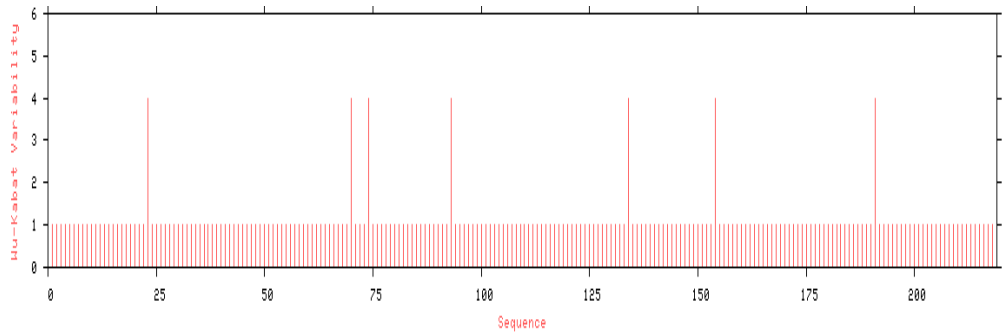
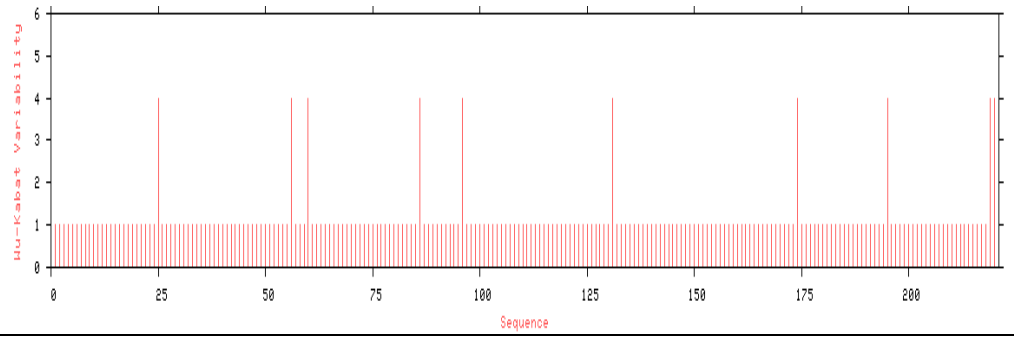
Region	Wu-Kabat protein variability plot
VP1	
VP2	
VP3	

Figure 3.4c: Wu-Kabat protein variability plots of VP1, VP2 and VP3 proteins between FMDV BAN/TA/Dh-301/2016 and O/India/R2/75.

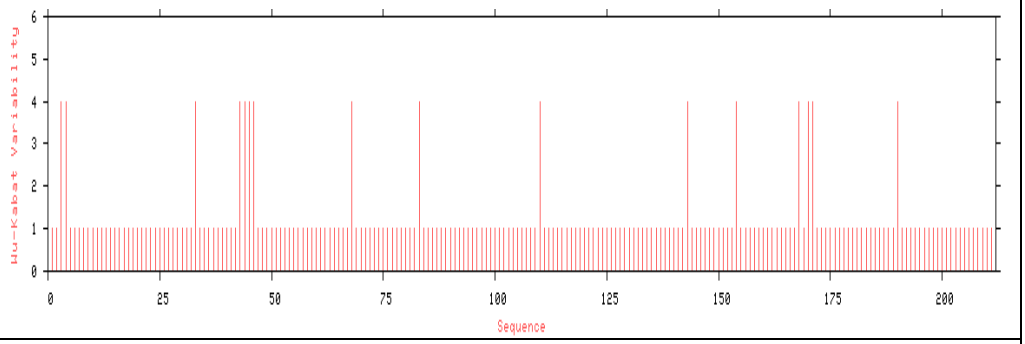
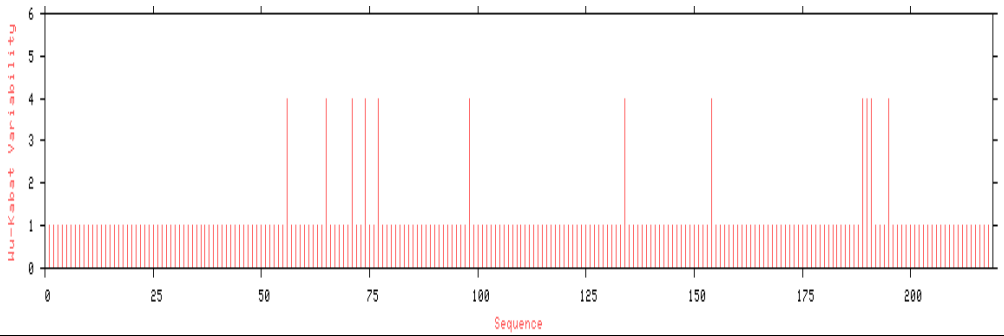
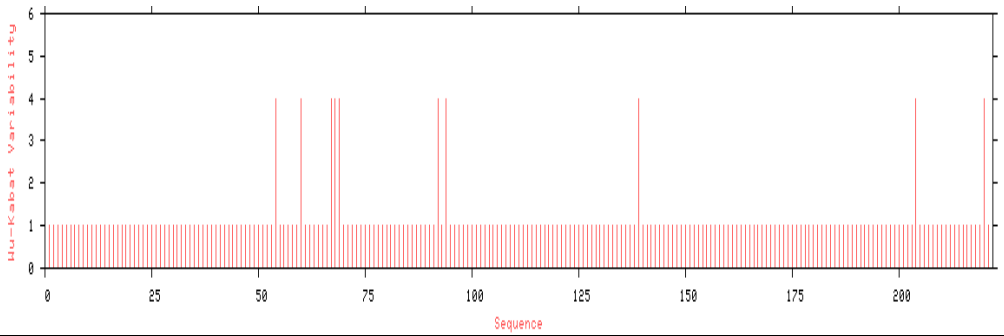
Region	Wu-Kabat protein variability plot
VP1	
VP2	
VP3	

Figure 3.4d: Wu-Kabat protein variability plots of VP1, VP2 and VP3 proteins between FMDV BAN/TH/Sa-304/2016 and IND40/00.

Predicted three Dimensional (3D) structures of VP1 proteins of BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 showed variations with those of respective current vaccine strains. When the predicted 3D structure of BAN/TA/Dh-301/2016 superimposed on 3D structure of O/India/R2/75 in PyMOL, significant differences in their structures were found. Similar result was found by superimposing 3D structure of BAN/CH/Sa-304/2016 on that of IND40/00. The PyMOL view of the superimposed VP1 proteins are delineated in **Figure 3.4e**.

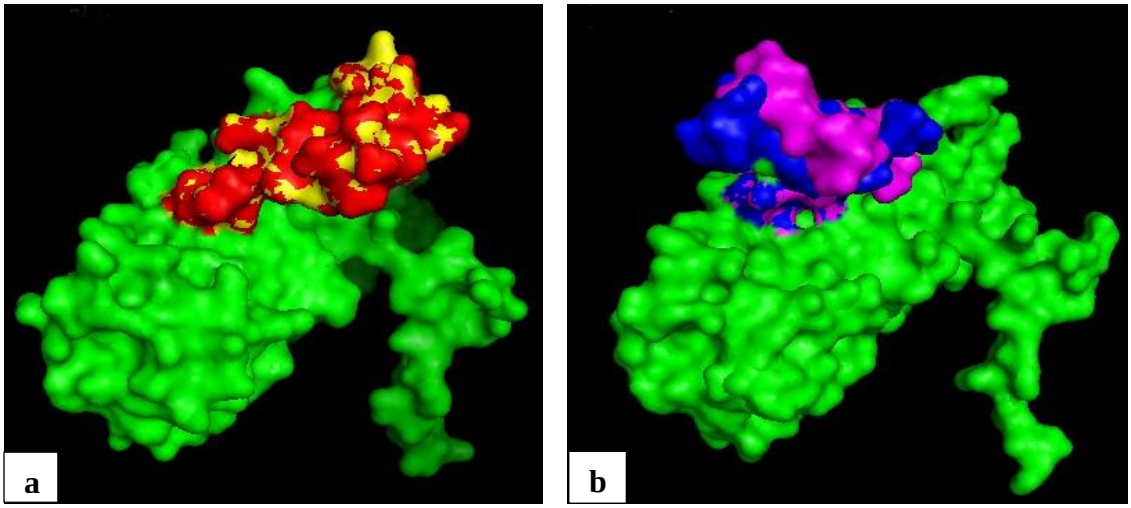


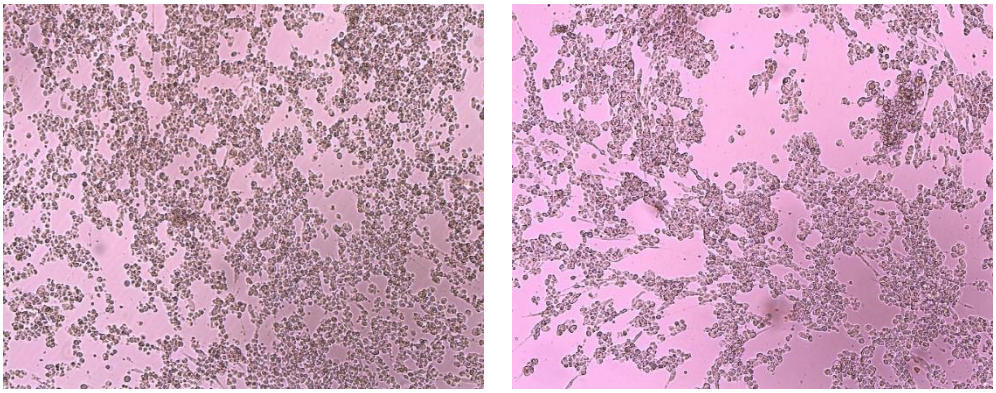
Figure 3.4e: **PyMOL view of superimposed G-H loop of VP1 protein of BAN/TA/Dh-301/2016 (yellow color) on O/India/R2/75 (red color) [a]; and that of BAN/CH/Sa-304/2016 (blue color) on IND40/00 (magenta color) [b]. PyMOL view confirmed structural variations between current and proposed vaccine candidates of each serotype.**

3.5 Manufacture of Mono- and Bi-valent Vaccine

During the period of 2014 to 2017, FMD outbreaks by serotype Asia1 was not reported in Bangladesh. Based on this epidemiological finding, the present study was confined to manufacturing mono- and bi-valent vaccine against circulating FMDV serotype O and A only. However, the re-emergence of serotype Asia1 in 2018 warranting a tri-valent FMD vaccine in Bangladesh. Our research group has already developed a tri-valent vaccine and efficacy testing in cattle has been completed.

3.5.1 Propagation of BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 in Cell Culture

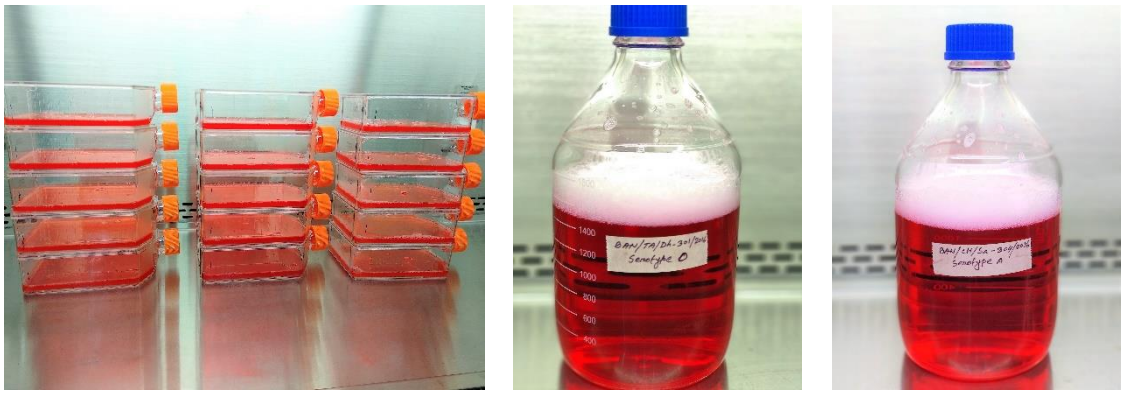
The selected FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016 and serotype A vaccine candidate BAN/CH/Sa-304/2016 were adapted to produce CPE rapidly in BHK-21 cell monolayers. In first passage both the isolates produced little but remarkable CPE in 48 hours. With the increase in the number of passages the isolates became capable of producing CPE rapidly and at passage number 6 complete CPE with sloughing off cells were found within 18-20 hours of inoculation **(Figure 3.5.1a)**.



(i) (ii)

Figure 3.5.1a: Cytopathic effects (CPE) produced at passage 6 by FMDV isolates BAN/TA/Dh-301/2016 (i); and BAN/CH/Sa-304/2016 (ii). CPE was marked by rounding and sloughing off of cells in BHK-21 cell monolayers.

Once the vaccine candidates were well adapted in the BHK-21 cell line, they were cultured in 175 cm^2 flasks to propagate bulk amount. Virus of 5^{th} passage was used to infect monolayers. An amount of 1.5L of virus fluid was harvested from 15 flasks for each serotype **(Figure 3.5.1b)**.



(i) (ii) (iii)

Figure 3.5.1b: (i) Propagation of virus in 175 cm^2 cell culture flasks; (ii) 1.5L clarified virus fluid of BAN/TA/Dh-301/2016 and (iii) BAN/CH/Sa-304/2016.

3.5.2 Inactivation of Virus and Quality Assurance

The virus cultures were inactivated by 3mM BEI and the pH of the BEA-NaOH solution before and after incubation at 37^0 C for 1hour was 12.32-12.84 and 8.45-8.64 respectively. Reduction of pH of the solution is the resultant of cyclization of BEA to form quality

assured BEI. The log_{10} $TCID_{50}$ of all hourly samples of both the serotypes were calculated and presented in **Table 3.5.2**.

Table 3.5.2: Reduction in log_{10} $TCID_{50}$ titer of virus with progression of inactivation process

Post Inactivation Hour	Log_{10} $TCID_{50}$ Titer									
	BAN/TA/Dh-301/2016 (Serotype O)					BAN/CH/Sa-304/2016 (Serotype A)				
	Run 1	Run 2	Run 3	Mean	SE	Run 1	Run 2	Run 3	Mean	SE
0	7.55	7.175	6.8	7.18	0.12	7.05	6.925	7.05	7.01	0.02
1	6.675	6.3	5.8	6.26	0.14	6.425	6.3	6.55	6.43	0.04
2	6.05	5.725	5.3	5.69	0.12	5.675	5.8	5.8	5.76	0.02
3	5.8	5.175	4.925	5.30	0.14	5.425	5.05	5.05	5.18	0.07
4	4.675	4.8	4.55	4.68	0.04	4.8	4.675	4.675	4.72	0.02
5	4.3	4.3	4.05	4.22	0.05	4.3	4.05	4.05	4.13	0.05
6	2.8	3.05	2.925	2.93	0.04	3.175	3.05	3.3	3.18	0.04
7	2.3	2.175	2.05	2.18	0.04	2.05	1.8	2.175	2.01	0.06
8	2.05	1.925	1,8	1.99	0.03	1.55	1.675	2.05	1.76	0.09
9	1.55	1.425	1.55	1.51	0.02	0	0	0	0.00	0.00
10	0	0	0	0.00	0.00					

In case of FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016 the initial mean log_{10} $TCID_{50}$ titer was 7.18±0.12 and reached to 0 at 10^{th} post inactivation hour and in case of serotype A vaccine candidate BAN/CH/Sa-304/2016 the initial mean titer was 7.01±0.02 and reached to 0 at 9^{th} hour. The mean log_{10} $TCID_{50}$ titer of each hourly sample was plotted against their respective hour and inactivation kinetics for each serotype was drawn and presented in **Figure 3.5.2a** and **3.5.2b**.

