Evolution and molecular epidemiology of foot and mouth disease virus in Bangladesh

PhD. Thesis

DEPARTMENT OF MICROBIOLOGY
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Evolution and molecular epidemiology 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….

"Keep your face always

Towards the sunshine

-and shadows will

Fall behind you"

Walt Whitman

Certification

It is hereby certified that student bearing Reg. No. 137, Session 2018-2019 has carried out the research work entitled **"Evolution and molecular epidemiology of foot and mouth disease virus in Bangladesh"** for the fulfillment of his PhD Degree from University of Dhaka, Bangladesh, under our academic supervision in the Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka.

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Abstract

Foot and mouth disease (FMD) in cloven-hoofed animals is considered an economically devastating disease for livestock sector in Bangladesh and globally. The World Organization for Animal Health (OIE) and FAO proposed a strategy of 5-step FMD eradication process from the epidemic's regions. Of which 0-2 steps are identification of epidemiology studies and identification appropriate causing FMDV circulating in the regions and selection of effective vaccine as it is the only appropriate step to control the FMD. On the background our laboratory has been working science 2011 and this thesis presents last 10 years (2012-2021) FMDV epidemiology and identification of a new genotype MYMBD21 under the lineage SA- 2018 most recently circulating in Bangladesh from 2021. This research including 32 districts and 71 outbreaks to reveal epidemiological patterns and evolutionary trends of FMDV over the past 10 years (2012-2021). Isolation of FMDV in cell culture BHK 21 cell line, VP1 gene and whole viral genome sequencing techniques combined with bioinformation tools are used for epidemiological studies and characterization of new novel genotype MYMBD21. The findings revealed that 54.7% prevalence of FMD with the majority of outbreaks occurring during the rainy season. Different risk factors such as age, gender, farming system and vaccination status demonstrated a significant association with FMD cases which was confirmed ($p<0.05$). Genotype O the most predominated over the variant A (11%) and variant Asia1 (4%). Emergence of Novel sublineages, Ind-2001BD1(Ind-2001e) and Ind-2001BD2 were reported under serotype O, the G-IX lineage of serotype Asia1 emerged in 2018, and most recently in 2021, a new genotype named MYMBD21 under the lineage SA-2018 was detected for the first time in Bangladesh. Until now, Ind-2001e (Ind-2001BD1) sublineage under serotype O became the predominant sublineage in Bangladesh. From the mutational trend analysis, highly variable sites were observed at positions 138 and 140 within the G-H loop for serotype O. For serotype A and Asia1, 45th and 44th remains in the B-C loop revealed the highest amino acid variations, respectively. A changing mutational pattern among the 2012-21 FMDV O, A and Asia 1 isolates were also observed. The whole genomes of ten FMDVs were sequenced from 2012 to 2021. In 2018, A new era O/ME-SA/2018 was detected in India which was also found to circulate in Bangladesh in 2021 with a significant Virus Protein 1 (VP1) nucleotide divergence (5-6%) suggesting the evolution of a novel sublineage, MYMBD21. This study reported the first complete genome sequence of the FMDV isolate, BAN/MY/My-466/2021 (shortly named My-466) of the MYMBD21 sublineage under the O/ME-SA/2018 lineage. The genome is 8,216 nucleotides long with 6,996 nucleotides open reading frame flanked by 5′UTR (1-1100) and 3′

UTR (8097-8216). More stability has been identified in non-structural proteins as compared to proteins with structural characteristics. Mutation analysis against available field vaccine and proposed local vaccine strains revealed that VP1 was highly variable among the structural proteins with crucial mutations in the major antigenic region, G-H loop. These mutations were responsible for antigen-specific polymorphism regarding both current and proposed vaccine strains. And that was evidenced by the G-H loop displacement in a superimposed 3D model of VP1 of the isolate against vaccine strains. In conclusions, these findings of the studies are crucial to understand the FMD situation and designing necessary preventive steps according to the Progressive Control Pathway for FMD Control (PCP-FMD) in Bangladesh. The complete genome information of the circulating strains would be valuable for revealing the evolutionary pattern which is also necessary to formulate whole virus vaccines using appropriate strain to control the FMDs.

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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 Phosphate

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

1. Introduction

1.1 General Introduction

Foot and Mouth Disease (FMD) is an apprehensive, incredibly transmissible viral infection that affects both domesticated and wild creatures with cloven hooves, notably cattle, pigs, sheep, goats, and water buffalo. It is imposed on by the legendary Foot-and-Mouth Disease Virus (FMDV). The virus is antigenically diverse with a high mutation rate which makes the control and eradication of the virus extremely difficult. FMD is ubiquitous throughout every continent, but has the highest incidence in Bangladesh and portions of Asia as well as Africa. The disease with its high morbidity rate poses a great economic loss to these endemic regions as the majority of the national economy is related to the livestock sector. The recovery program for the disease demands expertise, constant surveillance, and prolonged time.

Foot-and-Mouth Disease Virus (FMDV), the accountable substance is an ancestor of the genus *Aphthovirus* and the family *Picornaviridae*. (Ullah *et al*. 2014). The tiny nonenveloped virus recognised as FMDV comprises a hexagonal capsid along with pseudo-T3 equilibrium. The positive-sense RNA with a single-stranded genome of the FMDV has a length of approximately 8.5 kb. Like other picornaviruses, it has just one big open reading frame (ORF) that is complemented by exceptionally organised 5' and 3' UTRs (5' UTR and 3' UTR, respectively). Each of the four main structural proteins identified in the virus's capsid, VP1, VP2, VP3, and VP4, is present in sixty copies in the capsid even though VP4 is completely internalised (Mittal *et al*. 2005). The N-terminal half of the ORF codes for the four capsid proteins 1A, 1B, 1C, and 1D (also known as VP4, VP2, VP3, and VP1, respectively). Lpro, 2A, 2B, 2C, 3A, 3B, 3C pro, and 3D pol are non-structural proteins which collectively make up around two-thirds of the ORF (Carrillo *et al.* 2005). There are seven unconventional FMDV serotypes, of which A, O, and Asia 1 propagate in Bangladesh. The remainder of three are South African Territories (SAT) serotypes SAT1, SAT2, and SAT3. Serotype O was prevalent among them, and it was in charge of approximately 82% of the occurrences in Bangladesh (Siddique *et al.*, 2018). The distinct serotypes do not confer cross-protective immunity. Sometimes vaccine escape mutations are evident within the same serotypes even after vaccination. This necessitates epidemiological studies and a search for the emergence of newer subtypes of the virus.

Bangladesh is inhabited to 24.5 million cattle, 26.6 million goats, 3.7 million sheep, and 1.5 million buffaloes, all of which are vulnerable to FMD. Approximately 20 % of rural households depend completely on livestock production while an additional 50% of the people have an indirect connection to the livestock sector. Livestock contributes to 1.44% of the national

economy in Bangladesh and it is growing at 3.47% annually (Department of Livestock Services, Bangladesh,2021–2022). In Bangladesh, FMD is one of the most detrimental economic livestock-related illnesses, with annual consequences anticipated to be approximately US $125 million. The disease results in a reduction of milk yield by 66.6% and 51% mortality in calves. Over the course of a year, a variety of episodes take place in various regions throughout Bangladesh, predominantly during the winter and just before the monsoon. In accordance with Rahman *et al*. (2020), there were approximately 5.5 million FMD instances in the cattle and buffalo populations in Bangladesh. The cattle population was reported to be more susceptible than buffaloes and sheep/goats. FMD recurrence was discovered to be notably higher in older cattle in comparison to younger calves and in males than females (Mostary et al., 2018). In accordance with research reports, a substantial route for FMDV spread from India, Nepal, and Myanmar to Bangladesh incorporates massive cross-border transit of animals. Transboundary dissemination has also been suggested by molecular evidence of FMDV serotypes in Bangladesh, India, Nepal, and Myanmar (Nandi *et al*., 2015).

The detrimental outbreaks which take place throughout the year in Bangladesh were a result of the widespread distribution of immunogenically divergent FMDV strains with inadequate cross-protective immunity. To control the outbreak, vaccination against circulatory strains is crucial. But in Bangladesh, imported vaccines are more commonly used which may provide partial protection or no protection against local strains. This phenomenon increases the risk of selective pressure and the emergence of novel strains. To avoid this risk, research on developing local vaccine strains is necessary. An important step to facilitate vaccine research is the culture of the virus. In the case of FMDV, the virus is cultured via inoculation of the mammalian cell line. The isolation of the virus from the cell line is required for total RNA extraction and genome-wide analysis of the virus. The genomic characterization is necessary for selecting local vaccine candidates. Again, virus culture facilitates the observation of the cytopathic effect of the vaccine strain, determination of infective dose, and effective concentration of vaccine. The continuous search for local vaccine candidates is important for the prevention of outbreaks for which culture and isolation of virus must be practiced regularly.

High mutation rate, poor surveillance, uncontrolled animal movement, and unplanned vaccination complicates FMD control and prevention program in Bangladesh. Therefore, epidemiological investigation of outbreaks, molecular typing, and culture of the circulatory FMDV is crucial to designing effective vaccination and FMD control programs in Bangladesh.