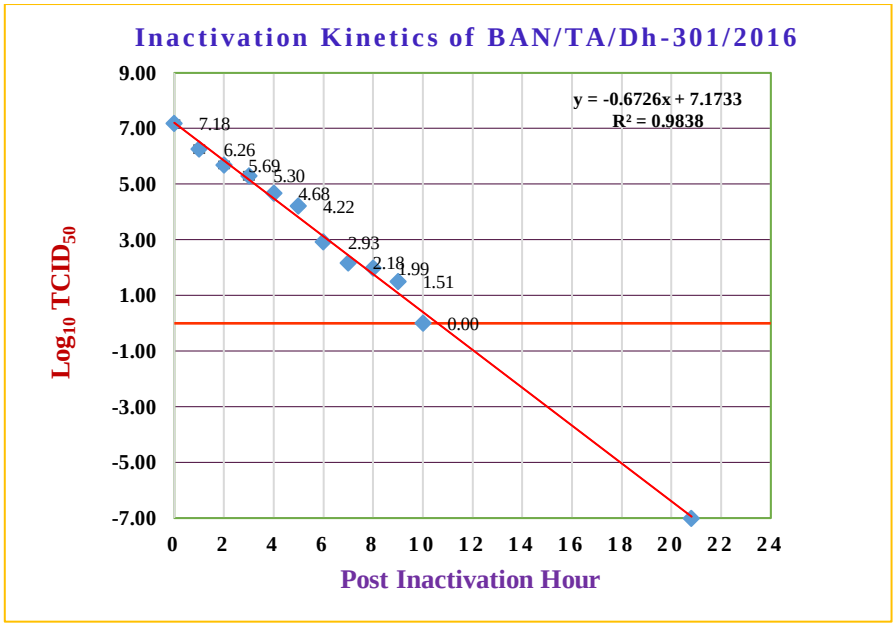


Figure 3.5.2a: Inactivation kinetics of FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016. The initial mean log_{10} $TCID_{50}$ titer of virus was 7.18 and zeroed at 10^{th} post inactivation hour. Statistical extrapolation showed a 10^4 L batch will be inactivated at 20.81^{th} post inactivation hour.

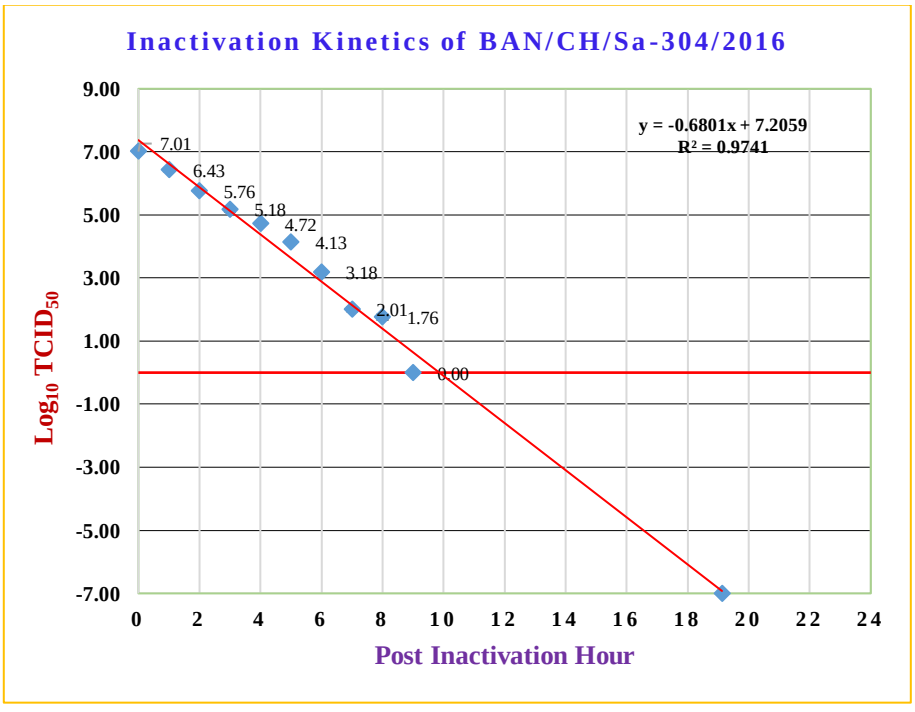
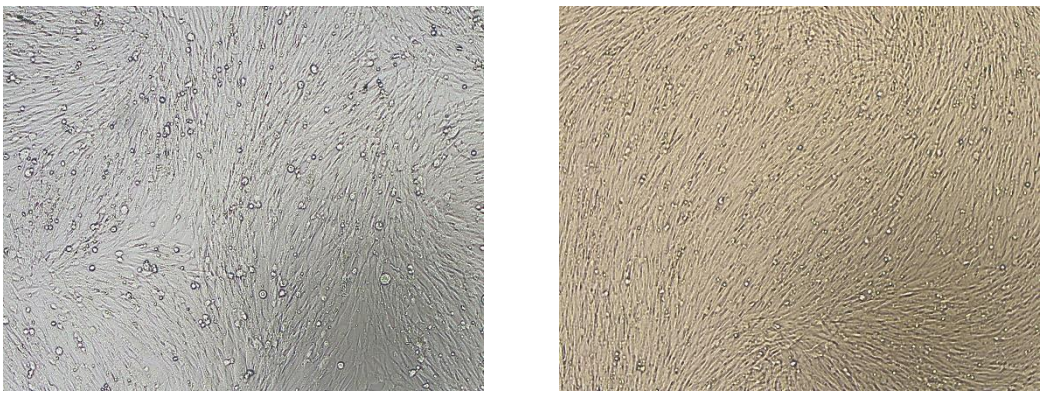


Figure 3.5.2b: Inactivation kinetics of FMDV serotype A vaccine candidate BAN/CH/Sa-304/2016. The initial mean log_{10} $TCID_{50}$ titer of virus was 7.18 and zeroed at 9^{th} post inactivation hour. Statistical extrapolation showed a 10^4 L batch will be inactivated at 19.12^{th} post inactivation hour.

The mean inactivation rates for O/BAN/TA/Dh-301/2016 and A/BAN/CH/Sa-304/2016 were 0.72±0.038 and 0.78±0.008 log_{10} $TCID_{50}$ per hour respectively. Statistical extrapolation revealed that a 10000-liter batch of FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016 would be inactivated in 20.81 hours after commencement of inactivation and at 24^{th} post inoculation hour log_{10} $TCID_{50}$ titer will be -8.97, that is, there would be 0.01 infectious particle per 10000 liters of liquid preparation at the end of the inactivation period. Likewise, the extrapolated inactivation kinetics of serotype A vaccine candidate BAN/CH/Sa-304/2016 would inactivate a 10000-liter batch in 19.12 hours with -10.29 log_{10} $TCID_{50}$ titer and 0.0005 infectious particle at 24^{th} post inoculation hour. The R-squared values of 0.9838 and 0.9741 of the inactivation curves of serotype O and A respectively are indicative of high linearity of both the inactivation kinetics.

Innocuity of the inactivated virus was tested in BHK-21 cell monolayers and in third serial passage there was no virus CPE found in cell sheets. The results were same for both the serotype as delineated in **Figure 3.5.2c**.



(a) **(b)**

Figure 3.5.2c: (a) Third passage of BHK-21 cell monolayers in innocuity testing of inactivated virus fluid of FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016 and (b) serotype A vaccine candidate BAN/CH/Sa-304/2016. CPE was not found at third passage of both the virus.

Innocuity test confirmed that the inactivated virus fluids of both serotypes were safe for further processing like concentration and vaccine formulation.

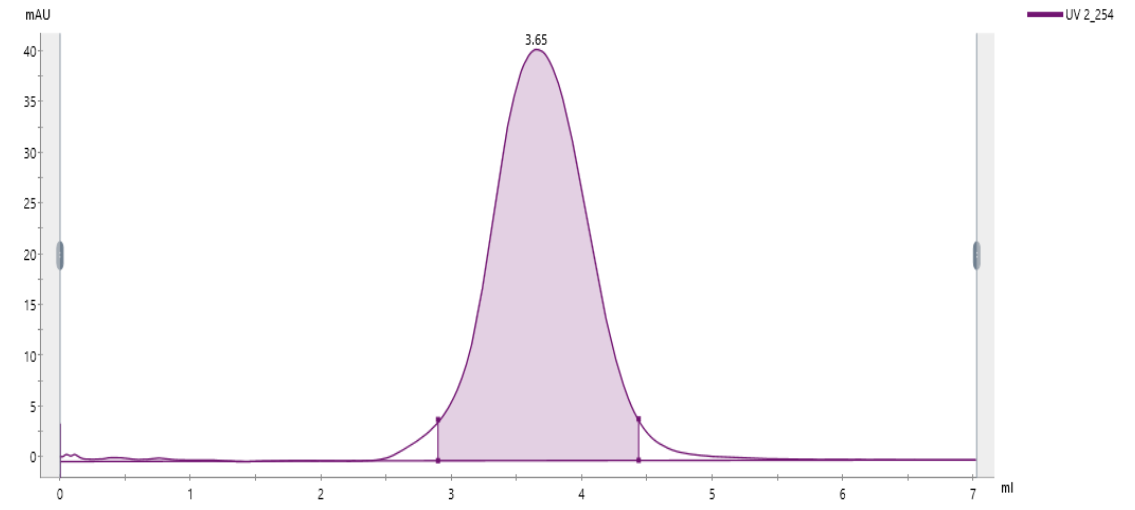
3.5.3 Virus Particles Concentration and Quantification

The inactivated virus particles in cell culture fluid was concentrated by 100 KDa hollow fiber cartridge. One hundred fifty (150) ml concentrate was collected from the feed tank

when the original volume of virus suspension reached to its 1/10th volume. Permeate was collected from permeate-pump outlet. Both concentrate and permeate were preserved at -80^{0} C in autoclaved glass bottle **(Figure 3.5.3a)**.

Figure 3.5.3a: Representative figure of virus particles concentration. Increased turbidity in concentrate than permeate indicates successful concentration.

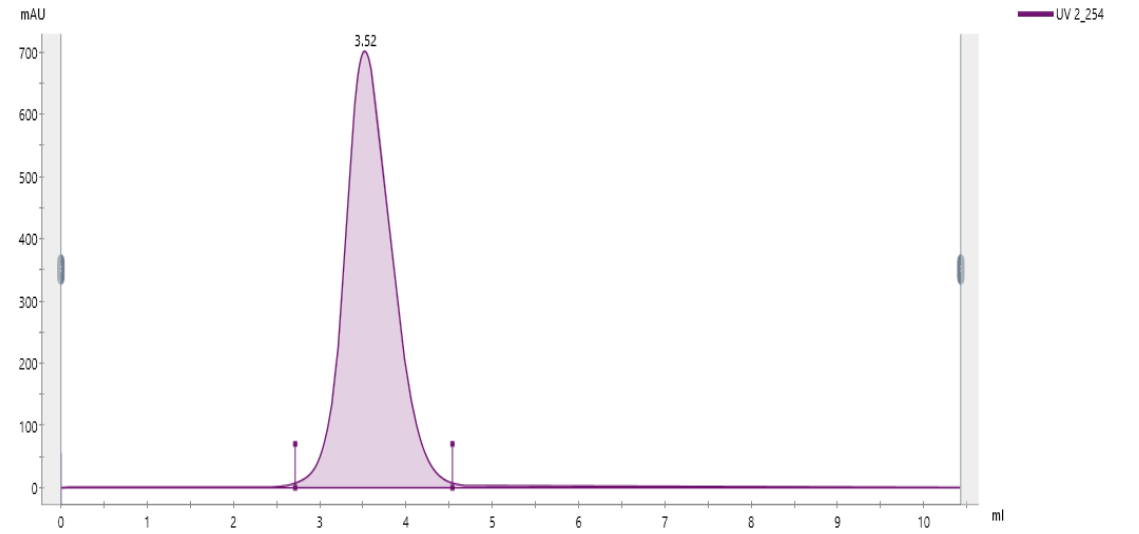
The amount of FMD viral particles were quantified before and after concentration of virus fluid. The concentration of virus particles in peak area **(Figure 3.5.3b and 3.5.3c)** was quantified from the Unicorn® 7.0 software.



Peak Table - UV 2_254

Peak	Retention ml	Area ml*mAU	Area %	Ext coeff. mg ml⁻¹ cm⁻¹	Amount mg	Concentration mg/ml
Peak A	3.647	34.34	100	72.000	0.002	0.002

Figure 3.5.3b: Representative peak areas given by none-concentrated virus particles.



Peak Table - UV 2_254

Peak	Retention ml	Area mlᵃmAU	Area %	Ext coeff. mg ml⁻¹ cm⁻¹	Amount mg	Concentration mg/ml
Peak A	3.522	448.5	100	72.000	0.031	0.017

Figure 3.5.3c: Representative peak areas given by concentrated virus particles.

The readings of Akta Pure were recorded and presented in **Figure 3.5.3d**. The mean virus particle contents of FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016 before and after concentration were 2.33±0.57 µg/ml and 16.33±1.15 µg/ml respectively. Those of FMDV serotype A vaccine candidate BAN/CH/Sa-304/2016 were 1.66±0.57 µg/ml and 14.33±0.57 µg/ml respectively.

Comparison of the inactivated virus particle contents before and after concentration revealed that virus particles were statistically significantly concentrated in case of serotype O ($p=0.002$) and serotype A ($p=0.003$).