1.2 Literature Review

In Bangladesh as well as overseas nations, the Foot and Mouth disease is a concerned economic challenge. The livestock sector is an indispensable component of agriculture, and consequently the primary propellant of Bangladesh's economy. Livestock boasts the average individual with essentials like meat, milk, fat, and skins as well as a handy source of income to support their daily needs. Nowadays many young people are interested in the livestock business as a means of self-employment. But several bacterial, viral, parasitic, and metabolic illnesses are ongoing concern to this industry. The most prominent concern among those is foot-and-mouth disease (FMD). The appearance of the illness possesses the potential to ruin many herd owners' fortunes and leading to substantial monetary damages.

1.2.1 Foot and Mouth Disease (FMD)

Severe along with exceptionally transmissible viral disease, FMD predominantly impacts animals having cloven hooves, that includes cattle, buffalo, pigs, sheep, and goats. Fever and vesicular events in the mouth, muzzle, foot, teats, and other hairless sensitive locations of the body are its most distinguishing characteristics. Different names for the illness have been used to defines it, including aphthous fever, epizootic aphthae, infectious aphthous stomatitis, aftosa (in Italian and Spanish), severe aphthous (in French), maul, and klavenseuch (in German) (Hagan *et al*., 1988). The enormous international literature on every aspect of the disease reveals that the greatest improvements in our comprehension of FMD and its regulation have been carried out in the last century or so. Inevitably we will only be able to briefly discuss a handful of the most substantial findings here. Loeffler and Frosch's (1897) demonstrated that the causative agent was a filterable particle was one of the primary and most important findings, thereby making FMD the initial animal disease to be linked to a virus. Aristotle may have been referring to the deadly bovine diseases rinderpest or foot-and-mouth disease when he wrote of a cattle plague in 350 B.C. Hieronymus Fracastorius, an Italian physician, offered the first clarified explanation of foot-and-mouth disease in 1546.

1.2.2 Clinical manifestations

The severity of the signs can be mild or inapparent or severe and depend on the dose of exposure, virus strain, age and species of animal, and also on host immunity. Signs and symptoms most often arise 2 to 14 days after the virus has been introduced. Vesicles or blisters developing on the nose, in or around the mouth, on the tongue, across toes, prior to the hooves, on the teats, or at pressure areas on the skin are common manifestations.

1.2.3 Transmission of FMD

Either direct or indirect interaction between infected and healthy animals might result in the transmission of FMD viruses. All of the excretions and secretions of sick animals contain the virus. When infected animals exhale, the virus is also shed in the form of an aerosol. As a result, the virus can spread by inhalation, contact with bodily fluids of infected animals, and aerosols containing contaminated items. FMDV was discovered in farm shedding, inanimate objects, and other environmental sources, demonstrating the validity of environmental sampling for FMD surveillance in the endemic region (Brown *et al*., 2021). According to Charleston *et al*. (2011), the infectious period for FMD in cattle is just 1.7 days, and animals are not contagious until 0.5 days following the onset of clinical symptoms.
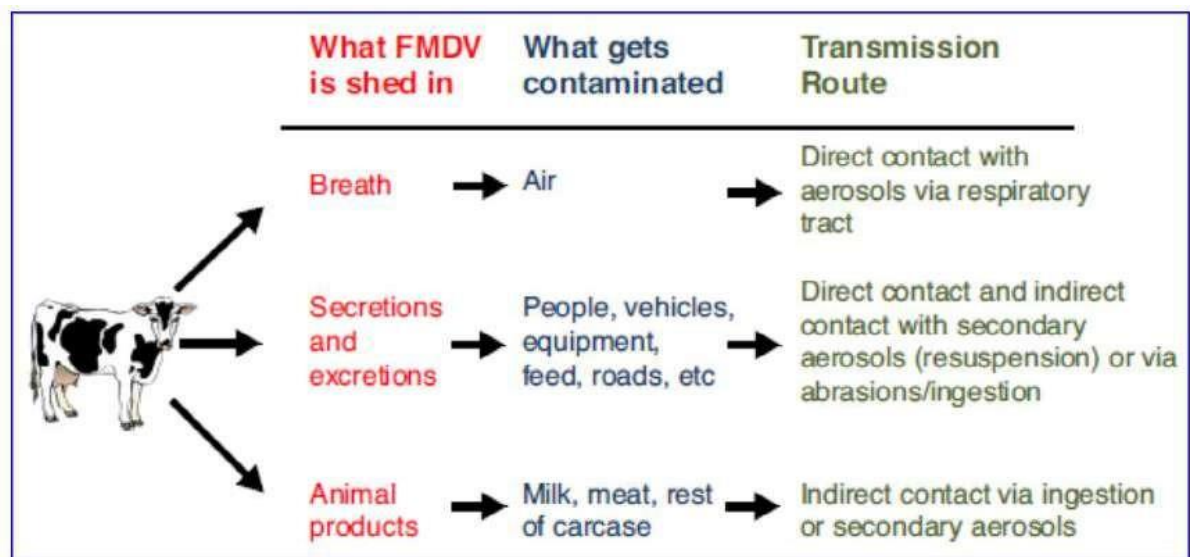
Figure 1.1: the main strategies through which the FMD virus transmits throughout animals (Paton et al., 2018).

1.2.4 Economic Losses due to FMD

The Office International des Epizooties' (OIE) "A" list of infectious animal diseases includes FMD, which is known as the primary barrier for trading in animals as well as animal-related goods internationally (Grubman & Baxt, 2004). The economic loss due to the emergence of this disease is mainly for losses in meat and milk production, losses in animal draught power, etc. Losses can be direct because of lower productivity as well as alterations in the herd layout, or indirect considering of the expenditure of FMD control, challenging market access, and

insufficient utilisation of newer production technologies. Giasuddin et al.,2021 studied with 850 households suffered a total financial loss of Tk. 53.17 million, or US$ 0.63 million, as a result of the FMD incident. The biggest percentage of loss was experienced when affected animals died (63.47%), followed by veterinary expenses (10.71%), weight loss in cattle used for
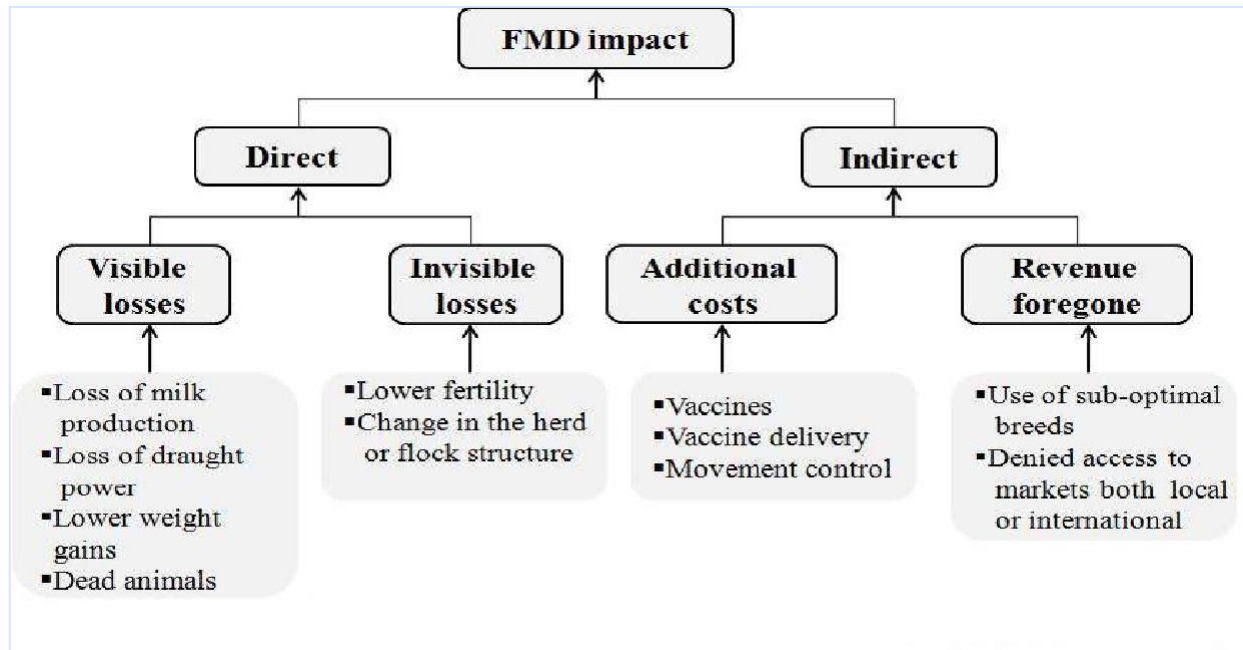
Figure 1.2: Economic consequences of FMD (Rushton, 2008).

fattening (10.68%), a decrease in milk production (9.17%), and a loss of manpower for caring for afflicted cattle (5.98%). According to this figure, Bangladesh's estimated annual financial loss from the FMD outbreak would be Tk. 188.57 billion, or US$ 2.22 billion (Giasuddin *et al.*, 2021). Mortality losses, abortion losses, FMD treatment costs, weight losses, an overal drop in milk output, and an impairment of draught capacity are just a few of the direct losses caused by FMD that result in productivity losses for an extended period of time.; while indirect losses due to FMD include salvage sale losses, cattle marketing losses, local government revenue losses and most importantly, trade embargo.

1.2.5 Foot-and-Mouth Disease Virus (FMDV)
1.2.5.1 Taxonomy of FMDV

The International Committee on Taxonomy of Viruses (ICTV) identified FMDV as an Aphthovirus in the Picornaviridae family in 1963. The phrase Picornaviridae, which relates to the size and genome type of the virus, is derived from the Latin acronyms "Pico" and "RNA,"

where Pico imply "very small" and "RNA" for RNA in English. The vesicular lesions that develop in animals with cloven feet are known as 'Aphthovirus' (OIE, 2009).

1.2.5.2   Morphology of FMDV

The FMD viruses feature non-enveloped, ether-resistant, hexagonal nucleocapsids (protein shells) with proportion of 27–28 nm in width, resulting in some of the smallest known RNA viruses (Melnick *et al.,* 1975). The FMD virion seems to be a sphere-shaped particulate with a smooth exterior under a 350,000X transmission electron microscope (TEM). FMDV differs from other Picornaviruses by insufficient a surface canyon and a place where entero and cardioviruses can connect to their receptors. Another distinctive feature is the existence of an opening at a fivefold axis that allows tiny molecules like CsCl to enter the capsid. Among the Picornaviruses, FMDV has the largest buoyant density (Grubman & Baxt, 2004).
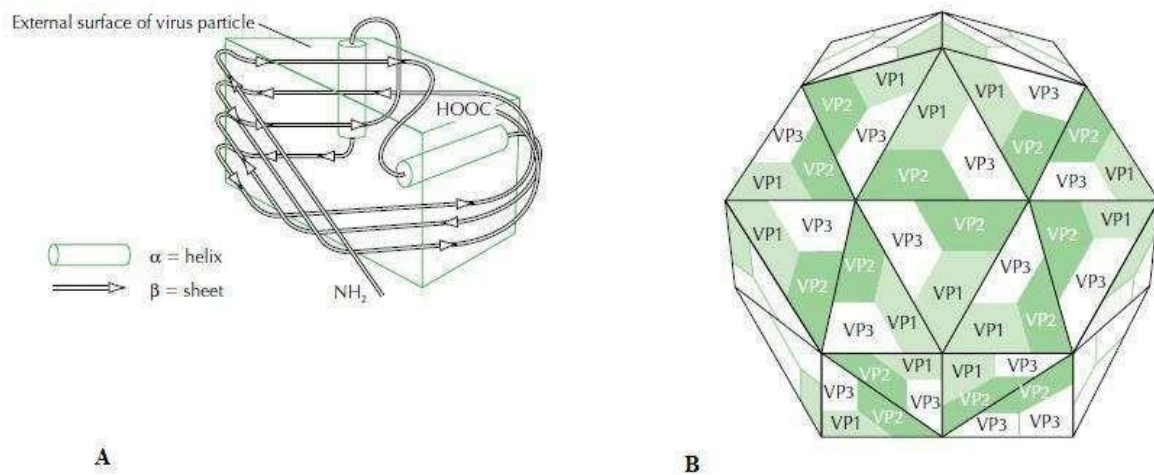
Figure1.3: Presentation of the Picornavirus capsid proteins structurally.

A: The 'eight-strand antiparallel b-barrel' component structure that is present in all T = 3 icosahedral RNA virus capsids. B: The three-dimensional number T of picornavirus particles, which are icosahedral in appearance, is 3. The surface of the particle is made up of the VP1, VP2, and VP3 viral proteins. VP4, a fourth protein, is present in each of the 60 repeating units that make up the capsid but is not exposed on the surface of the virion. (Cann, 2005)

1.2.5.3   Virion structure of FMDV

FMD virions are spherical, have an icosahedral symmetry, and have a diameter of 27–28 nm. According to Putnak and Phillips (1981), the virion is composed of 70% protein, 30% RNA, and a trace quantity of lipid. The molecular weight of the viral genome, which has 8000 nucleotides, is 2.6x10 daltons. According to Grubman and Baxt (2004), the virion proteins have the following sizes: VP1(1D) = 29 nm, VP2(1B) = 30 nm, VP3(1C) = 22 nm, and VP4(1A) = 8 nm. Another well-known FMDV characteristic is the precipitation standard of virions in sucrose gradients

(146S), which is frequently utilised in vaccine production to identify intact virions. The four structural proteins VP1-4 make up 60 copies of each in the non-enveloped icosahedral shell (Figure 1.3). The G-H loop (residues 134–160) and C-terminus (amino acids 200–213) of VP1 are exposed surface components of the FMDV attachment site. Furthermore, unlike other picornaviruses, FMDV's G-H loop, a key antigenic site, generates a noticeable, extremely accessible projection. In the variant viruses, this loop is disrupted. Thus, it appears that this virus employs a unique escape mechanism whereby the integrity of the epitope is destroyed by an induced conformational shift in a key antigenic loop (Parry *et al.,* 1990).

1.2.6 FMDV's genomic arrangement

The FMDV is a 146S particle rendering of 60 copies of each of the four structural proteins (VP1, VP2, VP3, and VP4) and an 8500 nucleotide single-stranded RNA genome. Three distinct genomic sections have been identified in the FMDV genome: (i) the 5' Untranslated region (5'-UTR), (ii) the protein-coding region (ORF), and (iii) The 3' UTR, commonly known as the non-coding region, has a poly (A) tail and roles in regulation. (160 nucleotides).

1.2.6.1   5' Un-translated Region (5' UTR)

The 5' UTR, which is between 1100 and 1300 nucleotides long, is crucial for FMDV replication. Genome-linked viral protein (VPg), a virus-encoded protein, is structurally connected to the 5' end of it (Sangar *et al*., 1987). Three distinct variants of the protein 3B—3B1, 3B2, and 3B3 have been identified (Sangar *et al*., 1987; Belsham, 1993). These three related, but distinct, VPg-encoding genes are only found in tandem in aphthoviruses, making them special. The 5' UTR is made up of the genomic long fragment (LF), that is approximately 720 bases long and anticipated to form three tandemly duplicated pseudoknots (Clarke *et al*., 1987). The CRE, also known as a 3B-uridylylation site (bus), stem-loop, and type II internal ribosome entry (Si) are the components of the CRE. The short S-fragment, which is 400 bases long and capable of forming a large hairpin structure. However, it is unknown how specifically the S-fragment, poly C tract, and L fragment pseudoknots contribute to FMDV biology. The core contains a conserved AAACA motif that in aphthovirus serves as a template for 3D-pol-mediated uridylation of 3B and is crucial for picornaviral replication (Murray & Barton, 2003).

1.2.6.2   ORF Encoded FMDV proteins

The Leader Protease (L^{pro}): The leader (L) protein is a papain-like cysteine proteinase that cleaves itself from the viral polyprotein at G479/R480 by acting as a trans-proteinase and initiation factor eIF4G (Glaser *et al*., 2001). It is found at the 5' end of the ORF and contains two

in-frame initiation codons (84 nt in distance, Lab/Lb). It plays a significant role in determining animal pathogenicity as well.

1.2.6.3 FMDV Structural Proteins

VP4 (1A): It stretches from 1662 to 1916 position on the FMDV RNA genome and plays role in forming capsid.

VP2(1B): The second antigenic site for the virus is located in the region of the viral genome that runs from position 1917 to 2570 nucleotides and contains the B-C loop, G-H loop, and E-F loop.

VP3(1C): It has a tentative position just after VP2 and it positions in 2571-3230 in viral RNA. Additionally, it has the G-H loop, which contributes to antigenicity.

VP1(1D): the major antigenic site, occupy the position 3231-3863 containing ~630 nucleotides. The FMD virus has at least four G-H loops, and these loops are essential for attachment and antigenicity (Bai *et al*., 2010). The well-known sections of 133-160 (the G-H loop) and 200-213 (the C-terminus region) of VP1 include trypsin-sensitive neutralizable antigenic sites 1. Amino acid residues 43, 44, 45, and 48 are involved in antigenic site 3 in the region 43–60 of VP1.

1.2.6.4 FMDV Non-Structural Proteins (NSPs)

2A: a peptide of 18 amino acids that modifies the cellular translation machinery.

2B: An essential membrane protein that increases flexibility of the membrane, obstructs protein secretion routes, inhibits apoptosis by influencing intracellular Ca2+ homeostasis, and is thought to be responsible for the cytopathic effects of viruses (Jecht et al., 1998)

2C: According to Tesar et al. (1989), 2C concentrates in membrane-associated virus-replicating complexes and affects the start of minus-strand RNA synthesis in the cytoplasm.

3A: In relation to virus pathogenicity and host range, FMDV 3A has been implicated.

3B: During virus replication, 3B primes the synthesis of genomic RNA (Falk *et al*., 1992).

3C: The majority of the viral polyprotein's proteolytic processing is carried out by it. The 3C also changes numerous cellular proteins; for example, this protease has been demonstrated for separating the histone H3 (Falk *et al*., 1992).

3D: The 3D gene produces minus- and-plus sense genomic RNA and encodes an RNA-dependent RNA polymerase.