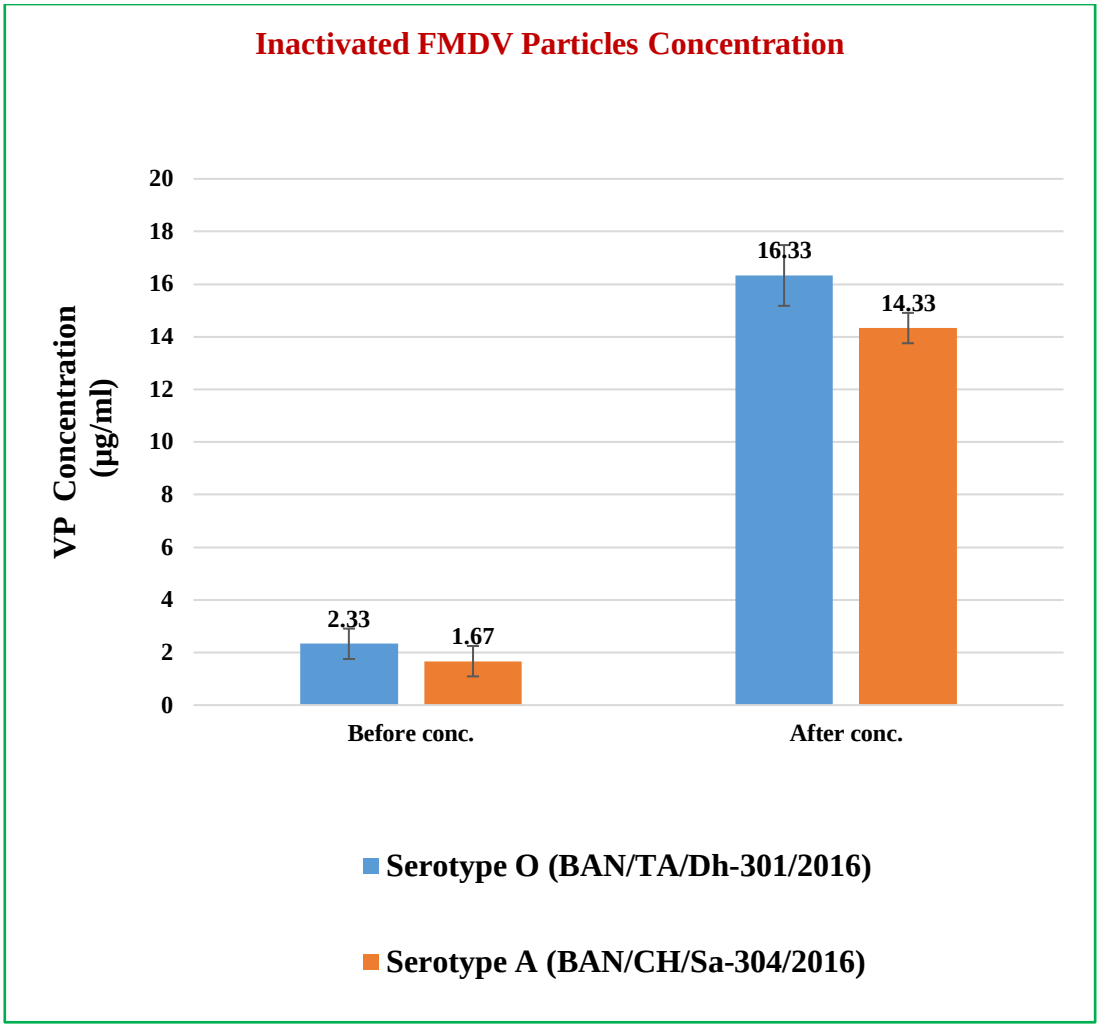
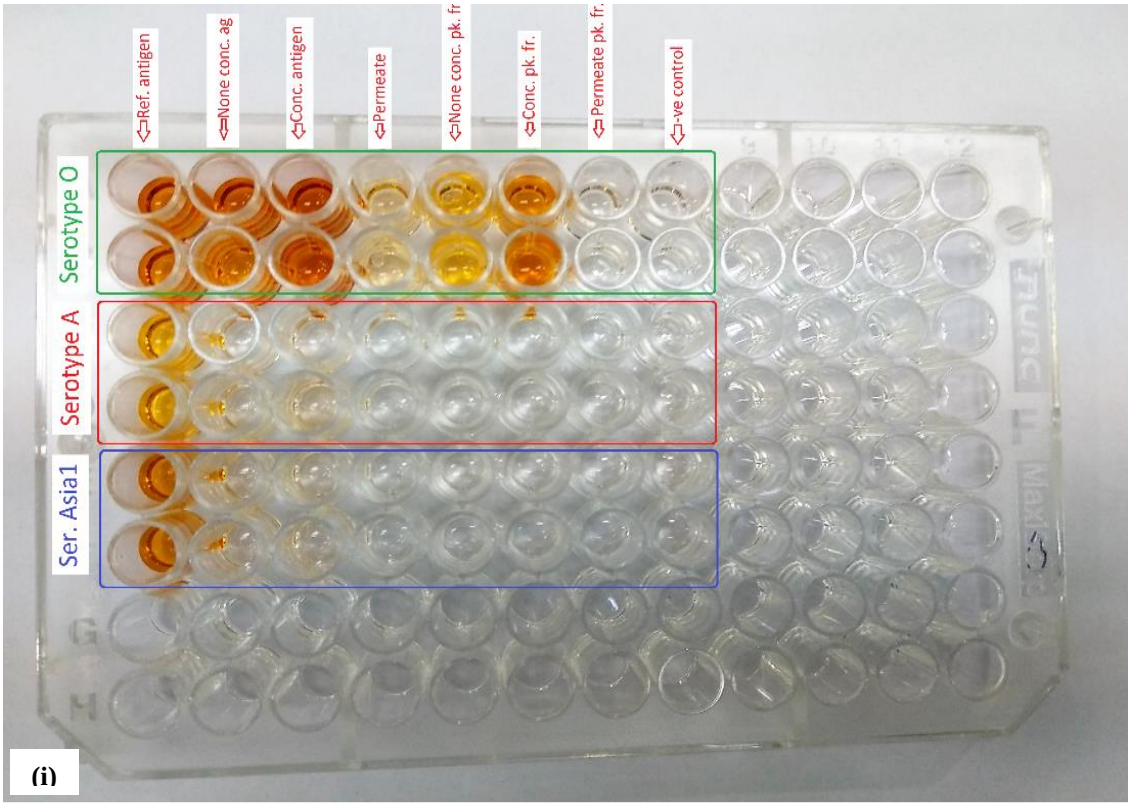


Figure 3.5.3d: Graphical representation of comparison of the inactivated virus particle contents before and after concentration.

3.5.4 Detection of FMDV Antigens

FMDV antigens in inactivated virus suspension, concentrate, permeate and their respective peak fractions were successfully detected by serotype specific antigen detecting sandwich ELISA. The optical density (OD) value >0.15 was considered positive for FMDV antigen **(Figure 3.5.4a and 3.5.4b)**.



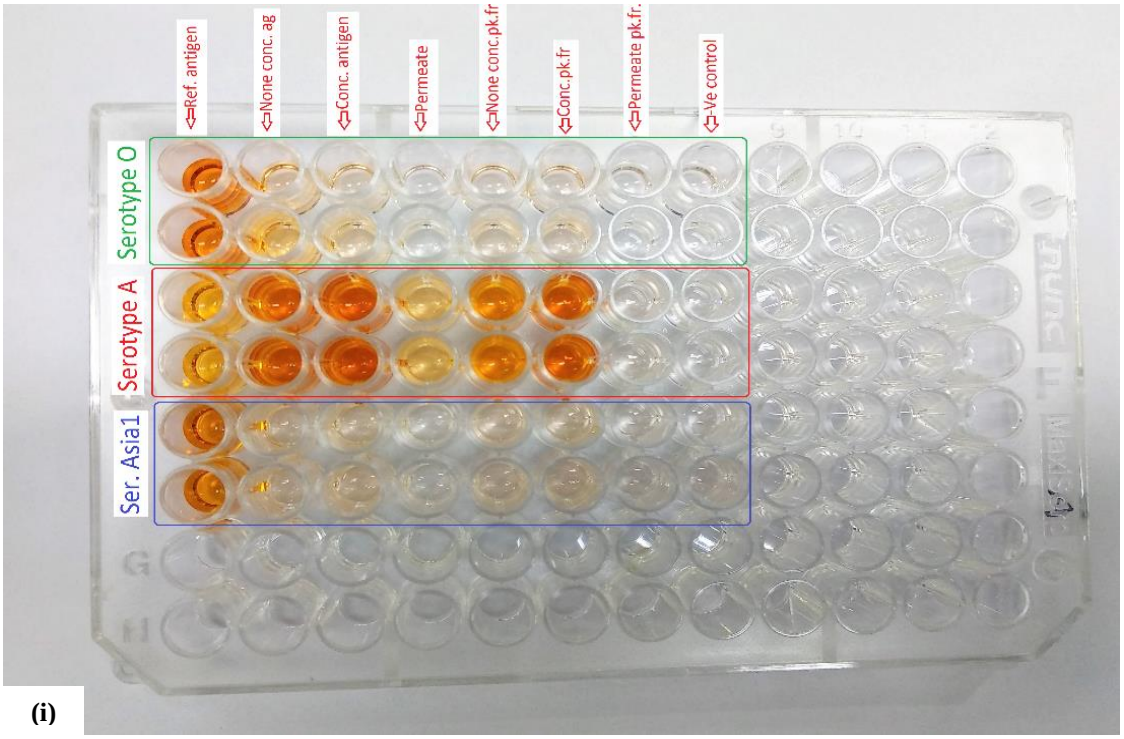
(ii)

	1	2	3	4	5	6	7	8	9	10	11	12	
A	3.71	3.85	4.58	0.37	1.05	2.77	0.11	0.10					492
B	3.31	2.56	4.35	0.33	1.06	3.83	0.11	0.10					492
C	1.46	0.12	0.13	0.09	0.09	0.09	0.09	0.14					492
D	1.30	0.12	0.15	0.08	0.08	0.09	0.09	0.11					492
E	2.48	0.13	0.12	0.09	0.10	0.10	0.09	0.12					492
F	2.42	0.14	0.13	0.09	0.09	0.09	0.08	0.13					492
G													

Figure 3.5.4a: Detection of FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016 antigens. (i) Color development in ELISA plate. First column showing OD of three reference FMDV antigens; second, third and fourth columns are for none concentrated, concentrated and permeate antigens respectively. Fifth, sixth and seventh columns are showing OD of peak fractions of none concentrated, concentrated and permeate antigens respectively. Eighth column is for negative control. (ii) OD values of the ELISA plate.

In case of serotype O vaccine candidate BAN/TA/Dh-301/2016 the virus particles developed color only in wells that were coated with FMDV serotype O specific coating serum. This finding further confirms the BAN/TA/Dh-301/2016 as serotype O isolate. Higher OD values (2.77 and 3.83) by peak fraction of concentrate (column 6) than those (1.05 and 1.06) of none-concentrated virus particles' peak fraction indicate effective

concentration of inactivated BAN/TA/Dh-301/2016 virus particles. Negative OD value (<0.15) by permeate peak fraction indicates absence of FMDV antigen in permeate.



(i)

	1	2	3	4	5	6	7	8	9	10	11	12	
A	3.92	0.13	0.10	0.11	0.11	0.12	0.11	0.11					492
B	3.71	0.13	0.11	0.12	0.11	0.07	0.09	0.09					492
C	1.47	4.23	4.37	0.53	2.82	4.02	0.13	0.11					492
D	1.36	4.22	4.38	0.56	2.73	4.01	0.13	0.10					492
E	3.03	0.13	0.11	0.12	0.10	0.11	0.12	0.13					492
F	2.75	0.13	0.12	0.12	0.11	0.12	0.13	0.14					492
G													

(ii

Figure 3.5.4b: Detection of FMDV serotype A vaccine candidate BAN/CH/Sa-304/2016 antigens. (i) Color development in ELISA plate. First column showing OD of three reference FMDV antigens; second, third and fourth columns are for none concentrated, concentrated and permeate antigens respectively. Fifth, sixth and seventh columns are showing OD of peak fractions of none concentrated, concentrated and permeate antigens respectively. Eighth column is for negative control. (ii) OD values of the ELISA plate.

The findings of serotype A vaccine candidate BAN/CH/Sa-304/2016 were similar with serotype O vaccine candidate. The isolate was further confirmed as serotype A FMDV as developed color only in serotype A specific coated wells. OD values in concentrate peak fraction wells were 4.02 and 4.01 compared to 2.82 and 2.73 of non-concentrated virus

particles' peak fraction and thereby effective concentration of antigen was also confirmed by comparing OD values. Absence of color development in permeate peak fractions indicated ~100% virus particle recovery by 100KDa hollo fiber cellulose membrane cartridge.

3.5.5 Vaccine Formulation

Thirty (30) ml of monovalent vaccine against serotype O was prepared by mixing 13 gm (13 ml) of concentrated virus fluid with 13 gm (17ml) of adjuvant. The yielded 30 ml vaccine contained 14.15 µg antigen/2 ml dose vaccine. Monovalent vaccine against serotype A was prepared by same procedure but antigen content in vaccine was 12.41 µg/2 ml dose.

3.5.6 Vaccine Sterility Testing

For sterility testing of experimentally developed vaccine, 100 µl of each type of formulated vaccine was streaked on individual nutrient agar plate and overnight incubation of the plates did not show any growth of bacteria **(Figure 3.5.6)**.

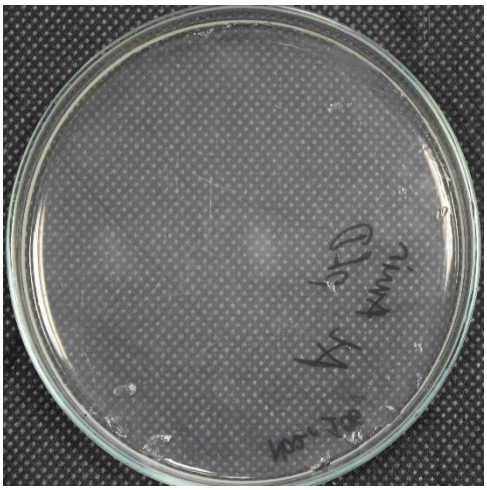


Figure 3.5.6: Representative vaccine sterility testing. Absence of bacterial growth after overnight incubation at 37^0C.

3.6 *In-Vivo* Vaccine Efficacy Testing in Guinea pig Model

Among the laboratory animals, upon adaptation, guinea pigs develop lesions to FMDVs similar to natural hosts. Moreover, immune response of guinea pigs to FMDV is similar with cattle. Additionally, there is no prior chance of natural infection by FMDV in guinea

pigs (Habiela *et al.*, 2014). Considering these things, guinea pig model was used for vaccine efficacy testing.

3.6.1 Adaptation of FMDV in Guinea pigs

The selected vaccine candidates BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 were adapted in guinea pigs to prepare homologous challenge virus. In first passage primary lesions were found only in the injected foot pad within 5 days post injection in case of serotype O and 4 days in case of serotype A (**Figure 3.6.1**). Secondary lesions started to develop in second passage of serotype O and third passage of serotype A. With the progression of passage numbers, the incubation period gradually reduced but intensity of lesions increased. In the fifth passage both the viruses were completely adapted in guinea pigs and secondary lesions developed within 2-3 days of injection **(Table 3.6.1)**. Anorexia, increased body temperature and weight loss were associated symptoms recorded in each passage of both the viruses.

(a) **(b)**

Figure 3.6.1: (a) Swelling of foot pad after first injection of FMDV; (b) swollen foot pads of fore legs after sixth injection indicates proper adaptation of FMDV in guinea pigs.

Table 3.6.1: Gradual adaptation of selected FMDV vaccine candidates in guinea pigs

Passage No.	BAN/TA/Dh-301/2016 (Serotype O)		BAN/CH/Sa-304/2016 (Serotype A)	
	Developed lesions	Time to develop lesions	Developed lesions	Time to develop lesions
1	Edematous injected foot pad	5 days	Swollen injected foot pad	Swelling started on 4th day, completed on 6th day
2	Swelling and vesicles formation in all foot pads	Swelling started on 4th day,	Swelling and vesicles formation in injected foot pad	Lesions started to develop on 3rd day,

		completed on 7th day		completed on 6th day
3	Swelling and vesicles formation in all foot pads	Swelling started on 3rd day, completed on 6th day	Swelling and vesicles formation in all foot pads	Lesions started to develop on 4th day, completed on 5th day
4	Swelling and vesicles formation in all foot pads	Swelling started on 2nd day, completed on 5th day	Swelling and vesicles formation in all foot pads	Lesions started to develop on 3rd day, completed on 4th day
5	Swelling and vesicles formation in all foot pads	Swelling started on 2nd day, completed on 4th day	Swelling and vesicles formation in all foot pads	Lesions started to develop on 3rd day, completed on 4th day

3.6.2 Vaccination and Challenge of Guinea pigs

The guinea pigs of first four groups were vaccinated with a different dose of vaccine and placebo was injected in guinea pigs of fifth group. The guinea pigs were observed after vaccination and challenge and data were recorded as presented in **Table 3.6.2a** and **Table 3.6.2b.**

Table 3.6.2a: Recorded data of vaccination and challenge test of monovalent vaccine against FMDV serotype O

Group	No. of guinea pigs	Swelling at injection site	Rise in body temperature (^{0} C)	Lesion development	No of protected animals	No of un-protected animals
2 ml (Full dose)	5	-	-	-	5	o
500 µl	5	-	-	-	5	o
125 µl	5	-	-	2	3	2
31.25 µl	5	-	-	5	0	5
Placebo (2 ml)	5	-	-	5	0	5

Table 3.6.2b: Recorded data of vaccination and challenge test of monovalent vaccine against FMDV serotype A

Group	No. of guinea pigs	Swelling at injection site	Rise in body temperature (^{0} C)	Lesion development	No of protected animals	No of un-protected animals
2 ml (Full dose)	5	-	-	-	5	o
500 µl	5	-	-	-	5	o
125 µl	5	-	-	3	2	3
31.25 µl	5	-	-	5	0	5
Placebo (2 ml)	5	-	-	5	0	5

'Full dose' and '1/4 dose' vaccine of both types could protect all the guinea pigs, whereas, '1/16 dose' vaccine of serotype O and A could protect 3 and 2 number of guinea pigs respectively from virus challenge. No guinea pigs in '1/64 dose' vaccine and 'Control' groups of both vaccines were protected after virus challenge. Absence of adverse reactions in case of both type of monovalent vaccines indicate vaccine and adjuvant are safe for use in animals. The calculated Protective Dose$_{50}$ (PD$_{50}$) of serotype O monovalent vaccine is 18.19 and that of A is 13.8.