1.2.6.5 3' Un-translated Region (UTR)

Following the ORF termination codon in the 3' UTR is a brief RNA segment that folds into a particular stem-loop structure (Pilipenko *et al.,* 1996), which is followed by a variable-length poly-A tract carried on the genome. In terms of genome replication, the 3' UTR also seems to be significant (Rohll *et al*., 1995; Pilipenko *et al.,* 1996). In vitro translation of the viral RNA was

not affected by antisense RNA hybridization to the FMDV 3' UTR, but RNA replication in infected cells was inhibited, as shown by Gutierrez and colleagues (1994).

1.2.7 Category of Foot-and-Mouth Disease Virus

Based on the nucleotide sequence of the VP1 (1D) structural protein, the FMDV exists as seven immunologically different serotypes, namely A, O, C, Asia 1, Southern African Territories (SAT)-1, SAT-2, and SAT-3. In addition, each serotype has a number of subgroups or topotypes that are frequently connected to the geographic area in which the disease first manifested itself (Domingo *et al*., 2002; Knowles *et al.,* 2016).

1.2.7.1 Serotype O

Of the seven FMDV serotypes, serotype O is the most common and is traditionally broken down into 10 or 11 antigenic subtypes. Serotype O has a significantly higher level of genetic variety, allowing for the classification of numerous separate lineages (Knowles *et al.,* 2016). Eight separate genetic lineages that correspond to geographically varied places have been identified by researchers.

1.2.7.1.1 Topotypes, Lineages, and Sub-lineages of Serotype O

FMDV serotype O has been reported in 11 topotypes with 15% nucleotide distinctions among them. These topotypes are from Europe-South America (Euro-SA), the 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). Three lineages have so far been recognised as corresponding to the ME-SA topotype: Ind2001, Ind2011, and PanAsia (Hemadri *et al*., 2002; Subramaniam *et al*., 2013). PanAsia-2 lineage was introduced by Knowles *et al*. in 2016 under which ANT–10, BAL–09, FAR–09 PUN–10, SAN–09, TER–0 sub-lineage was reported (Knowles *et al*., 2016). Ind2001 lineage was thought to get emerged from variants of PanAsia strains in 2001(Hemadri *et al*., 2002) and has later diversified into four sub-lineages (Ind2001a, b, c, and d) (Subramaniam *et al*., 2015). Two novels sub-lineages Ind2001BD1 and Ind2001BD2 were reported from Bangladesh in 2018 (Siddique *et al*., 2018)

1.2.7.1.2 Antigenic Sites on Capsid of FMDV Serotype O

In total, there are five reported, experimentally proved antigenic locations on the capsid of FMDV serotype O. The G-H loop and C terminus of VP1 (residues 140–160, 137–155, or 130–160) together create the antigenic site one's linear and structural epitopes (Momtaz *et al*., 2014).

Within antigenic site 1, positions 144, 146, 147, 148, 154, 206, and 208 have been reported to be antigenically critical (Aktas & Samuel, 2000; Kitson *et al.,* 1990; Xie *et al*., 1987). Interestingly, ruminant species respond to epitopes on the carboxy terminus end of VP1 more efficiently than pigs (Aggarwal & Barnett, 2002). The antigenic site 2 covers the B-C loop (31,70-80) and the adjacent E-F loop (132-135 or 131-134) of VP2 (Mateu *et al*., 1995). Residues of the B-C loop (43-59) of VP1 constitute the antigenic site three, where residues 43 and 44 are the most crucial for antigenicity (Aggarwal & Barnett, 2002). B-B knob (58-61) of VP3 has been reported to be critical for site four, wherein position 58 is thought to be most important in antigenicity (Aktas & Samuel, 2000; Kitson *et al*., 1990; Xie *et al*., 1987) Antigenic site five is formed by position 149 of VP1 and other surface located amino acids of the G-H loop (Crowther *et al*., 1993). Except for the antigenic site, which is linear and trypsin sensitive, all the other discovered sites are conformational and trypsin resistant.

1.2.7.2 Serotype A

Due to the high antigenic and genetic diversity of serotype A virus, three topotypes—AFRICA, ASIA, and EUROPE-SOUTH AMERICA (EURO-SA)—were identified by VP1 sequence analysis.

1.2.7.2.1 Topotypes, Lineages, and Sub-Lineages of Serotype A

Thirty-two subtypes have been described (Knowles & Samuel, 2003) and 26 genotypes of serotype A FMDV have been reported (Mohapatra *et al*., 2011) Some of these subtypes are genetically distinct (e.g. A22, A24). These are further divided into multiple lineage and sub-lineage. Topotype ASIA and AFRICA have seven distinct lineages: G-VII, A22, IRN-05, IRN-87, IRN-96, Thai-87, Sea-97 in ASIA, and G-I, G-II G-III, G-IV, G-V, G-VI, G-VII in AFRICA. EURO-SA topotype of Serotype A, geographically restricted to Europe and South America, has four lineages- A5, A12, A24, and A81.

Evolution of FMDV serotype A isolates of Bangladesh were grouped in Genotype VII of Asia Topotype (Amin *et al*., 2020).

1.2.7.2.2 Antigenic Sites on Capsid of FMDV Serotype A FMDV

According to N. Knowles *et al*. (2009), serotype A is one of the most antigenically varied serotypes. The five antigenic sites of FMDV serotype A strains that have been mapped using mAbs include the G-H loop, which is the most variable site on the capsid, as well as other surface-exposed areas of VP1, VP2, and VP3 (Crowther *et al*., 1993). Site 1 is the only one

among them that is linear and trypsin-sensitive; the others are conformation-dependent. Positions 144, 148, 154, and 208 of VP1's carboxy terminus and G-H loop contain crucial residues that contribute to antigenic site 1. Positions 70–73, 75, 77, and 131 of VP2's amino acid residues help to form antigenic site 2. The binding of site two mAbs has also been shown to be affected by amino acid residues at positions 79 and 134 (Mohapatra *et al*., 2008). The B-C loop of VP1's residues 43 and 44 contribute to the formation of antigenic site three, whereas the amino acid residues at positions 56 and 58 of VP3 have proven crucial for antigenic site 4. The interaction of the VP1 loop area with other surface-oriented amino acids is likely what creates antigenic site 5, which is identified by an amino acid at position 149 of VP1 (Mahapatra & Parida, 2018).

1.2.7.3   Serotype C

Type C was discovered by Waldmann & Trautwein in 1926 and seemed to have restricted to the Indian sub-continent. It has been traditionally observed in southern Asia, North Africa, North America, Europe, South America, and Eastern Africa. Although type C has not been observed since 1996, it is thought to still be in use in India. Recent research has demonstrated that FMD type C viruses can be divided into eight topotypes: EuroSA, Angola, the Philippines, ME-SA, Sri Lanka, EA, and Tajikistan.

1.2.7.4   Serotype Asia 1

From a Pakistani sample, the FMD serotype Asia 1 was originally discovered in 1957. Only a small number of FMD patients in the Asian region are caused by this serotype (Mohapatra *et al*., 2008). Even while VP1 nucleotide sequences showed a lot of variation, it wasn't enough to divide Asia 1 viruses into more than one topotype. These viruses have been categorised into six separate groups, I to VI, according to a recent study on viruses of the serotype Asia 1 from an outbreak that occurred in Asian nations between 2003 and 2007 (Valarcher *et al*., 2009).

1.2.7.5   Serotype SAT-1, SAT-2, and SAT-3

In 1948, Brooks reexamined various African field isolates acquired since 1931 and established an innovative strain from the South African Territories (SAT 1). SAT 2 and SAT 3 are two additional Southern African strains that have been found. For the SAT, there are five distinct topotypes: South Africa, Zimbabwe, Zambia, Namibia, Botswana, Malawi, and Uganda. Three of the five topotypes are found in various parts of Zimbabwe, whereas just one topotype is found within the borders of the other four nations. (Vosloo *et al*.;2002)

1.3 Epidemiological dispersion of foot-and-mouth disease virus
1.3.1 Global dispersion of foot-and-mouth disease virus:

FMD is widespread around the world, but it is more prevalent in South Africa, Asia, the Middle East, and South America. The FMD viruses circulating worldwide are divided into seven
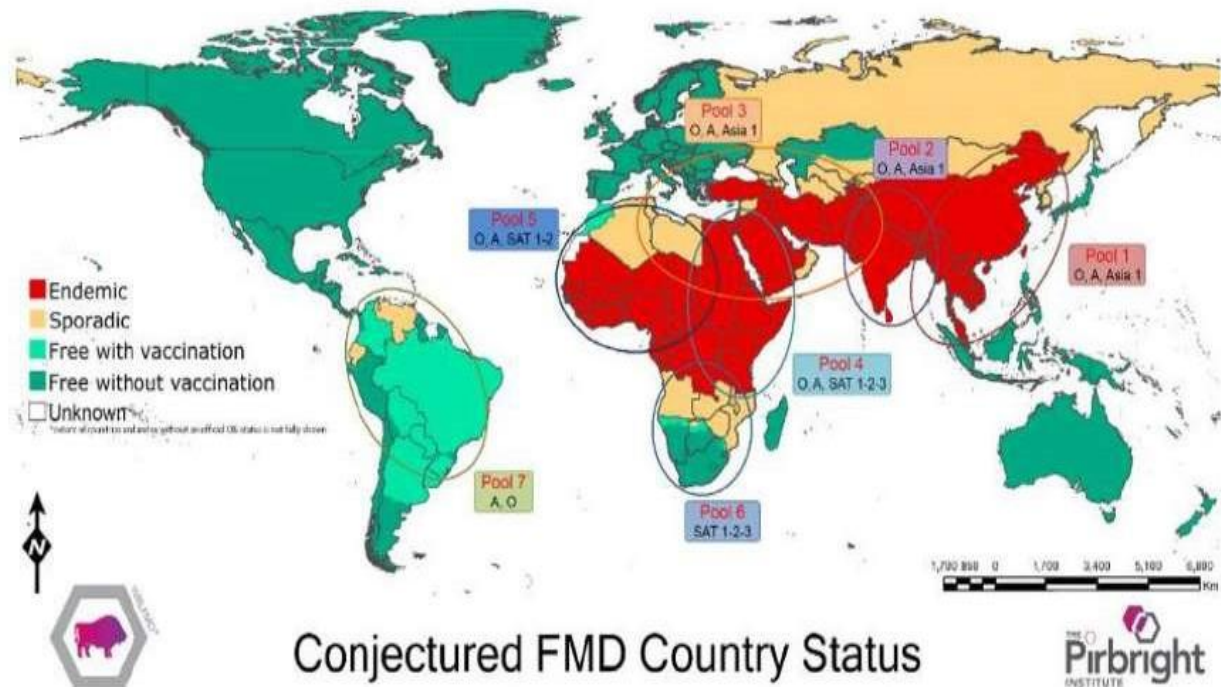
Figure1.4: Distribution of the seven endemic FMD pools illustrating the estimated prevalence of FMD in each nation in 2016 (FAO, 2018).

different pools. The virus pool distribution is depicted in **Figure 1.4.** The movement and emergence of viruses within these geographical virus pools shift the focus for properly suited vaccinations. Viruses occasionally travel between pools and free areas, and nations at the borders of pools (such those in North Africa and Central Asia) frequently experience FMD outbreaks from several regional origins. [FAO 2018].

Table 1.1 displays the infrequent incidence of several serotypes in different parts of the world. The most widespread serotype across various countries is O.

Table 1.1: List of nations that correspond to each viral pool from 2011 to 2015 (FAO, 2018).

Pool	Region/Countries	Serotypes
1.	**Central/East Asia** Cambodia, China (People's Republic of), China (Hong Kong, SAR), China (Taiwan Province), Korea (DPR), Korea (People's Republic of), Laos PDR, Malaysia, Mongolia, Myanmar, Russian Federation, Thailand, Viet Nam	O, A, Asia-1
2.	**South Asia** Bangladesh, Bhutan, India, Nepal, Sri Lanka	O, A, Asia-1
3.	**West Eurasia and the Middle East** Afghanistan, Algeria, Algeria, Azerbaijan, Bahrain, Bulgaria, **Egypt,** Georgia, Iran, Iraq, Israel, Jordan, Kazakhstan, Kuwait, Kyrgyzstan, Lebanon, **Libya,** Oman, Pakistan, Palestine Autonomous, Territories, Qatar, Saudi Arabia, Syrian Arab Republic, Tajikistan, Tunisia, Turkey, Turkmenistan, Uzbekistan	O, A, Asia-1
4.	**Eastern Africa** Burundi, Comoros, **Congo D. R.,** Djibouti, **Egypt,** Eritrea, Ethiopia, Kenya, **Libya,** Rwanda, Somalia, Sudan, South Sudan, Tanzania, Uganda, Yemen	O, A, SAT-1, SAT-2, SAT-3
5.	**West/ Central Africa** Benin, Burkina Faso, Cameroon, Cape Verde, Central African Republic, Chad, **Congo D. R.,** Congo, Cote 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, SAT-2
6.	**Southern Africa** Angola, Botswana, **Congo D. R.,** Malawi, Mozambique, Namibia, South Africa, Zambia, Zimbabwe	{O, A}, SAT-1, SAT-2, SAT-3
7.	**South America** Ecuador, Paraguay, Venezuela	O, A

1.3.2 Recent outbreaks in 7 endemic pools:

Pool 1 (Southeast Asia/Central Asia/East Asia):
An outbreak was reported in The Socialist Republic of Viet Nam in 2020. VP1 genotyping

identified ME-SA/Ind-2001e, ME-SA/PanAsia, and SEA/Mya-98 as circulatory strains.

Pool 2 (South Asia): No new outbreaks were reported.

Pool 3 (West Eurasia and the Middle East): From the Republic of Turkey, an outbreak

occured by FMDV serotype A, topotype ASIA, lineage G-VII was reported on 24 August 2020.

Pool 4 (North and Eastern Africa): On September 6, 2020, the State of Libya reported four

outbreaks of FMD type A in sheep at Qasabana Ghashir, Blair Althawthuh, and Tarabulus. On

June 22, 2020, the Kayonza District in the Eastern Province of the Republic of Rwanda reported

an outbreak of FMD type SAT 2 in cattle.

 Pool 5 (West/Central Africa): There were no new FMD occurrences noted.

Pool 6 (Southern Africa): On April 30, 2020, the Republic of South Africa announced a

livestock outbreak of FMD type SAT 2. On August 7 and August 10, respectively, outbreaks

have been identified from the Republics of Malawi and Mozambique. On July 28, 2020, the

Republic of Zambia reported an outbreak of FMD type O, EA-2 topotype.

Pool 7 (South America): There were no new FMD occurrences noted. **(FAO. 2020. Food-and-mouth disease, July–September 2020: Quarterly report.)**
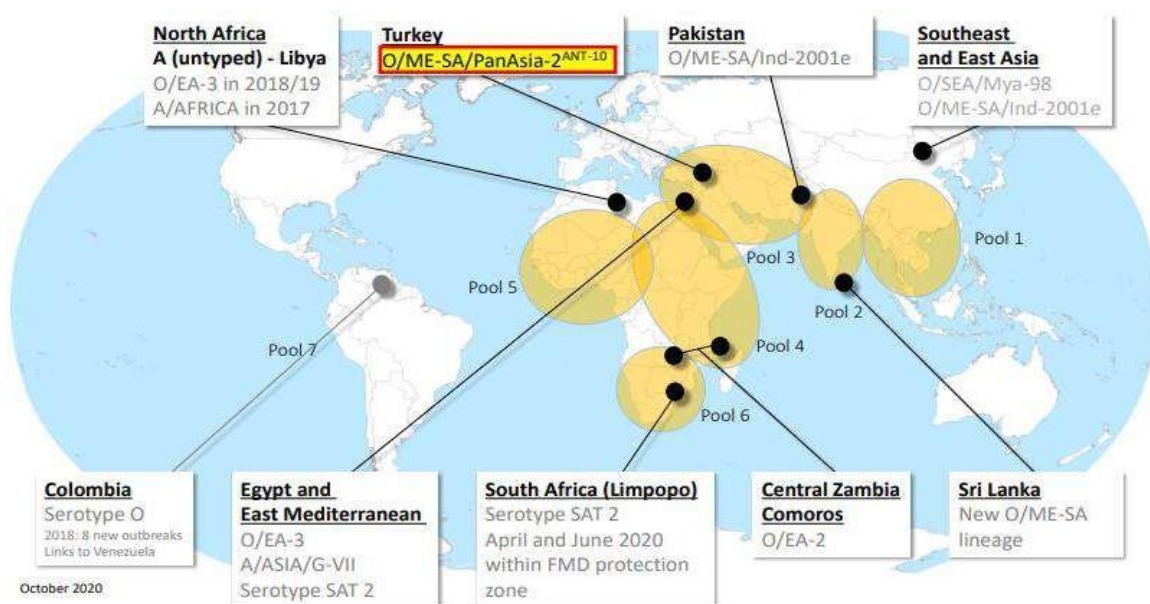
Figure 1.5: Recent newsworthy incidents with endemic pools (reported from July to September 2020) are marked in orange. Author: WRLFMD. The map is in accordance with the February 2020 United Nations World Map.