3.7 *In-Vitro* Vaccine Efficacy Testing

3.7.1 Virus Neutralization Test (VNT)

In-vitro quantitative virus neutralization tests revealed that the experimentally developed monovalent vaccines against FMDV serotype O and A are effective in eliciting protective immunity in vaccinated guinea pigs. The mean $\log_{10}$ SN$_{50}$ titer of prevaccinal sera of all the guinea pigs was 0±0.00SD for both type of vaccines. In case of serotype O monovalent vaccine, the elicited mean $\log_{10}$ SN$_{50}$ titers in sera of 'Full dose', '¼ dose' '1/16 dose' and '1/64 dose' vaccine group guinea pigs were 2.34±0.20, 2.19±0.27, 1.44±0.36 and 0.93±0.13 respectively. Those for serotype A monovalent vaccine were 2.34±0.13, 1.89±0.17, 1.11±0.17 and 0.78±0.07 respectively. For both type of vaccines, the guinea pigs of first two groups elicited $\log_{10}$ SN$_{50}$ titers above the protective titer 1.2 (Daoud *et al.*, 2013). In the third group, 2 and 3 number of guinea pigs could not elicit protective SN$_{50}$ titers against Serotype O and A vaccine respectively. No guinea pigs of fourth group

elicited SN_{50} titer beyond the protective level. Guinea pigs of control groups of both vaccines did not elicit neutralizing antibody and the mean log_{10} SN_{50} titer was 0±0.00. Minimum virus particles content that protected all the guinea pigs were 3.53 µg and 3.10 µg for FMDV serotype O and A vaccine respectively. The results are delineated in **Figure 3.7.1a** and **Figure 3.7.1b.**

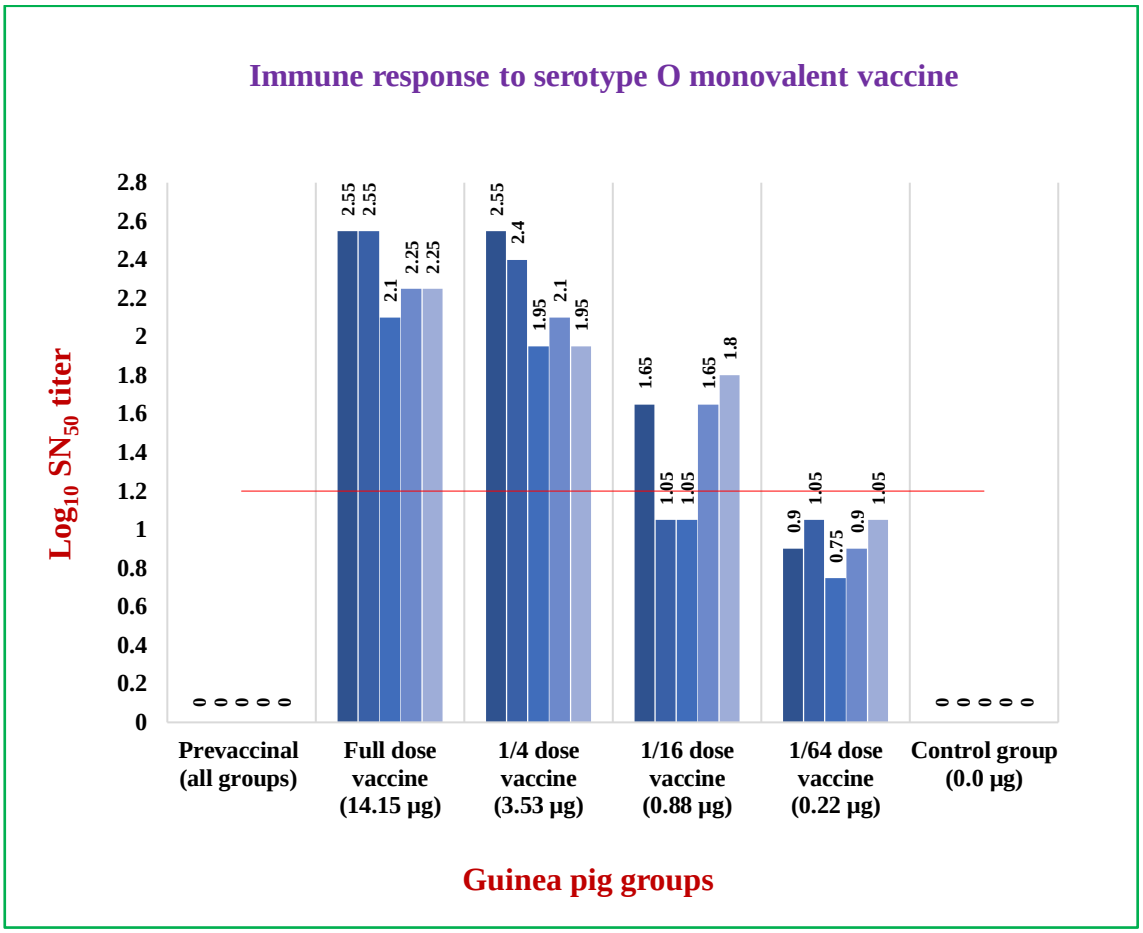


Figure 3.7.1a: Immune response of guinea pigs to FMDV serotype O monovalent vaccine. All the guinea pigs of 'Full dose', '¼ dose' and 3 guinea pigs of '1/16 dose' vaccine group elicited log_{10} SN_{50} titer above the protective level. The log_{10} SN_{50} titers of all prevaccinal sera, '1/64 dose' vaccine group and 'Control group' sera were below the protective level.

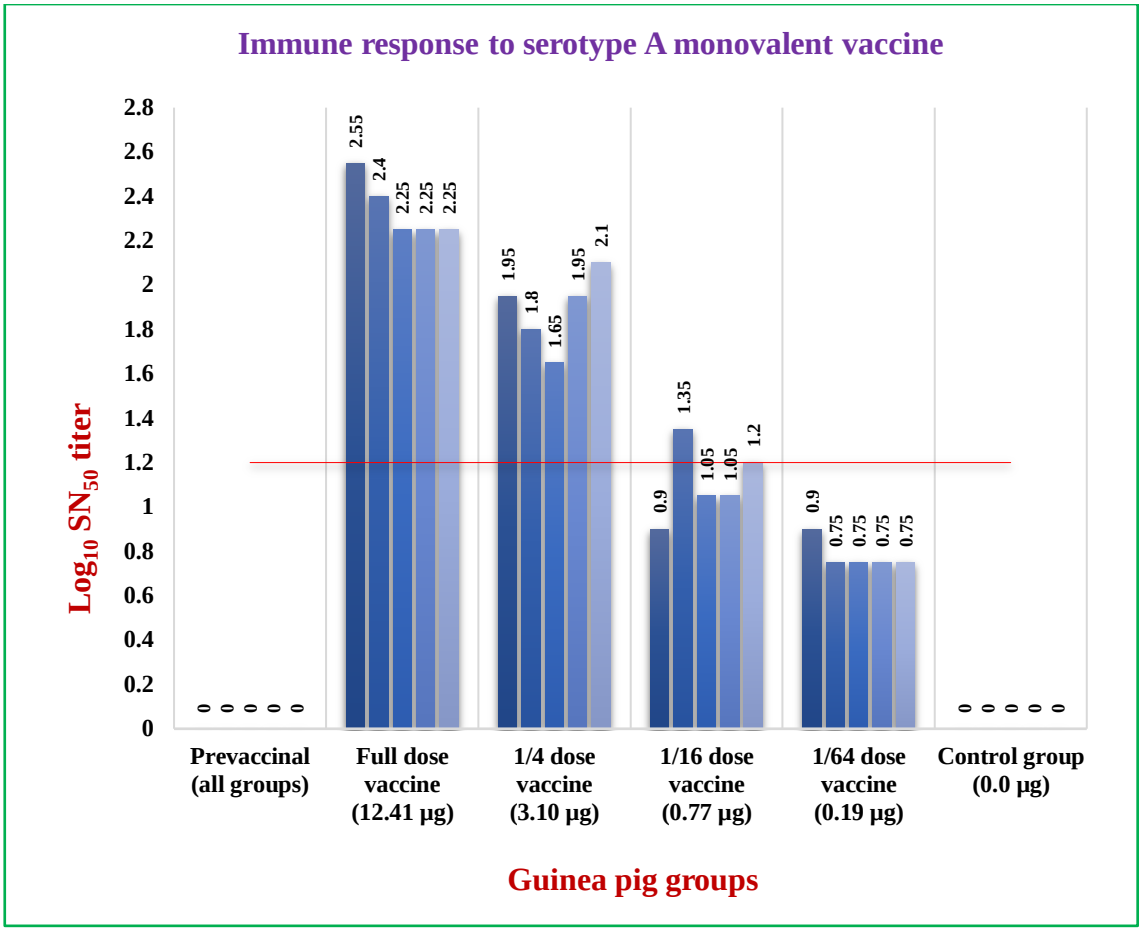


Figure 3.7.1b: Immune response of guinea pigs to FMDV serotype A monovalent vaccine. All the guinea pigs of 'Full dose', '¼ dose' and 2 guinea pigs of '1/16 dose' vaccine group elicited log_{10} SN_{50} titer above the protective level. The log_{10} SN_{50} titers of all prevaccinal sera, '1/64 dose' vaccine group and 'Control group' sera were below the protective level.

3.7.2 Two Dimensional Micro Neutralization Test (2D-MNT)

Two Dimensional Micro Neutralization Test (2D-MNT) was used to estimate antigenic relationship value ('r' value) of FMDV vaccine virus with respective field isolates.

For FMDV serotype O isolates BAN/TA/Dh-301/2016 (vaccine virus), BAN/MA/Ku-269/2015, BAN/Go/Ka-236/2015(Pig), BAN/GA/Ka-212/2014, BAN/JA/Me-180/2013, BAN/BO/Na-161/2013 and BAN/NA/Ha-156/2013 the mean r values were 1.0±0.00, 2.22±0.08, 2.48±0.22, 3.18±0.06, 0.95±0.02, 1.7±0.56 and 1.15±0.01 respectively. Whereas, for serotype A isolates BAN/CH/Sa-304/2016 (vaccine virus), BAN/DH/Sa-307/2017, BAN/DH/Sa-310/2017 and BAN/GA/Sa-197/2013 the values were 1.0±0.00, 0.42±0.09, 0.95±0.02 and 1.37±0.21respectively. The estimated mean r values of all the

selected homologous and heterologous field isolates of FMDV serotype O were above 0.3 even greater than 1.0. The findings are similar in case of serotype A. the results are delineated in **Figure 3.7.2a** and **Figure 3.7.2b**.

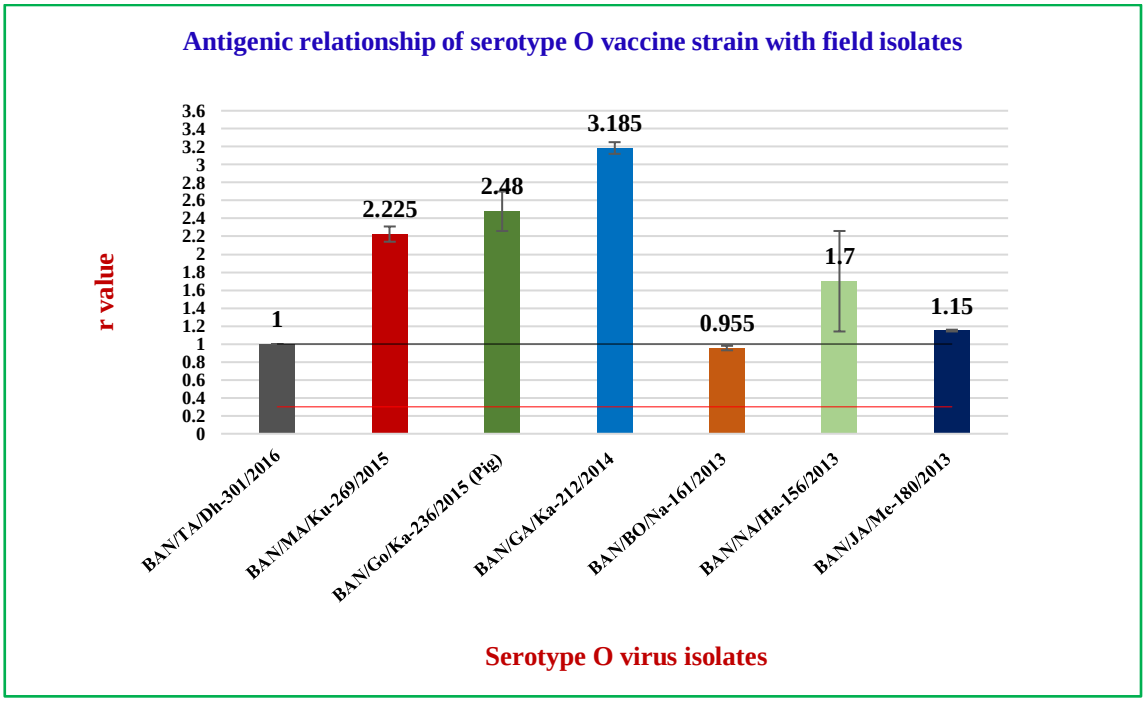


Figure 3.7.2a: r values of serotype O field isolates of different year, district and sublineages. The calculated mean r values of all the field isolates were >0.3.

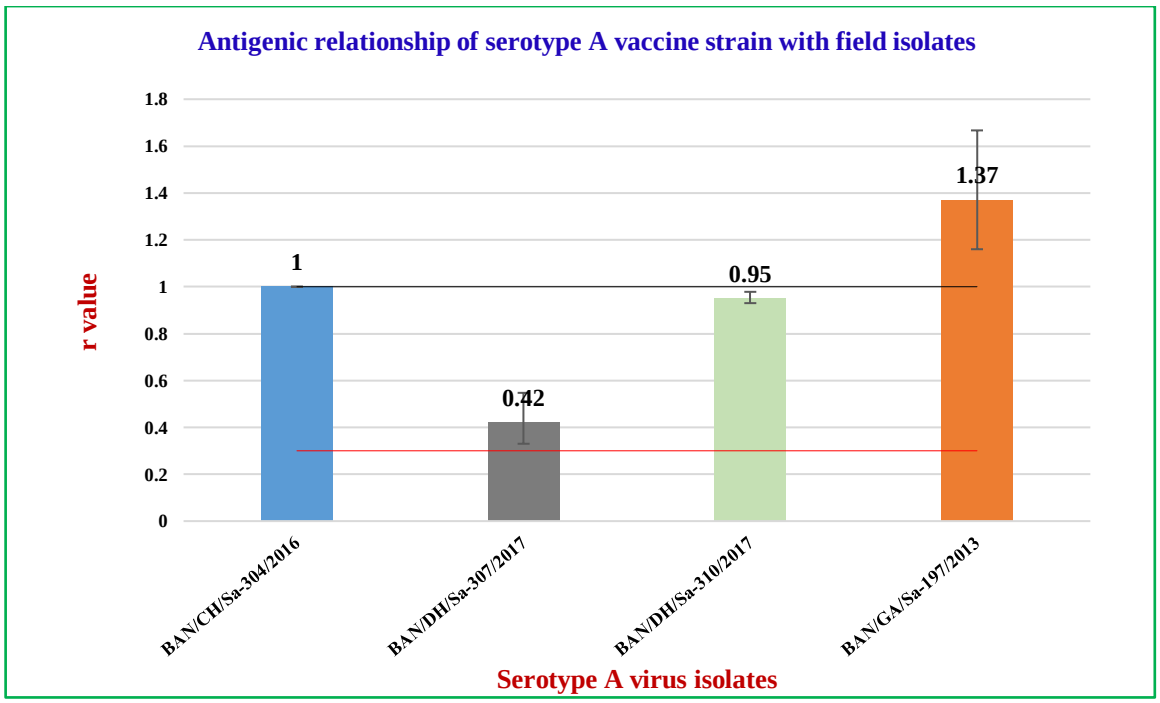
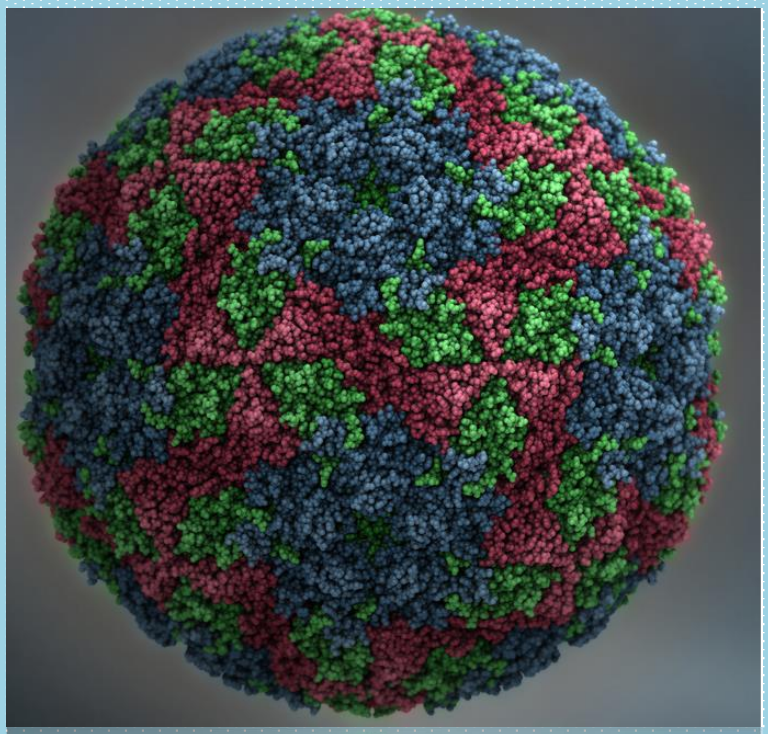


Figure 3.7.2b: r values of serotype A field isolates of different year, district. The calculated mean r values of all the field isolates were >0.3.