1.3.3 Foot-and-mouth disease virus in Bangladesh

The first FMD cases were formally recorded in Bangladesh in 1958 (Pirbright Laboratory 2010) (Figure: 1.6) during widespread outbreaks across the nation.
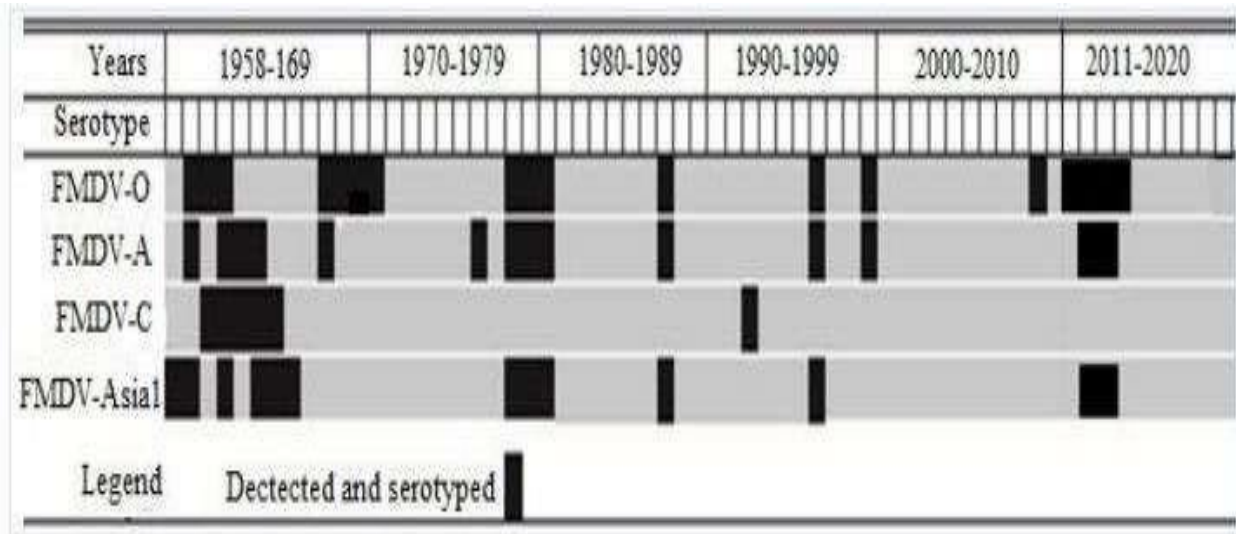
Figure 1.6: Occurrence of FMDV serotypes in Bangladesh. [Credit: FAO]
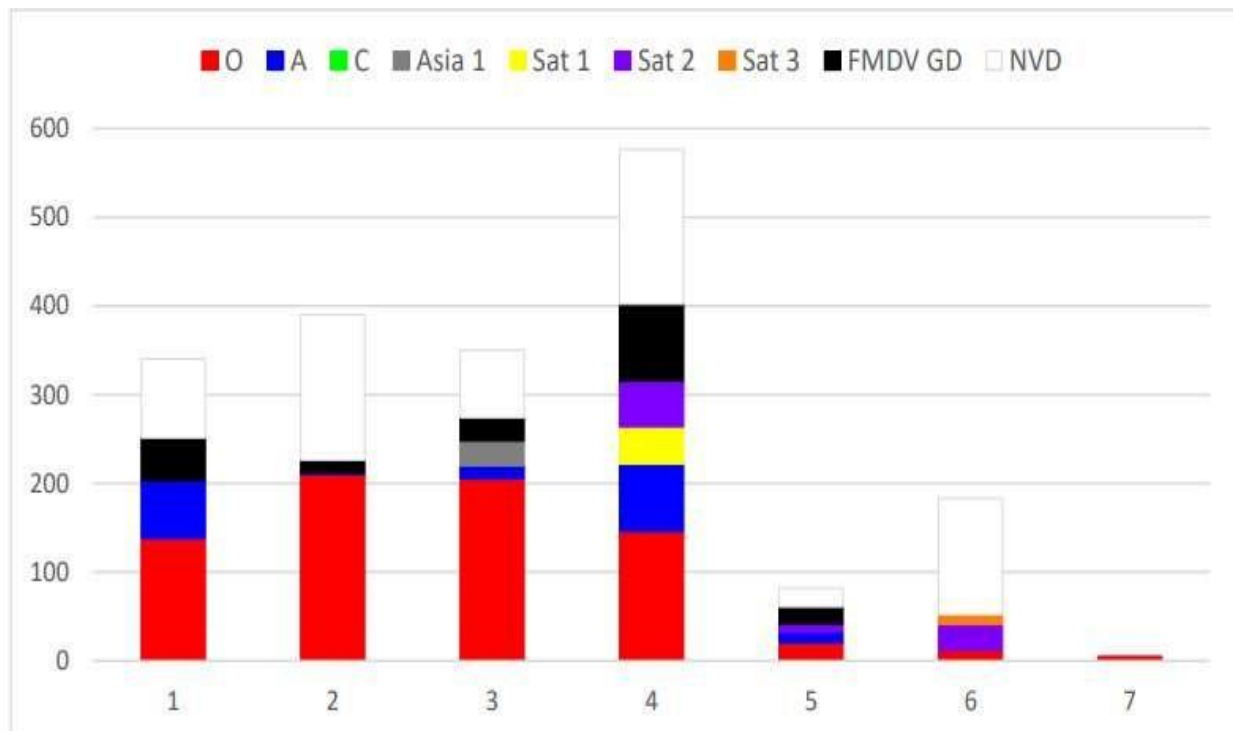
Figure 1.7: The OIE/FAO FMD Laboratory Network's detection of several FMDV serotypes in samples taken from the endemic pools for FMD in 2019 (proposed data). (GD = genome discovered; NVD = no virus detected) Source: 2019 (draught data) OIE/FAO FMD Laboratory Network.

In Bangladesh, FMDV serotypes O, A, and Asia1 are in circulation. After 1990, no reports of serotype C were made. First discovered in Bangladesh between 1987 and 1997, serotype O virus was later discovered there between 1998 and 2000 (Islam et al., 2001), 2008-2009 (Loth *et al.*, 2011), and between 2011 and 2013 (Nandi *et al.,* 2015; Sultana *et al.*, 2014). All of the isolates from 2008 and 2009 belonged to the ME-SA topotype, according to the phylogenetic restoration, however they belonged to two different sub-lineages, one of which is known as Ind-2001 and the other is unnamed. The viruses from Nepal that were collected in 2008 and 2009 were more closely linked to the 2009 Bangladesh isolates within both sub-lineages. Both sub-lineages also contained older viruses that were gathered in India in 2000 and 2001 (Loth *et al.*, 2011). Ind2001 sub-lineage of the Middle East-South Asia (ME-SA) topotype previously identified circulating in Bangladesh was shown to be closely related to serotype O sequences through sequencing and phylogenetic analysis of VP1 sequences of 2011–2013 isolates (Nandi *et al.,* 2015). A subsequent investigation using samples of FMD collected in Bangladesh between 2012 and 2016 and VP1 coding area sequences found that 82% of the outbreaks there were caused by FMDV serotype O, demonstrating its dominance over serotype A and Asia1. Two unique sub-lineages of serotype O, designated Ind2001BD1 and Ind2001BD2, within the Ind2001 lineage also appeared in Bangladesh around this time, and the Ind2001d sub-lineage's distribution in the region persisted. Most recent isolates of FMDV serotype O were reported to fall within the sub-lineage Ind2001BD1, which demonstrated dominance over other sub-lineages (Siddique *et al.*, 2018). Giasuddin *et al.* (2016) claimed that the PanAsia lineage circulated in Bangladesh between 2011 and 2014, but Siddique *et al.* (2018) determined that the isolates belonged to the Ind2001 lineage through phylogenetic analysis using detailed data sets for the VP1 sequence and conservative alignment, supporting the dominance of the Ind2001 lineage in Bangladesh between 2011 and 2014. Later, a different investigation showed an isolate that grouped within the FMDV serotype O lineage PanAsia and was closely connected to an isolate discovered in Nepal in 2009 (Ali & Giasuddin, 2020). There were no further PanAsia cases reported in Bangladesh. Bangladesh never received any reports of the PanAsia-2 lineage. Alongside serotype O viruses, serotype A viruses have also been discovered in Bangladesh (Islam *et al.*, 2001; Nandi *et al.*, 2015; Siddique *et al.,* 2018). FMDV According to Nandi *et al.* (2015), serotype A viruses found in Bangladesh at various times belonged to the genotype VII of the ASIA topotype, which has been prevalent in India for the past ten years. FMDV Asia-1 serotype circulation in Bangladesh is irregular. In Bangladesh, the serotype was initially discovered between 1987 and 1996, again between 1996

and 2000, and again in 2012 and 2013. The sequences of every local circulatory serotype Asia1 acquired between 2012 and 2013 clustered under the genetic lineage C, which reemerged in India in 2005, according to the VP1 phylogeny (Ullah *et al*., 2015).