The r values of different field isolates of both FMDV serotype O and A indicate that inactivated vaccines produced by selected vaccine candidates BAN/TA/Dh-301/2016 and BAN/CH/Sa-304/2016 will protect all genetically divergent virus strains of respective serotypes circulating in Bangladesh.



Chapter 04:

Discussion

4. Discussion

Foot and mouth disease (FMD) is endemic in Bangladesh and seriously affects livestock productivity and trades. FAO and European Commission for the control of Foot-and Mouth Disease (EuFMD) have developed 5 step-Progressive Control Pathways for Foot-and-Mouth Disease (PCP-FMD) and later, the World Organization of Animal Health (OIE) also endorsed the proposal. This working tool mainly focused on progressive reduction of FMDVs load through risk assessments by epidemiological studies and prevention of the disease by using appropriate vaccine programing. Bangladesh also endorsed this program and expected to complete stage 2 by 2019, but due to poor reporting of the disease outbreak to appropriate authorities and lack of proper epidemiological studies, we are trailing far behind to achieve the goal. Thus, the present studies have addressed- first, on a systemic epidemiological study of FMD and characterization of etiological FMDVs types circulating in Bangladesh; and secondly, development of effective vaccine(s) using the circulatory FMDVs which give maximum protection.

Three FMDV serotypes O, A and Asia1 are circulating in Bangladesh:

In the present study VP1 gene sequence based homology search using BLAST algorithm in NCBI GenBank indicated that FMDV serotypes O, A and Asia1 were responsible for the FMD outbreaks in Bangladesh during 2012 and 2013, although majority of the outbreaks occurred by serotype O. The FMDV virus VP1 gene sequences obtained from the samples of outbreaks during 2014 to mid of 2016 had maximum homology only with FMDV serotype O. The re-emergence of serotype A and Asia1 were observed in September 2016 and January 2018 respectively, although serotype O continuously maintained its circulation. The findings confirmed the circulation of FMDV serotypes O, A and Asia1 as causative agents of foot-and-mouth disease in Bangladesh and are in consistent with previous studies reported by Nandi *et al.*, 2015 and Zinnah *et al.*, 2010. The FMD outbreaks analyses during the period of 2012-2018 further confirmed the serotype O as the most abundant (81%) serotype followed by serotype A (13%) and Asia1 (6%) as stated earlier by Siddique *et al*., 2018 (**Figure 3.1a**).

Among the 27 districts of Bangladesh under the present study area, the circulations of all three serotypes O, A and Asia1 were identified from outbreaks of Dhaka and Gazipur districts (**Figure 3.1b**). This may be imputed to the excess cattle movement towards Dhaka for slaughter purpose from all around the country because unrestricted animal movement

is very risky and associated with long distance spread of FMDV (Paton *et al.*, 2018). Circulations of more than one serotypes (O & A) were also recorded in Chittagong district attributable to same cause as in Dhaka district. Noteworthy, during the period of 2014 to 2017, no FMD outbreaks in Bangladesh by serotype Asia1 was recorded by our group and from neighboring countries from which intrusion of the disease occurred by uni-directional animal movements, hence, this thesis restricted to report mono- and bivalent vaccine development. It should be noted that we have also developed a trivalent vaccine after isolation of a new FMDV serotype Asia1 late of early quarter 2018 which at present is circulating in Bangladesh.

Circulatory FMDVs in Bangladesh diverse and maintain distance from Indian vaccine strains:

The analyses of the evolutionary history of the FMDV isolates of Bangladesh were restricted to only serotype O and A. VP1 gene based phylogeny debunked the closeness of Bangladeshi FMDV isolates of serotype O and A to their respective Indian strains. The phylogenetic analysis of VP1 gene sequences of FMDV serotype O isolates of Bangladesh confirmed that most of the isolates are placed in Ind2001 lineage of Middle East-South Asia (ME-SA) topotype except few which are clustered in an unnamed group (**Figure 3.2.1a**). These findings supported earlier reports from Bangladesh (Loth *et al.*, 2011; Nandi *et al.*, 2015; Siddique *et al.*, 2018). The serotype O isolates of Bangladesh further clustered into three different sublineages Ind2001d, Ind2001BD1 and Ind2001BD2 of Ind2001 lineage and maximum number of isolates along with the recent isolates are under sublineage Ind2001BD1. This finding confirms the continuity and dominance of sublineage Ind2001BD1 over other sublineages as reported earlier by Siddique *et al.*, 2018. The concomitant circulation of genetically divergent virus strains within the same serotype making the epidemiology of FMD in Bangladesh very complex. This epidemiological complexity is further resulting in FMD control program failures. Moreover, the current Indian serotype O vaccine candidate O/India/R2/75 did not cluster with any of the 3 sublineages; rather distantly placed from the isolates of Bangladesh (**Figure 3.2.1a**). Frequent Indian FMD vaccine failure may be imputed to the use of this genetically divergent strain in vaccine.

The phylogenetic analysis of FMDV serotypes A isolates of Bangladesh placed all of them with VP3^{59} aa deletion strains under genotype VII of Asia topotype (**Figure 3.2.1b**). The

circulation of this unique genotype is indicating the less genetic heterogeneity among the FMDV serotype A isolates of Bangladesh and is in conformity with previous reports by Ullah *et al*., 2014; Nandi *et al*., 2015 and Bachanek-Bankowska *et al*., 2018. The VP3^{59} aa deletion FMDV serotype A strains are also constantly circulating in India since 2007 (Rudreshappa *et al.*, 2012). The circulation of FMDV strains in Bangladesh similar to those in India indicates outstripping of these strains from the neighboring countries specially India due to unidirectional cross-border movement of animals.

Though the current Indian FMDV serotype A vaccine strain IND40/00 clustered in genotype VII of Asia topotype, it is a member of VP3^{59} aa none-deletion group (Mohapatra *et al.*, 2008). In phylogeny, IND40/00 strain clustered with other Indian strains at a distant place from serotype A isolates of Bangladesh. VP1 protein coding gene based phylogeny debunked the genetic heterogeneity of current Indian FMDV serotype O and A vaccine strains from respective isolates of Bangladesh. This finding established a basis for selecting new vaccine strains from indigenous FMD virus strains.

FMDV serotype O isolate BAN/TA/Dh-301/2016 and serotype A isolate BAN/CH/Sa-304/2016 considered as vaccine candidates:

Analyzing the evolutionary trees of both FMDV serotype O and A isolates circulating in Bangladesh, the most recent (at the time of selection) serotype O isolate BAN/TA/Dh-301/2016 and serotype A isolate BAN/CH/Sa-304/2016 were selected as vaccine candidates.

The rationales for selecting the vaccine candidates from indigenous virus isolates were further supported by genetic distance analysis. The current FMDV serotype O Indian vaccine strain India/R2/75 has significantly higher ($p=0.001$) genetic distance from respective isolates of Bangladesh compared to proposed vaccine candidate BAN/TA/Dh-301/2016. Similarly, the serotype A vaccine strain IND40/00 also has significantly higher ($p=0.002$) genetic distance than the selected vaccine candidate (**Table 3.2.2e)**. The genetic distance analysis revealed that the selected vaccine candidates are genetically closer to respective isolates of Bangladesh, thus increasing the probability of antigenic relatedness among them.

Significant positive selection ($p<0.05$) pressure was detected by several algorithms on five antigenically important codon positions of VP1 region of serotype O. This region has also

been reported being under strong selection pressure in case of Indian type O viruses (Mahapatra *et al.*, 2015). The positive selection on these codons indicating that they are prone to mutation and this might be the underlying cause of evolution of genetically heterogenic sublineages within a single lineage of FMDV serotype O. Furthermore, changes in these positions may be associated with changes in the antigenicity of the virus (Mahapatra *et al.*, 2016). Absence of positive selection pressure on any codon position of VP1 region of FMDV serotype A isolates of Bangladesh is itself exponential for the circulation of a unique genotype in Bangladesh. The Z-test for selection could not detect any significant positive selection ($p>0.2$) among serotype O and A isolates of Bangladesh (**Table 3.2.3)** and overruled the chances of extensive antigenic heterogeneity among the isolates of both serotypes in Bangladesh. Absence of significant evolutionary selection in Z-test indicates antigenic relatedness among the isolates of data set (Mahapatra *et al.*, 2015). Thus, the findings of Z-test predict that the vaccines prepared by selected candidates will work on respective FMDV strains circulating in Bangladesh.

As new strains have been emerging constantly within each serotype owing to high mutation rate and the quasi-species nature of FMD virus (Tosh *et al.*, 2002), complete nucleotide information of the selected vaccine candidates is a prerequisite for an authenticated approach to vaccine development. The length of complete genome of both the vaccine candidates O/BAN/TA/Dh-301/2016 and A/BAN/CH/Sa-304/2016 were found to be around 8.2 kbp with an ORF of 6999 nt. These findings are similar to previous reports on complete genome sequencing of FMDV serotype O and A isolates of Bangladesh (Ali *et al.*, 2016; Sultana *et al.*, 2014; Ullah *et al.*, 2014). Complete genome sequencing and annotation of each gene (**Table 3.3.2a** and **3.3.2b**) has deciphered the molecular and structural organization of the proposed vaccine candidates. This information will assist in investing mutation in field virus as well as in exploring the vaccine failure cases if any in the future.

The principal aim of the present study was to develop an effective vaccine and therefore the comparative genome analysis was confined predominantly to only VP1, VP2 and VP3 structural protein coding regions because these regions are only associated with eliciting protective immunity in susceptible hosts (Reeve *et al.*, 2010).

Nucleotide sequence comparison revealed noticeable divergence at nt level in VP1, VP2 and VP3 structural protein coding regions of selected FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016 and A vaccine candidate BAN/CH/Sa-304/2016 compared to O/India/R2/75 and A/IND40/00 respectively (**Figure 3.4a & 3.4b, Table 3.4)**. These changes lead to the structural variation in the stated proteins.

In case of serotype O FMDVs VP1 137-141 and 197 residues form the antigenically important sit 1, whereas VP2 134 forms the site 2 and VP1 45 forms the site 3. In addition, VP2 191 residues is related to antigenic site 2 (Lloyd-Jones *et al.*, 2017). Whereas in serotype A FMDVs, 36 antigenically critical amino acid residues (VP1 83, 137, 139, 141, 143, 145, 147–154, 159, 170, 173, 199, 201, 205 and 209; VP2 72, 79, 80, 132, 133, 196; VP3 58, 59, 61, 70, 136, 139, 175, 178, 195) across the three structural proteins VP1-3 have been identified (Rudreshappa *et al.*, 2012). Any alteration of critical residues can confer antigenic specificity to the FMD viral variants (Martinez *et al.*, 1991). Escaping of vaccine induced immunity by circulatory FMDV serotype Asia1 due to aa substitutions in G-H loop at VP1 protein was also reported by Ullah *et al.*, 2015. In this study VP1, VP2 and VP3 structural proteins of selected vaccine candidates were found to be variable than respective regions of current Indian vaccine strains with aa substitutions in antigenically critical sites **(Table 3.4)**. This protein variability was confirmed by concomitant use of three different algorithms. Hence, it is presumed that the structural variations in critical antigenic sites of indigenous FMD strains from the current vaccine strains might account for incomplete protection.

The divergence at nt and aa level across the antigenically important VP1, VP2 and VP3 regions of currently circulating FMDV serotype O and A provided strong bases in favor of selecting new vaccine candidate O/BAN/TA/Dh-301/2016 and A/BAN/CH/Sa-304/2016.

Developed mono- and bi-valent vaccines successfully meet OIE guidelines:

The inactivation kinetics of both FMDV serotype O vaccine candidate BAN/TA/Dh-301/2016 and serotype A vaccine candidate BAN/CH/Sa-304/2016 were very linear and full filled a mandatory requirement of OIE (OIE, 2012) (**Figure 3.5.2a** and **3.5.2b)**. The mean inactivation rates for O/BAN/TA/Dh-301/2016 and A/BAN/CH/Sa-304/2016 were 0.72±0.038 and 0.78±0.008 $\log_{10}$ $TCID_{50}$ per hour respectively. Aarthi *et al.*, 2004 reported an inactivation rate of 0.62 $\log_{10}$ $TCID_{50}$ per hour using 2mM BEI for both serotype O and

A. Ismail *et al.*, (2013) noticed a higher inactivation rate (1.15 $\log_{10}$ $TCID_{50}$ per hour) for SAT-2 serotype. The finding in the current study is superior to that reported by Aarthi *et al*., 2004, though 3mM BEI has been used here.

For in-process quality control of FMD virus inactivation, the OIE set mandatory criteria is that there would be less than one infectious particle per 10^4 liters of liquid preparation at the end of the inactivation period (OIE, 2012). Bahnemann, H. G., 1990 also set similar parameter for safe and successful inactivation of FMD virus. The extrapolated inactivation kinetics for both O/BAN/TA/Dh-301/2016 and A/BAN/CH/Sa-304/2016 vaccine candidates confirmed that there would be <1 infectious particle in a 10^4 liter batch after 24 hours inactivation cycle (**Figure 3.5.2a** and **3.5.2b**). Therefore, the quality control parameters of FMDV virus inactivation confirmed the complete inactivation of vaccine candidate O/BAN/TA/Dh-301/2016 and A/BAN/CH/Sa-304/2016 according to OIE prescribed procedures.

Inactivated virus particles in the cell culture fluid is concentrated in order to obtain expected concentration of inactivated antigen as well as formulating vaccine in low dose volume like 2-3 ml/dose. In the present study ultrafiltration technique using 100KDa hollow fiber cellulose membrane cartridge significantly concentrated both FMDV serotype O and A inactivated virus particles to 7 and 8.6 times of its original concentration respectively (**Figure 3.5.3c**). The findings were in conformity with the findings of Adikane *et al*., 1997.

Quantification of the concentration of inactivated virus particles in the fluid is the crucial part for quality assured vaccine production. In this study, Fast Flow Liquid Chromatography (FPLC) was found effective for this purpose. This finding corroborated the findings of Hosseini *et al.*, 2016 and Spitteler *et al.*, 2011. The success of the chromatography system was further confirmed by the detection of FMDV antigens by ELISA in peak fractions (**Figure 3.5.4a and 3.5.4b**). The successful quantification of the concentration of inactivated virus particles in the fluid facilitated vaccine formulation with desired antigenic pay-load.

In-vivo efficacy of experimentally developed two monovalent vaccines were tested in guinea pig model. The bases for choosing the guinea pig model were-(i) the most accepted laboratory animal model for FMD vaccine efficacy testing (di Girolamo *et al.*, 1985;

Habiela *et al.*, 2014) (ii) guinea pigs are easy to maintain (iii) No prior chance of natural infection by FMDV (iv) response of guinea pigs to FMDV exposure has similarity with cattle (Richard C Knudsen *et al.*, 1979) and (iv) immune response to FMDV has a good correlation with the protection afforded to cattle (El-Sayed *et al.*, 2012).

Homologous serotype O vaccine virus BAN/TA/Dh-301/2016 and A vaccine virus BAN/CH/Sa-301 were adapted in guinea pigs to an average requirement of 6-7 passages for adaption and full expression of clinical signs.

The findings were almost in conformity with observations of Balamurugan, 2005 and Motamedi *et al.*, 2007 who adapted the FMDV serotype O and A87/IRN in 6th and 5th passages respectively. There are also reports of adaptation in as early as at second passage (El-Sayed *et al.*, 2012) and as high at 10^{th} passage (Núñez *et al.*, 2001).

The guinea pigs did not show any adverse reactions (**Table 3.6.2a** & **3.6.2b**) after subcutaneous injection of vaccines ensuring the safety of both FMDV inactivated antigens and Montanide ISA 201 adjuvant. The outcomes support the previous reports by Dar *et al.*, 2013 and Ibrahim *et al.*, 2015 who compared several adjuvants and found ISA 201 very safe for use in animals.

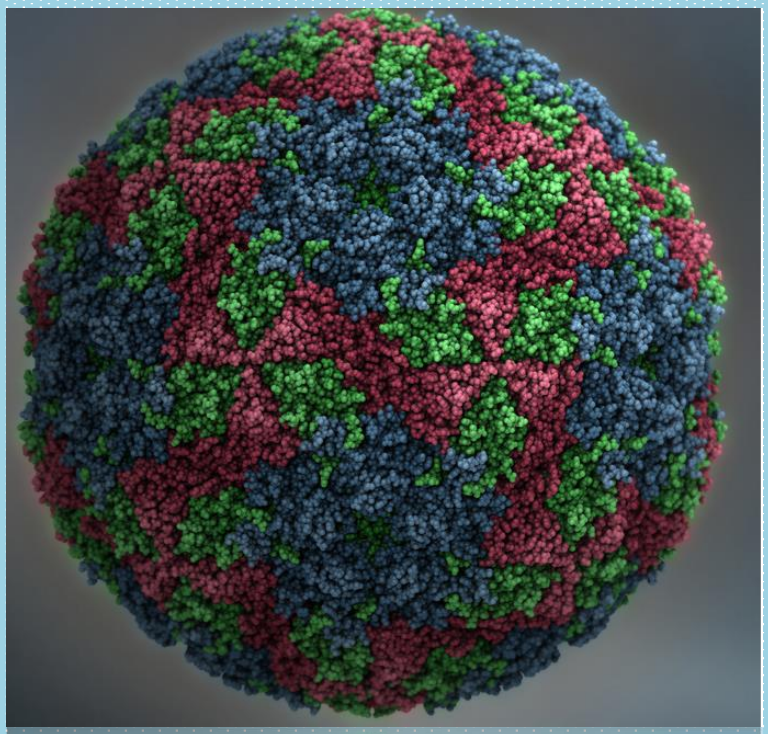
The challenge test unfurled that as low as 3.53 µg of FMDV O vaccine candidate and 3.10 µg of FMDV A vaccine candidate antigen could protect 100% animals from virus challenge (**Table 3.6.2a** & **3.6.2b**), though there was no significant correlation observed between antigenic payload and protection regarding FMD O vaccine ($p=0.185$) and A vaccine ($p=0.143$). The minimum antigen required for eliciting protective immunity was in the range of recommended antigenic pay load of 1-10 µg (Paul V Barnett *et al.*, 2003). The findings regarding antigen pay load were supported by the findings of Daoud *et al.*, 2013 who found 2.8 µg of FMDV antigen could protect 100% guinea pigs. The Protective $Dose_{50}$ (PD_{50}) of serotype O and A monovalent vaccine were found to be 18.19 and 13.8 respectively, higher enough the value of 7.06 found by Motamedi Sedeh *et al.*, 2007 but lower than those found by Daoud *et al.*, 2013. However, the PD_{50} values of the experimentally developed vaccines were substantially higher than the OIE recommendations of 3-6 PD_{50} (OIE, 2012). Thus, the higher PD_{50} values are indicatory of high effectiveness of the experimentally developed vaccines.

Virus neutralization test (VNT) was employed for *in-vitro* assessment of vaccine efficacy through determination of elicited antibody titer in sera of test guinea pigs. Noticeable seroconversions were obtained 21 days' post vaccination in guinea pigs in response to both type of vaccines. The results of VNT supported the findings of *in-vivo* challenge test. Minimal antigenic payload required to elicit SN_{50} titer significantly higher (p=0.001) than the protective level in 100% animals were 3.53 µg for FMDV O and 3.10 µg for FMDV A vaccine candidates (**Figure 3.7.1a & 3.7.1b)**. The results came parallel to the results described by Ibrahim *et al.*, 2015 who mentioned that Montanide ISA 201 adjuvanted vaccine could elicit protective antibody even after 2 weeks post vaccination. Significant correlations were not found between antigenic payload and SN_{50} titer elicited by FMD monovalent vaccine against serotype O (p=0.062) and A (p=0.093). The findings corroborated the findings of Barnett *et al.*, 2003.

Among the seven FMDV serotype O field isolates selected for 2D-MNT, three were from most abundant and dominating sublineage Ind2001BD1, one from Ind2001BD2 and the rest three from Ind2001d (**Table 2.15.2.2**). Furthermore, of the seven isolates, one was porcine origin and six were bovine origin of different years. All the serotype O isolates used in the study had r value >0.3 indicating they have close antigenic relationship with the vaccine virus (**Figure 3.7.2a**). The findings corroborate the findings of Yuvaraj *et al.*, 2013 who found that the FMDV isolates within Ind2001 lineage in India had close antigenic relationship among them with r values even >1.0. Negussie *et al.*, 2013 also reported same features of FMDV serotype O isolates in Ethiopia.

For serotype A, four isolates of different year and geographical location were selected. Noteworthy, all the isolates were genetically very close and phylogenetically under the Genotype VII of Asia topotype (**Table 2.15.2.2**). Strong antigenic relationship was observed among the isolates with r value >0.3, of which two isolates hade r value ≥1.0 (**Figure 3.7.2b**). The findings corroborated with the finding of Mohapatra *et al.*, 2008 who found that A/IND81/00 and A/IND40/00 belonging to genotype VII had the best antigenic relationship with contemporary serotype A isolates in India.

The r values of different field isolates of both FMDV serotype O and A indicated that, inactivated vaccines produced by selected vaccine candidate O/BAN/TA/Dh-301/2016 and A/BAN/CH/Sa-304/2016 will protect all genetically divergent virus strains of respective serotypes circulating in Bangladesh.

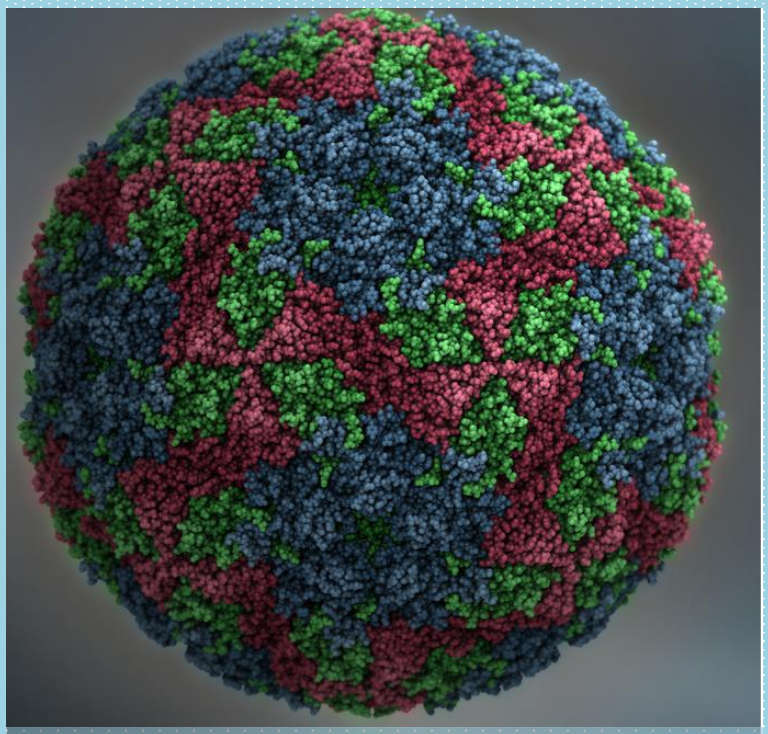


Chapter 05:

Conclusion

5. Conclusion

The focus of this study was to develop effective FMD vaccine using local isolates of FMDV to mitigate the repeated vaccine failure instances in Bangladesh. Additionally, optimization of a sustainable and fecund vaccine production process was also considered. A sequence based molecular approach was used to predict appropriate vaccine candidates. Marked genetic and structural differences between present and selected vaccine strains provided concrete rationales for new FMD vaccine in Bangladesh. Ultrafiltration, and fast flow liquid chromatography (FPLC) techniques were adopted, optimized and found very effective for quick concentration and quantification of inactivated virus particles in the liquid preparation. These two techniques played key roles behind formulation of vaccine at 2ml/dose. The developed vaccines protected the test guinea pigs from virus challenge and elicited antibody titer significantly higher than the protective level. Serology revealed that the developed vaccines would be able to protect all genetically divergent virus groups of each serotypes contemporarily circulating in Bangladesh. The newly developed FMD vaccine will contribute to effective control of the disease in livestock population and at the same time the optimized vaccine production technology will play a role in faster and efficient vaccine production thereby in mitigating vaccine deficit in Bangladesh.



Chapter 06:

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6.0 References

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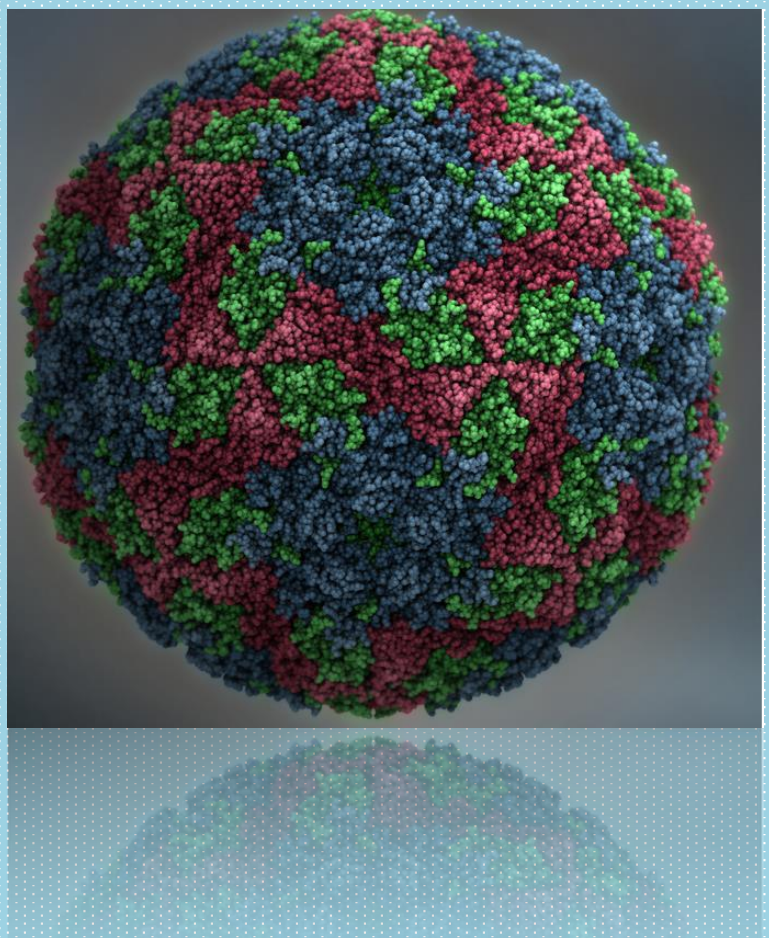

Appendices

Appendix-I

List of Positive Samples

Sample ID	PCR Amplification of VP1 gene			Serotype		Accession Number
	16F:16R (426bp)	16F:NK61 (714bp)	VP1UP:NK61 (1141bp)	Specific primer	BLAST search	
Jessore (February 2012)						
BAN/JE/Mf-01/2012	+ve	+ve	+ve	Not done	Asia1	KJ175170
BAN/JE/Mf-01/2012	+ve	+ve	+ve	Not done	Asia1	KJ175171
BAN/JE/Mf-01/2012	+ve	+ve	+ve	Not done	Asia1	KJ175172
BAN/JE/Mf-01/2012	+ve	+ve	+ve	Not done	Asia1	KJ175173
BAN/JE/Mf-01/2012	+ve	+ve	+ve	Not done	Asia1	KJ175174
BAN/JE/Mf-01/2012	+ve	+ve	+ve	Not done	Asia1	KJ175175
Chittagong (May 2012)						
BAN/CH/Ra-01/2012	+ve	-	-	A	-	-
BAN/CH/Ra-02/2012	+ve	-	+ve	Not done	A	KC795960
BAN/CH/Ra-08/2012	+ve	+ve	-	Not done	A	KC795949
BAN/CH/Ra-10/2012	+ve	-	+ve	Not done	A	KC795961
BAN/CH/Ra-13/2012	+ve	-	+ve	Not done	A	KC795962
BAN/CH/Ra-14/2012	+ve	+ve	-	Not done	A	KC795950
BAN/CH/Ra-15/2012	+ve	+ve	-	Not done	A	KC795951
BAN/CH/Ra-16/2012	+ve	+ve	-	Not done	A	KC795952
BAN/CH/Ra-18/2012	+ve	-	+ve	Not done	A	KC795953
BAN/CH/Ra-26/2012	+ve	+ve	+ve	Not done	A	KC795954
BAN/CH/Ra-28/2012	+ve	+ve	-	Not done	A	KC795955
BAN/CH/Ra-31/2012	+ve	-	-	Not done	A	KC795964
BAN/CH/Ra-39/2012	+ve	-	+ve	Not done	A	KC795965
Munshigonj (June 2012)						
BAN/MU/Lo-02/2012	+ve	-	-	O	-	-

Sample ID	PCR Amplification of VP1 gene			Serotype		Accession Number
	16F:16R (426bp)	16F:NK61 (714bp)	VP1UP:NK61 (1141bp)	Specific primer	BLAST search	
BAN/MU/Lo-04/2012	+ve	+ve	-	O	-	-
BAN/MU/Ra-07/2012	+ve	-	-	O	-	-
Tangail (July 2012)						
BAN/TA/Gh-01/2012	+ve	+ve	-	O	-	-
BAN/TA/Gh-02/2012	+ve	+ve	-	O	-	-
Faridpur (July 2012)						
BAN/FA/Bh-01/2012	+ve	-	-	O	-	-
BAN/FA/Kh-05/2012	+ve	+ve	-	Not done	O	KC795947
Chittagong (July 2012)						
BAN/CH/Sa-01/2012	+ve	-	-	O	-	-
BAN/CH/Sa-02/2012	+ve	+ve	-	O	-	-
Tangail (September 2012)						
BAN/TA/Sa-02/2012	+ve	-	-	O	-	-
BAN/TA/Sa-03/2012	+ve	-	-	O	-	-
Faridpur (October 2012)						
BAN/FA/Ka-01/2012	+ve	-	-	Not done	O	KC795956
BAN/FA/Ka-01/2012	+ve	+ve	+ve	Not done	O	KC795947
BAN/FA/Do-11/2012	+ve	+ve	-	Not done	O	KJ175178
BAN/FA/Do-12/2012	+ve	+ve	-	Not done	O	KJ175179
Pabna (October 2012)						
BAN/PA/Ra-05/2012	+ve	+ve	+ve	Not done	O	KC795957
BAN/PA/Sa-12/2012	+ve	+ve	+ve	Not done	O	KC795958
BAN/PA/Kg-16/2012	+ve	-	-	Not done	O	KC795959
BAN/PA/Kg-20/2012	+ve	+ve	-	Not done	O	KJ175180
Gazipur (November 2012)						
BAN/GA/To-01/2012	+ve	-	-	A	-	-