In Bangladesh, there were 5.5 million FMD cases in cattle and buffalo between 2014 and 2017, according to a study by Rahman *et al*. Five space-time clusters, nine local clusters, and fourteen hotspots were found after doing a spatiotemporal analysis. The majority (31.5%) of cases were reported following the monsoon season, and the division with the greatest percentage (29.2%) was Chattogram. In general, eastern Bangladesh was regularly expected to have greater cumulative FMD incidences (Rahman *et al*., 2020). The spatial distribution of FMD cases (pooled proportional prevalence) in Bangladesh from 2015 to 2019 was also released by the Department of Livestock Services (DLS), Bangladesh (Figure 1.8).
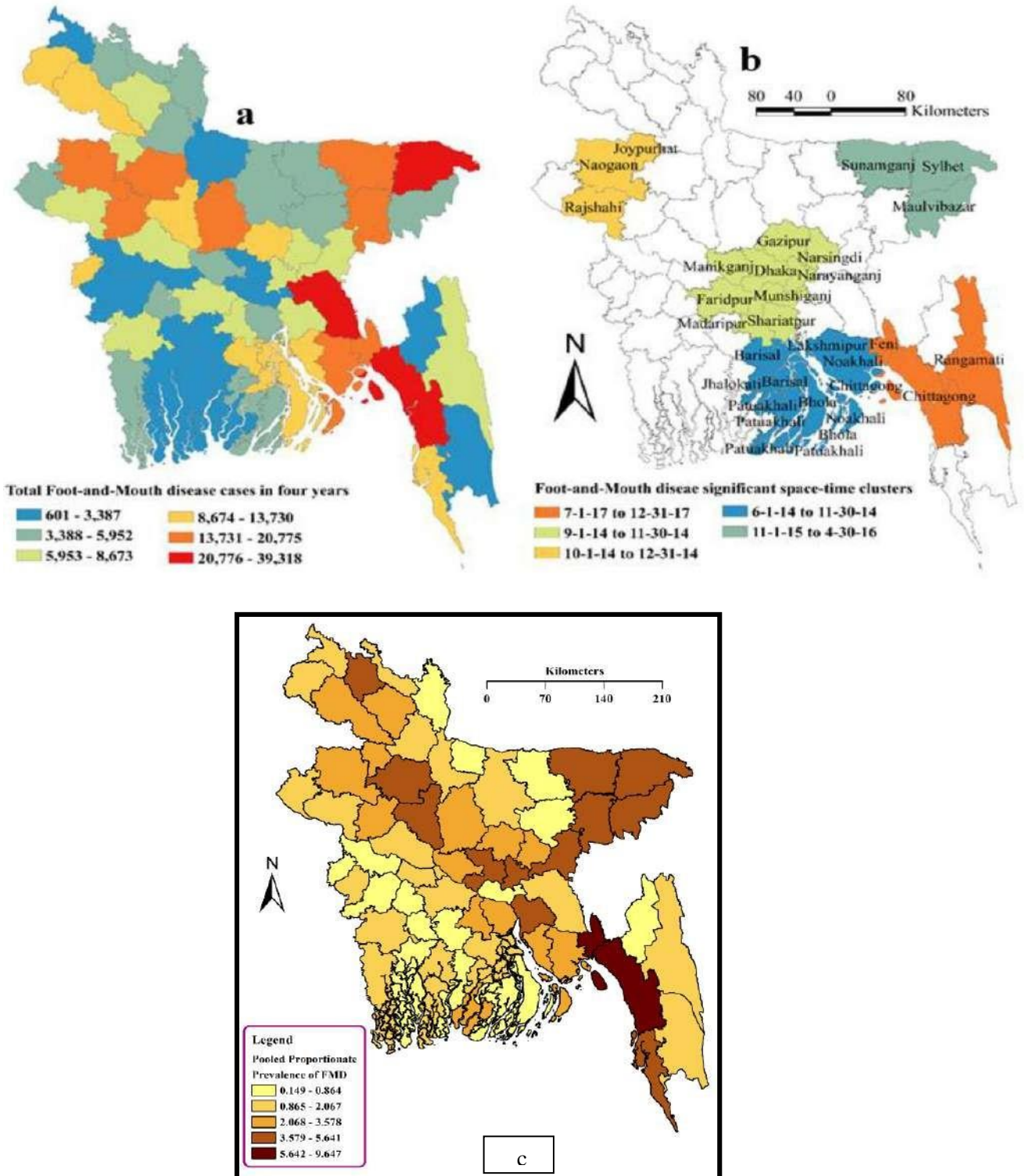
Figure1.8: Map of Bangladesh showing (a) Over all FMD cases and (b) major space-time cluster of foot-and-mouth disease (FMD) in 2014-17. (Rahman et al., 2020) (c) Spatial distribution of FMD cases (pooled proportionate prevalence) throughout the Bangladesh during 2015-19(DLS

1.4 Molecular Characterization of FMDV

1.4.1 Nucleotide Sequencing

The genetic information of FMDV is embedded in its RNA that can be retrieved through sequencing methods. Although the FMD RNA genome can be directly sequenced, it is first reverse transcribed into cDNA and then sequenced because the RNA is unstable. Reverse transcription (RT), in conjunction with polymerase chain reaction (PCR), offers a quick and effective method for analysing various RNA genomes. Nucleotide sequencing of the VP1 gene has been widely employed in epidemiological studies of the FMD virus to ascertain the links between the field isolates. The European FMD viruses type A and type O obtained from several epidemics were subtyped using the nucleotide sequence of the primary immunogenic protein, VP1 (Beck & Strohmaier, 1987). They claimed that nucleotide sequences can be used to distinguish between variants of a certain subtype in addition to being a quick and accurate method for subtyping FMD viruses. Additionally, they showed that a single nucleotide alteration could be found in the nucleotide sequencing of the strain O1 Kaufbeuren and the isolation from Germany in 1984.

1.4.2 Phylogenetic Analysis

At the moment, phylogenetic approaches are used to analyse genetic sequence data in order to study the genetic links and evolution of viruses. The genetic relatedness of the viruses is inferred via phylogenetic trees, which are created (Abdul-Hamid *et al*., 2011). Phylogenetic analysis consists of two main parts: By randomly choosing the original character matrix to construct new matrices with the same size as the original, bootstrapping is a technique for evaluating the dependability of phylogenetic trees. The bootstrap proportion, which represents the frequency with which a specific branch is discovered, can be used as a gauge of reliability.

1.5 Containment and prevention strategies of Foot-and-mouth disease virus (FMDV)

Diagnostics, surveillance, and routine mass immunisation are used to control FMD in endemic areas. Depopulation of diseased and infected animals, together with limitations on the transportation of animals and animal products, are all part of control efforts in FMD-free zones. When an outbreak is severe and it is impossible to controlably depopulate a significant number of animals in a timely manner, emergency vaccination is also an option. Although they do not prevent the primary infection of the nasopharyngeal mucosa (Cox *et al*., 2006; Stenfeldt *et al.*,

2016), the currently available FMDV vaccines offer protection against clinical FMD, and persistent FMDV infection affects both vaccinated and unvaccinated cattle at a similar rate (Cox *et al*., 2006; Pacheco *et al*., 2015; Steinfeldt *et al.,*2016). Consequently, vaccination is an effective way to prevent clinical FMD and has demonstrated to be a very effective tactic in reducing the spread of FMD outbreaks (Parida, 2009), as demonstrated during the 2001 FMD outbreak in Europe when the Netherlands chose to vaccinate all FMDV vulnerable species. However, as part of a "vaccination-to-kill policy" to quickly ease trading restrictions and recover FMD-free status, vaccinated animals were put to death.

1.6   The Progressive Control Pathway for FMD Control (PCP-FMD)

The FAO has created the Progressive Control Pathway for Foot and Mouth Disease (PCP-FMD) to help and facilitate nations where FMD is still endemic in gradually reducing the effects of FMD and a load of FMD virus. Additionally, FAO devised a rough timeline for achieving the stated objective in each of the FMD-endemic nations. Bangladesh has a deadline to meet the established objective because it is a country with an FMD epidemic. A comprehensive epidemiological data based on FMD outbreaks must be gathered by regular sampling and analysis at the molecular level for scheming the control plan by 2015. Bangladesh should logically proceed to Stage 2 by 2019 and Stage 3 by 2020, under the FAO's recommendations.
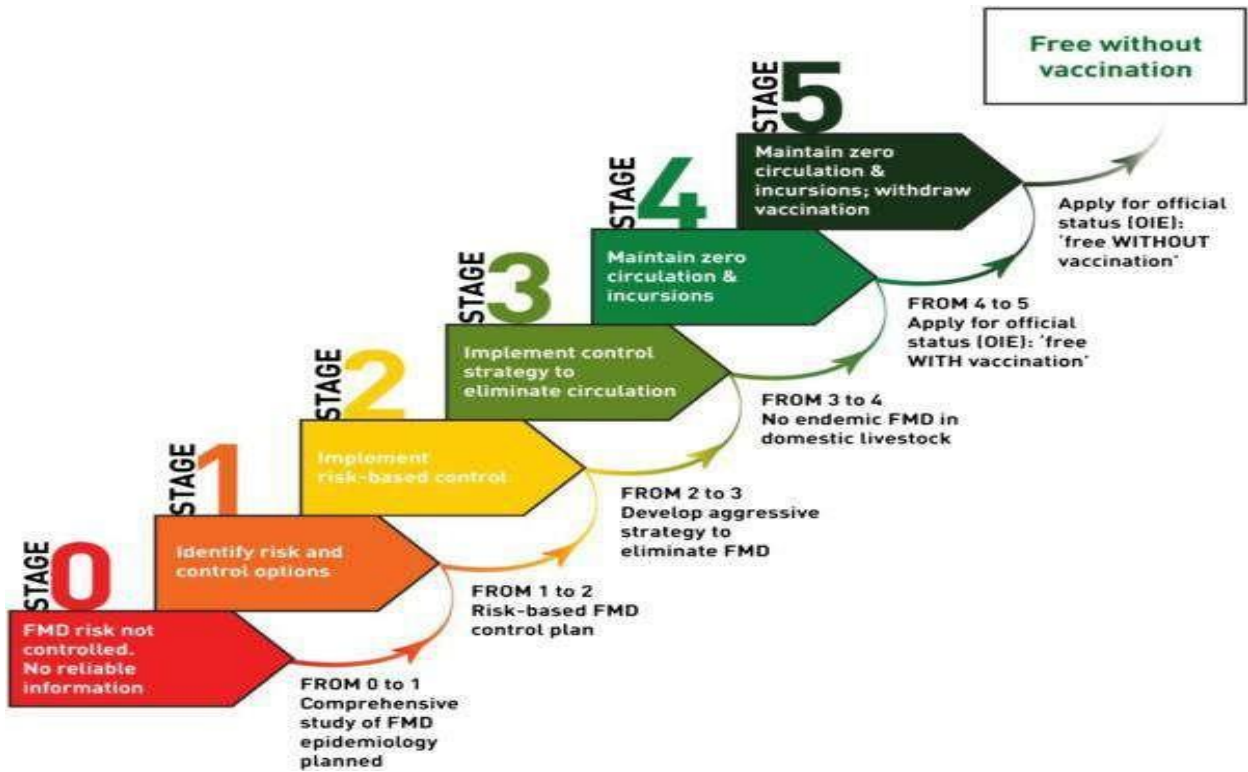
Figure 1.9: The Progressive Control Pathway for FMD control (PCP-FMD) stages (adapted from FAO, 2018).