Sample ID	PCR Amplification of VP1 gene			Serotype		Accession Number
	16F:16R (426bp)	16F:NK61 (714bp)	VP1UP:NK61 (1141bp)	Specific primer	BLAST search	
BAN/GA/To-02/2012	+ve	+ve	+ve	Not done	A	KC795948
BAN/GA/To-03/2012	+ve	-	-	A	-	-
BAN/GA/To-04/2012	+ve	+ve	-	A	-	-
Tangail (November 2012)						
BAN/TA/Sa-01/2012	+ve	-	-	O	-	-
BAN/TA/Mi-04/2012	+ve	+ve	-	O	-	-
BAN/TA/Mi-05/2012	+ve	-	-	O	-	-
BAN/TA/Mi-06/2012	+ve	+ve	-	O	-	-
Lalmonirhat (March 2013)						
BAN/LA/Ch-129/2012	+ve	-	-	O	-	-
BAN/LA/Du-135/2012	+ve	+ve	+ve	Not done	O	KJ175181
BAN/LA/Sa-137/2012	+ve	+ve	-	Not done	O	KJ175182
BAN/LA/Ch-141/2012	+ve	-	-	O	-	-
Natore (July 2013)						
BAN/NA/Ra-151/2012	+ve	+ve	-	O	-	-
BAN/NA/Ra-156/2012	+ve	+ve	+ve	Not done	O	KF985189
BAN/NA/Ra-157/2012	+ve	-	-ve	O	-	-
Bogra (July 2013)						
BAN/BO/Na-158/2012	+ve	-	-	O	-	-
BAN/NA/Na-159/2012	+ve	+ve	-	O	-	-
Jamalpur (September 2013)						
BAN/JA/Sa-173/2012	+ve	+ve	-	O	O	-
BAN/JA/Me-180/2012	+ve	+ve	+ve	Not done	O	KJ175183
Tangail (October 2013)						
BAN/TA/Dh-184/2012	+ve	+ve	-	Not done	O	KJ175184
BAN/TA/Dh-185/2012	+ve	+ve	-	Not done	O	KJ175176

Sample ID	PCR Amplification of VP1 gene			Serotype		Accession Number
	16F:16R (426bp)	16F:NK61 (714bp)	VP1UP:NK61 (1141bp)	Specific primer	BLAST search	
BAN/TA/Dh-186/2012	+ve	+ve	-	Not done	O	KJ175185
Gazipur (October 2013)						
BAN/GA/Sr-187/2012	+ve	+ve	+ve	Not done	Asia1	KJ175186
Rangamati (October 2013)						
BAN/RA/Sa-189/2012	+ve	+ve	-	Not done	O	KJ175177
Gazipur (October 2013)						
BAN/GA/Kk-190/2012	+ve	+ve	-	O	-	-
BAN/GA/Kk-191/2012	+ve	-	-	O	-	-
BAN/GA/Kk-192/2012	+ve	+ve	-	O	-	-
Gazipur (December 2013)						
BAN/GA/Sa-193/2013	+ve	-	-	A	-	-
BAN/GA/Sa-194/2013	+ve	+ve	-	A	-	-
BAN/GA/Sa-195/2013	+ve	+ve	-	A	-	-
BAN/GA/Sa-196/2013	+ve	+ve	-	A	-	-
BAN/GA/Sa-197/2013	+ve	+ve	+ve	Not done	A	KJ754939
Tangail (March 2014)						
BAN/TA/Ma-198/2014	+ve	+ve	-	O	-	-
BAN/TA/Ma-199/2014	+ve	-	-	O	-	-
BAN/TA/Ma-200/2014	+ve	+ve	+ve	Not done	O	KY077604
Narayangonj (March 2014)						
BAN/NA/Ru-202/2014	+ve	-	-	O	-	-
BAN/NA/Ru-203/2014	+ve	-	-	O	-	-
Gazipur (September 2014)						
BAN/GA/Ka-204/2014	+ve	+ve	-	O	-	-
BAN/GA/Ka-205/2014	+ve	-	-	O	-	-
BAN/GA/Ka-212/2014	+ve	+ve	+ve	Not done	O	KY077605.1

Sample ID	PCR Amplification of VP1 gene			Serotype		Accession Number
	16F:16R (426bp)	16F:NK61 (714bp)	VP1UP:NK61 (1141bp)	Specific primer	BLAST search	
BAN/GA/Ka-213/2014	+ve	+ve	+ve	Not done	O	KY077606
Gazipur (March 2015)						
BAN/GA/Ka-215/2015	+ve	-	-	O	-	-
Dhaka (June 2015)						
BAN/DH/Dh-216/2015	+ve	-	-	O	-	-
BAN/DH/Dh-217/2015	+ve	-	-	O	-	-
Pabna (August 2015)						
BAN/PA/Ch-220/2015	+ve	+ve	-	O		
BAN/PA/Ch-229/2015	+ve	+ve	+ve	Not done	O	Not submitted
Sirajgonj (August 2015)						
BAN/SI/Sh-233/2015	+ve	-	-	O	-	-
BAN/SI/Sh-234/2015	+ve	+ve	-	Not done	O	KY077610
Gopalgonj (August 2015)						
BAN/GO/Ka-236/2015	+ve	+ve	+ve	Not done	O	KX712091
BAN/GO/Ka-237/2015	+ve	-	-	O	-	-
BAN/GO/Ka-239/2015	+ve	+ve	-	O	-	-
Narail (August 2015)						
BAN/NL/Lo-241/2015	+ve	+ve	-	O	-	-
BAN/NL/Lo-245/2015	+ve	+ve	+ve	Not done	O	KY077611
Lakshmipur (October 2015)						
BAN/LK/Sa-247/2015	+ve	-	-	O	-	-
Noakhali (October 2015)						
BAN/NO/Be-251/2015	+ve	-	-	O	-	-
Dinajpur (October 2015)						
BAN/DI/Sa-252/2015	+ve	+ve	+ve	Not done	O	KY077616

Sample ID	PCR Amplification of VP1 gene			Serotype		Accession Number
	16F:16R (426bp)	16F:NK61 (714bp)	VP1UP:NK61 (1141bp)	Specific primer	BLAST search	
BAN/DI/Sa-254/2015	+ve	+ve	+ve	Not done	O	KY077617
Panchagar (October 2015)						
BAN/PG/At-262/2015	+ve	+ve	+ve	Not done	O	KY077618
BAN/PG/At-264/2015	+ve	+ve	+ve	Not done	O	KY077619
BAN/PG/At-265/2015	+ve	+ve	+ve	Not done	O	KY077620
Thakurgaon (October 2015)						
BAN/TG/Ba-268/2015	+ve	+ve	+ve	-	O	KY077621
Moulovibazar (December 2015)						
BAN/MA/Ku-269/2015	+ve	+ve	+ve	Not done	O	KY077622
Siragonj (December 2015)						
BAN/SI/Sa-273/2015	+ve	-	-	O	-	-
Magura (December 2015)						
BAN/MG/Sa-275/2015	+ve	-	-	O	-	-
Lalmonirhat (January 2016)						
BAN/LA/Ad-278/2015	+ve	+ve	+ve	Not done	O	KY077623
BAN/LA/Ad-279/2015	+ve	+ve	+ve	Not done	O	Not submitted
Kurigram (January 2016)						
BAN/KU/Fu-280/2015	+ve	+ve	+ve	Not done	O	Not submitted
BAN/KU/Fu-283/2015	+ve	+ve	+ve	Not done	O	KY077624
Magura (April 2016)						
BAN/MG/Sa-285/2016	Not done	+ve	-	Not done	O	-
BAN/MG/Sa-287/2016	Not done	+ve	-	Not done	O	KY077625
BAN/MG/Sa-289/2016	Not done	+ve	-	Not done	O	-
BAN/MG/Sa-292/2016	Not done	+ve	-	Not done	O	-
BAN/MG/Sa-294/2016	Not done	+ve	-	Not done	O	KY077626

Sample ID	PCR Amplification of VP1 gene			Serotype		Accession Number
	16F:16R (426bp)	16F:NK61 (714bp)	VP1UP:NK61 (1141bp)	Specific primer	BLAST search	
Tangail (August 2016)						
BAN/TA/Dh-299/2016	Not done	+ve	-	Not done	O	KY077627
BAN/TA/Dh-301/2016	Not done	+ve	-	Not done	O	KY077628
Chittagong (September 2016)						
BAN/CH/Sa-302/2016	Not done	+ve	-	Not done	A	KY077629
BAN/CH/Sa-304/2016	Not done	+ve	+ve	Not done	A	KY077630
Dhaka (December 2016)						
BAN/DH/Sa-307/2016	Not done	+ve	+ve	Not done	A	Not submitted
Pabna (March 2017)						
BAN/PB/St-308/2016	Not done	+ve	+ve	Not done	O	Not submitted
Dhaka (April 2017)						
BAN/DH/Sa-310/2016	Not done	+ve	-ve	Not done	A	Not submitted
Netrokona (May 2017)						
BAN/NT/Sa-315/2017	Not done	+ve	-ve	Not done	O	Not submitted
Dhaka (January 2018)						
BAN/DH/Sa-318/2018	Not done	+ve	+ve	Not done	Asia1	Not submitted
BAN/DH/Sa-319/2018	+ve	+ve	+ve	Not done	Asia1	Not submitted

Appendix-II

Solutions and Reagents used

Preparations of the stock solutions used in this work are given below: (all the working solutions used in this work were prepared from the stock solutions)

TAE Buffer

242 g of tris-base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) was taken and distilled water was added to the mixture to make 1L. 1X concentrated TAE buffer was made by adding 10 ml 50X TAE buffer with 490 ml distilled water and stored at room temperature.

Ethidium Bromide Solution

10 µl of ethidium bromide was dissolved in 100 ml TAE buffer to make a final concentration of 20 mg/ml and stored at 4°C in the dark.

Gel loading buffer

Ingredients	Amount (g/L)
Sucrose	6.7
Bromophenol blue	0.04
Distilled water	Up to 1 L

Maxwell® 16 Total RNA Purification Kit (Catalog No. AS1050, Promega, USA)

Reagents	
Maxwell® 16 RNA Cartridges	Lysis Buffer
RNA Dilution Buffer (RDB)	Clearing Agent (CAA)
Nuclease-Free Water	Mercaptoethanol, 97.4%
Clearing Columns	Collection Tubes
Plungers	Elution Tubes

GoScript™ Reverse Transcription System (Promega, USA)

Reagents

GoScript™ Reverse Transcriptase
GoScript™ 5X Reaction Buffer
MgCl2
dNTP Mix
Oligo(dT)15 Primer
Random Primers
1.2kb Kanamycin Positive Control RNA
Upstream Control Primer
Downstream Control Primer
Nuclease-Free Water
Recombinant RNasin® Ribonuclease Inhibitor

Wizard® SV Gel and PCR Clean-Up System (Catalog No. A9282, Promega, USA)

Reagents	Purpose
Membrane Binding Solution	Help in binding of PCR product
SV Minicolumn	For Binding of PCR product
Collection Tube	For collection of flow-through
Membrane Wash Solution	For washing purposes
Nuclease-Free Water	For elution of the purified DNA from the GD column

Dulbecco's Modified Eagle Medium (Catalog No. 11965092, Thermo Fisher Scientific, USA)

Components	Conc. (mg/L)	Components	Conc. (mg/L)
Amino Acids			

Glycine	30.0	L-Methionine	30.0
L-Arginine hydrochloride	84.0	L-Phenylalanine	66.0
L-Cystine 2HCl	63.0	L-Serine	42.0
L-Glutamine	584.0	L-Threonine	95.0
L-Histidine hydrochloride-H_2O	42.0	L-Tryptophan	16.0
L-Isoleucine	105.0	L-Tyrosine	104.0
L-Leucine	105.0	L-Valine	94.0
L-Lysine hydrochloride	146.0		
Vitamins			
Choline chloride	4.0	Pyridoxine hydrochloride	4.0
D-Calcium pantothenate	4.0	Riboflavin	0.4
Folic Acid	4.0	Thiamine hydrochloride	4.0
Niacinamide	4.0	i-Inositol	7.2
Inorganic Salts			
Calcium Chloride	200.0	Sodium Bicarbonate	3700.0
Ferric Nitrate	0.1	Sodium Chloride	6400.0
Magnesium Sulfate	97.67	Sodium Phosphate monobasic	125.0
Potassium Chloride	400.0		
Other Components			
D-Glucose (Dextrose)	4500.0		
Phenol Red	15.0		

Formulations for Cell Culture Media

Type of Media	FBS (Fetal Bovine Serum)	DMEM (Dulbecco's Modified Eagle Medium)	Penicillin and Streptomycin	Gentamycin
Growth Media	10%	90%	10000 µg/ml	10 mg/ml
Maintenance Media	2%	98%		

20% Sodium Thiosulfate

200 ml of 20% sodium thiosulfate solution was prepared by adding 40 g of sodium thiosulfate was taken in an autoclaved 250 ml glass bottle (Duran, Germany). Then Nano pure water was added to the bottle up to 200 ml, mixed thoroughly, filtered through 0.22 µm syringe filter assembly and cooled at 4^0 C prior to use.

0.1% Crystal Violet Stain

The stain was prepared by dissolving 1 g of crystal violet powder (Merck, India) in 900 ml of Nano pure water and adding 100 ml of 37% formaldehyde to make 1000 ml.

30 mM Tris buffer with 400 mM NaCl, pH 8.0

3.634 g of tris-base and 23.68 g of sodium chloride were weighed separately and taken in an autoclaved glass bottle. Water was added up to 1L and pH was adjusted to 8.0 by dropwise adding of 37% hydrochloric acid.

Appendix-III

Table 1: FMDV Serotype O Isolates used in Phylogeny, Genetic Distance Calculation and Selection Analysis

Name of Isolate	Accession No.	Name of Isolate	Accession No.	Name of Isolate	Accession No.
BAN/TA/Dh-301/2016	KY077628.1	BAN/9/2009	HQ630681.1	BAN/TG/Ba-268/2015	KY077621.1
BAN/NA/Ha-156/2013	KF985189	BAN/5/2009	HQ630680.1	BAN/TG/Ba-265/2015	KY077620.1
BAN/GO/Ka-236(Pig)/2015	KX712091.1	BAN/4/2009	HQ630679.1	BAN/PG/At-264/2015	KY077619.1
BD_SI_6_2013	KT037120.1	BAN/3/2009	HQ630678.1	BAN/PG/At-262/2015	KY077618.1
BD_Gh_2_2013	KT037119.1	BAN/2/2009	HQ630677.1	BAN/DI/Sa-254/2015	KY077617.1
BD_SI_5_2013	KT037118.1	BAN/1/2009	HQ630676.1	BAN/DI/Sa-252/2015	KY077616.1
BD_BAU_ML1_2013	KT960948.1	BD_AH_21_2011	KY595434.1	BAN/NO/Be-251/2015	KY077615.1
BAN_PA_Kg-16_2012	KC795959.1	BD_AH_20_2011	KY595433.1	BAN/NO/Be-250/2015	KY077614.1
BAN_PA_Sa-12_2012	KC795958.1	AH_19_2013	KY595432.1	BAN/LK/Sa-249/2015	KY077613.1
BAN_PA_Ra-05_2012	KC795957.1	BD_AH_18_2012	KY595431.1	BAN/LK/Sa-248/2015	KY077612.1
BAN_FA_Ka-01_2012	KC795956.1	BD_AH_17_2012	KY595430.1	BAN/NL/Lo-245/2015	KY077611.1
BAN_FA_Kh-05_2012	KC795947.1	BD_AH_16_2013	KY595429.1	BAN/SI/Sh-234/2015	KY077610.1
BAN/31/2009	HQ630692.1	BD_AH_15_2012	KY595428.1	BAN/PA/Ch-228/2015	KY077609.1
BAN/30/2009	HQ630691.1	BD_AH_14_2011	KY595427.1	BAN/DH/Dh-216/2015	KY077608.1
BAN/29/2009	HQ630690.1	BD_AH_13_2012	KY595426.1	BAN/GA/Ka-215/2015	KY077607.1
BAN/28/2009	HQ630689.1	BD_AH_12_2011	KY595425.1	BAN/GA/Ka-213/2014	KY077606.1
BAN/27/2009	HQ630688.1	BD_BAU_ML2_2013	KT982203.1	BAN/GA/Ka-212/2014	KY077605.1

Name of Isolate	Accession No.	Name of Isolate	Accession No.	Name of Isolate	Accession No.
BAN/26/2009	HQ630687.1	BAN/TA/Dh-299/2016	KY077627.1	BAN/TA/Ma-200/2014	KY077604.1
BAN/25/2009	HQ630686.1	BAN/MG/Sa-294/2016	KY077626.1	BAN/GA/Kk-192/2013	KY077603.1
BAN/24/2009	HQ630685.1	BAN/MG/Sa-287/2016	KY077625.1	BAN/GA/Kk-191/2013	KY077602.1
BAN/23/2009	HQ630684	BAN/KU/Fu-283/2016	KY077624.1	BAN/BO/Na-162/2013	KY077601.1
BAN/20/2009	HQ630683.1	BAN/LA/Ad-278/2016	KY077623.1	BAN/BO/Na-161/2013	KY077600.1
BAN/11/2009	HQ630682.1	BAN/MA/Ku-269/2015	KY077622.1	BAN/JA/Me-180/2013	KJ175183.1
BAN/TA/Dh-186/2013	KJ175185.1	BAN/TA/Dh-184/2013	KJ175184.1	BAN/LA/Du-135/2013	KJ175181.1
BAN/PA/Kg-20/2012	KJ175180.1	BAN/FA/Kh-05/2012	KC795947.1	BAN/FA/Do-11/2012	KJ175178.1
BAN/FA/Do-12/2012	KJ175179.1	BAN/25/2009	HQ630686.1	BAN/20/2009	HQ630683.1
BD_BAU_ML2 2013	KT982203.1	O/IND205/2013	KM264362.1	IND142/2013	KJ825801.1
O/IND22/2011	KC506491.1	O/IND163/2011	KC506501.1	O/IND187/2011	KC506505.1
O/IND132/2010	KC506472.1	O/IND102/2010	KC506466.1	O/IND27/2012	KC506510.1
O/IND128/2010	KC506540.1	O/IND56/2011	KC506443.1	O/IND182/2011	KC506553.1
O/India/R2/75	AF204276.1	BHU/1/2013	KJ206908.1	NEP/17/2013	MG983713.1
O/VIT/3/97	AJ294930.1	O/VIT/4/97	AJ294929.1	O/PHI/2/95	KM243042.1

Table 2: FMDV Serotype A Isolates used in Phylogeny, Genetic Distance Calculation and Selection Analysis

Appendices

Name of Isolate	Accession No.	Name of Isolate	Accession No.	Name of Isolate	Accession No.
BAN/CH/Sa-304/2016	KY077630.2	BAN_CH_Ra-18_2012	KC795953.1	BD_AH_10_2012_VP1	KY421678.1
BD_SI_16_2013	KR869773.1	BAN_CH_Ra-16_2012	KC795952.1	BD_BAU_ML4_2014	KT982205.1
BAN_CH_Ra-31_2012	KC795964.1	BAN_CH_Ra-15_2012	KC795951.1	BD_BAU_ML3_2013	KT982204.1
BAN_CH_Ra-30_2012	KC795963.1	BAN_CH_Ra-14_2012	KC795950.1	BD_SI_35_2013	KU684494.1
BAN_CH_Ra-02_2012	KC795960.1	BAN_CH_Ra-08_2012	KC795949.1	BAN/CH/Sa-302/2016	KY077629.1
BAN_CH_Ra-28_2012	KC795955.1	BAN_GA_To-02_2012	KC795948.1	BAN_GA_Sa-197_2013	KJ754939.1
BAN_CH_Ra-26_2012	KC795954.1	BD_AH_11_2013	KY421679.1	IND107/2013 (199)	KU612882.1
IND404/2012 (828)	KU612875.1	IND267/2012 (612)	KU612872.1	IND245/2007	HQ127704.1
IND17/2009	HQ127719.1	IND165/2008	KU612844.1	IND45/2010	HQ666880.1
IND250/2003	KU612831.1	IND249/2004	HQ127680.1	IND199/1995	KU737534.1
IND99/2006	HQ127695.1	IND40/00	HM854025.1	IND106/2006	HQ127698.1
IND61/1988	KU737533.1	IND340/2000	KU612820.1	A/BHU/27/2003	FJ755013.1
A/BHU/41/2002	EU414525.1	A/NEP/21/84	FJ755081.1	A/BAL/PAK/19/2011	KT832829.1
FMDV A/229-1/41SB/Pak-2012	KU641166.1	FMDV A/RAJ-13/43/Pak-2013	KU569949.1		

Appendix-IV

Foot and mouth Disease Virus Serotype O Vaccine Candidate BAN/TA/Dh-301/2016 Complete Genome (5`-3`)

TTGAAAGGGGGCGCTAGGGTCTCACCCTAGCACACCACCGGCAACTCCCGCGTTGCACTCCA
CACTTCGCCCGTGTACTCGCGGGAACCGATGGACTTTCGTTCACCCACCTACAGCTGGACTCA
CGGCACCGCGAGGCCATCTTAGCTGGACTGTGCGGACGAACACCGCTTGCGCATCTCGCGTG
ACCGGTTAATACTCTTACCACTTTCCGCCTGCTTGGTCATTAGCGCTGTCCTGGGCACTCCTGT
TGGGGGCCGTTCGACGCTCCACGGTCTCCCCCCGCGTAACGGACTACGGTGATGGGGCCGCCT
CGTGCGGGTTGGTCGCTTGGTCTGCTTCGGTTGTTGCTCGAAGCCCGCCTTTCACCCCCCCCCC
CCCCCCAAGTTTTACCGTCGTTCCCGACGTTAAAGGGGTGTAACCACAAGCTTGGAACCGTCT
TGCCCGACGTTAATGGGTTGTAACCACGCGCTTGTACCGCCTTTCCCAGCGCTACCGGGATGT
AACCAAAAGATGAACCTTCACCCGGAAGTAAAACGGCAACCACACTCAGTTTTGCCCGTTTT
CATGAGAAATGGGACGTCAGCGCACGAAACGCGCCGTCGCTTGAGGAAGATTTGTACAAACA
CGATCTATGCAGGCTTCCACAACTGACACGAACCGTGCAATTTGAAGCTCCGCCTGGTCTTTC
CAGGTCTAGAGGGGCAACACTTTGTACTGTGCTTGACTCCACGCTCGGTCCACTAGCGGGTGT
TAGTAACAGCACTGTTGTTTCGTAGCGGAGCATGATGGCCGCGGGAACTCCCCCTTGGTGACA
AGGACCCGCGGGGCCGAAAGCCACGTCCTAACGGACCCATCATGTGTGCAACCCCAGCACGG
CAACTCTACTGTGAAAACCACTTTAAGGTGACACTGATACTGGTACTCAACCACTGGTGACAG
GCTAAGGATGCCCTTCAGGTACCCCGAGGTAACAAGTGACACTCGGGATCTGAGAAGGGGAC
AGGGGCTTCTTTAAAAGCGCTCTGTTTAAAAAGCTTCTATGCCTGAATAGGCGACCGGAGGCC
GGCGCCTTTTCCACTAAACCACTACTGACTTTATGAATACAACTGACTGTTTTATCGCTCTGCT
GCACGCTCTCAGAGAGATAAAAACACTGTTTCTTTCACGAACACAAGGAAAGATGGAATTCA
CACTTCACAACGGTGAGAAAAAGACTTTCTATTCTAGACCCAACAACCACGACAATTGTTGG
CTGAATACCATCCTCCAATTGTTTAGGTACGTCGATGAACCTTTCTTTGACTGGGTCTATGAAT
CGCCTGAAAACCTCACTCTTGAGGCGATTAGGCAATTGGAAGAACTCACTGGTCTTGAGCTGC
ACGAGGGTGGGCCGCCCGCTCTCGTCATTTGGAACATCAAGCACTTGCTCCACACCGGAATC
GGCACTGCTTCGCGACCCAGCGAGGTGTGCATGGTTGATGGCACGGACATGTGTTTGGCTGA
CTTCCACGCTGGCATCTTCCTGAAAGGGCAAGAGCACGCTGTGTTCGCCTGCGTCACCTCCGA
CGGGTGGTACGCGATCGACGACGAGGACTTCTACCCCTGGACGCCGGATCCGTCCGACGTTC
TGGTGTTTGTCCCGTACGATCAAGAACCACTCAATGGAGGGTGGAAGACAAAGGTTCAAAAG
CGACTCAAAGGAGCCGGGCAATCCAGCCCGGCAACTGGGTCGCAGAACCAGTCAGGCAACA
CTGGAAGTATTATCAACAACTACTACATGCAGCAGTACCAGAACTCTATGGACACACAACTT
GGAGACAACGCCATCAGCGGAGGCTCCAACGAGGGGTCCACAGACACCACTTCCACCCACAC
AACCAACACACAAAACAATGATTGGTTCTCAAAACTGGCCAGTTCTGCCTTCAGCGGTCTGTT
TGGCGCTCTTCTCGCTGACAAGAAAACCGAGGAGACCACTCTCCTCGAGGACCGCATCCTCA
CTACCCGCAACGGACACACGATCTCGACAACCCAGTCGAGCGTCGGAGTTACTTACGGGTAC
GCAACAGCTGAGGATTTTGTGAGCGGGCCAAACACATCTGGTCTCGAGACCAGGGTTGTGCA
GGCAGAGCGGTTCTTCAAAACCCATCTGTTCGACTGGGTCACCAGCGATCCATTCGGGCGGTG
CCACCTGCTAGAACTTCCAACTGACCACAAAGGGGTCTACGGCGGTCTGACCGACTCTTATGC
TTACATGAGAAACGGTTGGGACGTTGAGGTCACTGCAGTGGGAAACCAGTTCAACGGAGGTT
GTTTGCTGGTGGCCATGGTGCCAGAACTTTGCTCCATCCAGAAGAGAGAGCTGTACCAACTTA
CGCTCTTCCCTCATCAGTTCATCAACCCACGCACGAACATGACGGCGCACATCACTGTGCCAT
TTGTTGGCGTCAACCGCTACGACCAATACAAGGTACACAAGCCTTGGACCCTTGTGGTCATGG
TCGTGGCCCCTTTGACTGTCAACAACGAAGGTGCCCCACAGATCAAGGTTTACGCCAACATCG
CCCCCACCAACGTCCACGTTGCGGGTGAGTTCCCTTCCAAAGAAGGGATCTTCCCCGTGGCGT
GCAGCGACGGTTACGGCGGTCTGGTGACCACTGACCCGAAGACGGCTGACCCCGCCTACGGG
AAAGTGTTTAACCCCCCTCGCAACATGTTGCCCGGGCGGTTCACCAACTTCCTTGATGTGGCT
GAGGCGTGTCCTACGTTTCTGCACTTTGAAGGTGACGTACCGTACGTGACCACGAAGACAGA
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Foot-and-mouth Disease Virus Serotype A Vaccine Candidate BAN/CH/Sa-304/2016 Complete Genome (5`-3`)

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Appendix-V

Figure 1: Questionnaire used during sample collection

Data collection sheet

SL No.: Date:

Owner's name and address: .....

Patient's data:

Present condition:

Breed..... Age..... Years Sex .....

Clinical signs

- ☐ Excessive salivation
- ☐ Fever
- ☐ Lameness
- ☐ Vesicle formation
- ☐ Loss of milk production (milking cow)

Treatment: Yes No

If yes:

Vaccination: Yes No

If yes:

Previous condition:

Infection with FMDV: Yes No

Breed..... Age..... Years Sex .....

Number of death cases:

Treatment: Yes No

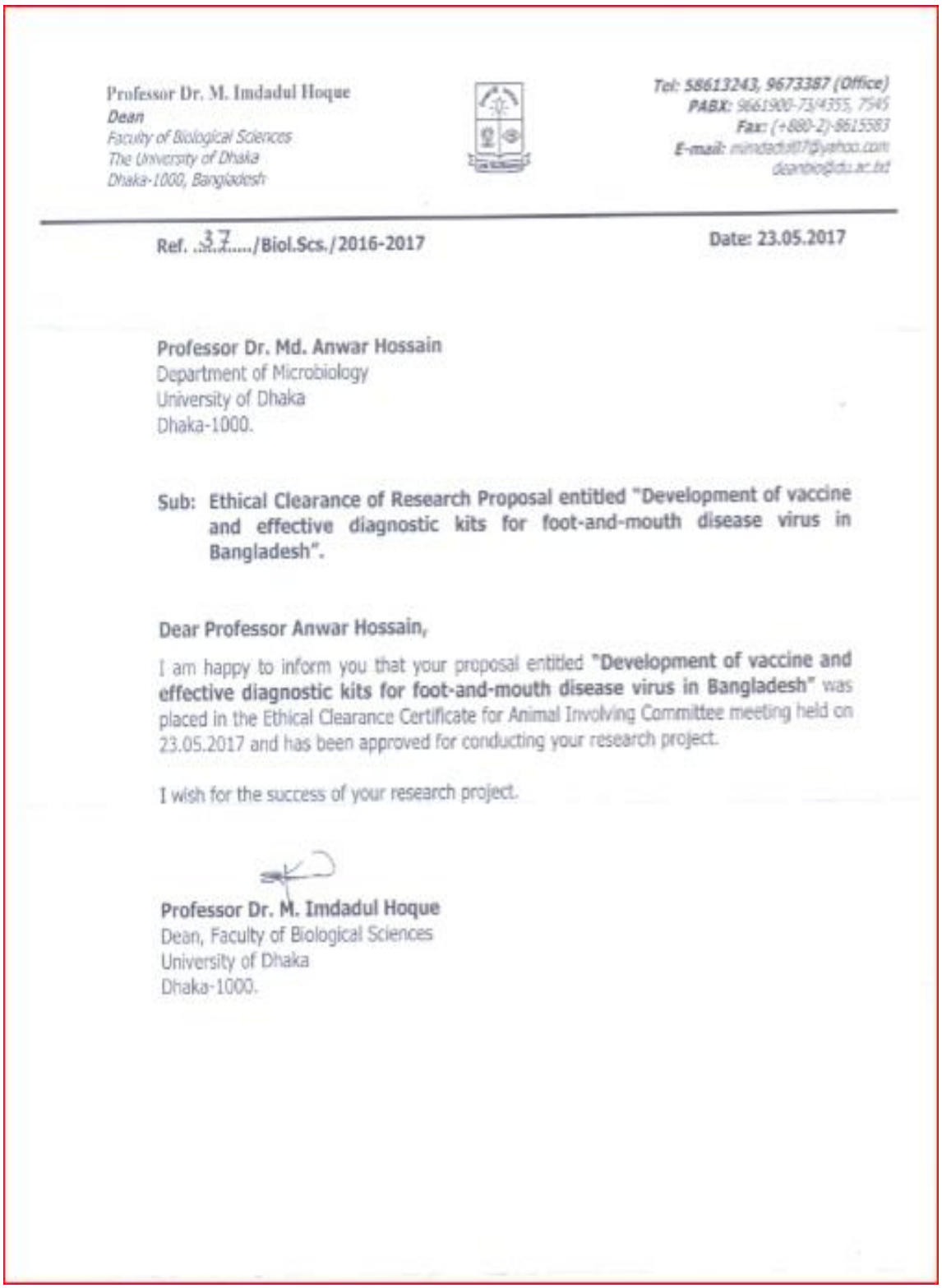
If yes: .....

Vaccination: Yes No

If yes: .....

Signature of collector

Figure 2: Ethical Clearance of Research Proposal



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Ref. 37...../Biol.Scs./2016-2017

Date: 23.05.2017

Professor Dr. Md. Anwar Hossain
Department of Microbiology
University of Dhaka
Dhaka-1000.

Sub: Ethical Clearance of Research Proposal entitled "Development of vaccine and effective diagnostic kits for foot-and-mouth disease virus in Bangladesh".

Dear Professor Anwar Hossain,

I am happy to inform you that your proposal entitled **"Development of vaccine and effective diagnostic kits for foot-and-mouth disease virus in Bangladesh"** was placed in the Ethical Clearance Certificate for Animal Involving Committee meeting held on 23.05.2017 and has been approved for conducting your research project.

I wish for the success of your research project.

Professor Dr. M. Imdadul Hoque
Dean, Faculty of Biological Sciences
University of Dhaka
Dhaka-1000.