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 1.10: Time frame for attaining the goal assigned by (FAO, 2020) for Bangladesh (marked area).

1.7   Problem statement

Being an FMD endemic country, Bangladesh experiences economically devastating outbreaks almost every year. It is necessary to control the spread of FMD outbreaks to allow the uninterrupted growth of the livestock industry. But the control and prevention strategies often fail due to the highly contagious nature of the FMDV. The inadequate monitoring system, unrestricted transboundary movement of animals, and unplanned vaccination programs complicate the FMD situation in Bangladesh. Again, the rapid evolutionary rate of FMDV raises questions about the effectiveness of the currently available vaccine.

Realistically preventing the disease requires a well-thought-out control approach, for which assessing the status of FMD comes first. The current study was planned to conduct comprehensive surveillance to monitor the current epidemiological situation of FMD in Bangladesh from 2019 to 21. Prevalence analysis was planned to find out the risk factors contributing to the uncontrolled FMD outbreak. The study focused on revealing the current circulatory outbreak strains and comparative VP1-based genomic and structural analysis to assess whether the current vaccine is effective enough against recent outbreak strains.

1.8   Objectives of the study

- ❖ Epidemiological monitoring of FMD cases between 2012 and 21 in order to understand the trend of current outbreaks and the impact of risk variables on the prevalence of FMD patients.

- ❖ Phylogenetic reconstruction for detailed genetic characterization of isolated samples during 2012-21 and finding predominating outbreak strains.

- ❖ Evolutionary divergence analysis of FMDV isolates with the available vaccine strain.

- ❖ Emergence of novel strains of FMDV over a period of many years, 2012–2021.

- ❖ Analysis of VP1 Amino Acid substitution during the period 2012–20211.

- ❖ Comparative VP1-based mutational and structural analyses of representative FMDV with reference strains and vaccine strains.

- ❖ Isolation and whole genome amplification of the representative isolates.

2 MATERIALS AND METHODS

2.1 Research plan

he objectives of this study included a review of risk factors, a pattern of FMD outbreaks in 2012–2021, VP1-based evolutionary analysis of circulatory FMDV isolates and whole genome based molecular epidemiology of FMDV of new isolates. **Figure 2.1** depicts the study strategy created for the epidemiological investigation, isolation, and thorough comparative genomic studies of FMDV outbreak strains in Bangladesh.
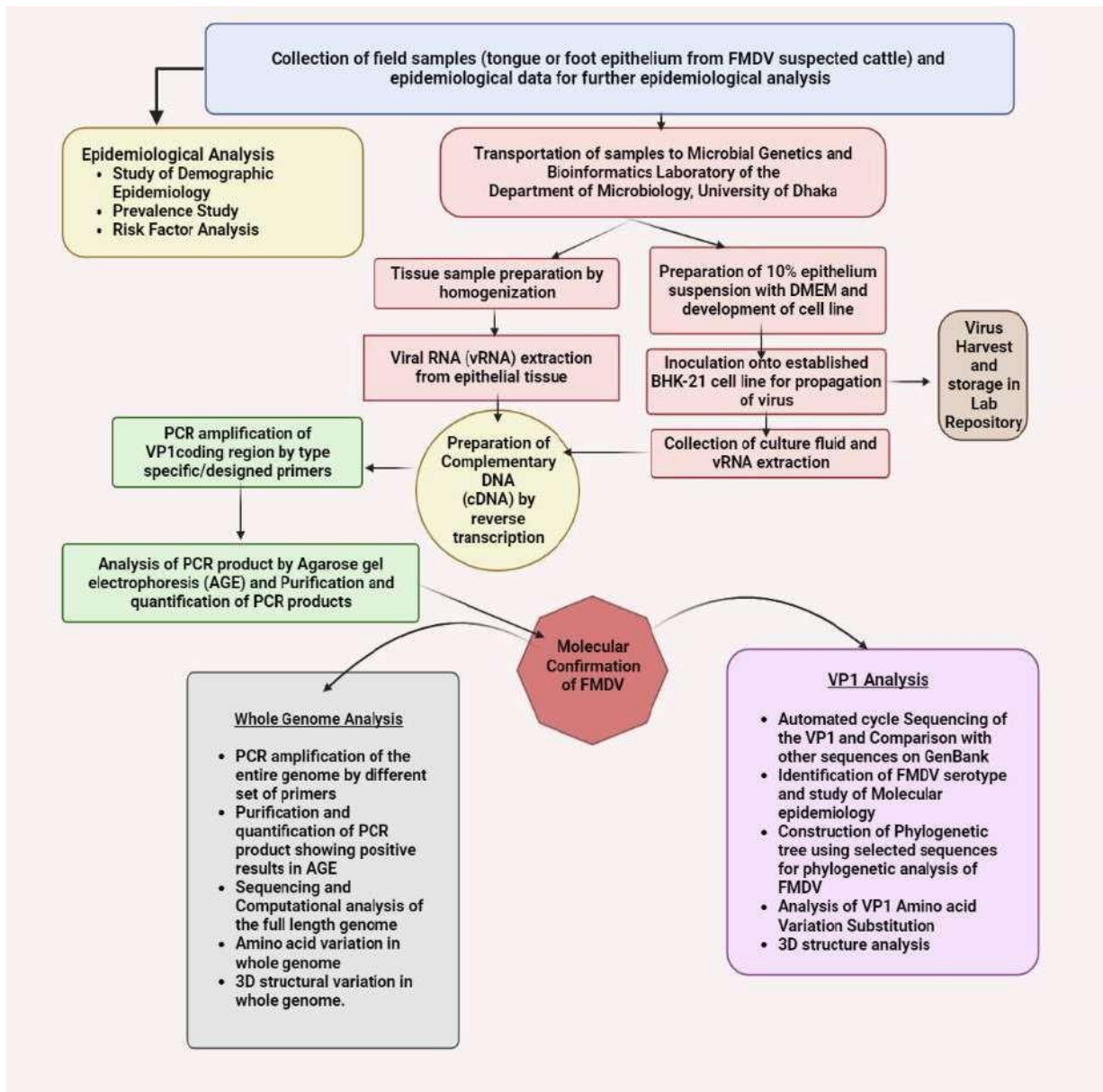
Figure 2.1: Research plan for Evolution and molecular epidemiology Foot and Mouth Disease Virus in Bangladesh.

2.2   Sample Collection

From 2012 to 2021, tissue samples from FMD epidemics across the nation were consistently collected by the Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka's FMD investigation group. Based on the clinical history provided by farmers and clinical observations of the cattle, buffalo, or pigs, a total of 481 tongue or foot tissue spacimens were obtained from FMD-suspicious cattle, buffalo, or pigs in 32 different districts throughout Bangladesh (Figure 2.2). In my study period (2019-2021) I myself collected 156 tongue or foot epithelium tissues from different districts. Following the procedure authorised (Ref.66/Biol.Scs./2018-2019, Date:14.11.18) by the Animal Experimentation Ethical Review Committee (AEERC), Faculty of Biological Sciences, University of Dhaka, all samples were taken by licenced veterinarians **(Appendix-XII)** with the permission of the herd owner.

During the sample collection, a pre-set questionnaire (Appendix-XI) that details the patients' backgrounds was developed and completed. The samples have been taken in cryo-vials and shipped in 20 hours from the collection site to the MGBL (Microbial Genetics and Bioinformatics Laboratory), where they were stored at -80°C until the next stage of the laboratory test. Three-letter nation codes (e.g., BAN for Bangladesh), two-letter district codes (e.g., MY for Mymensingh), Upazilla and laboratory record numbers (e.g., My-466), and finally the epidemic year were assigned to the samples. For instance, the Bangladesh FMDV was collected in 2021 and is represented by the Mymensingh Mymensingh-laboratory record number 466, BAN/MY/My-466/2021.
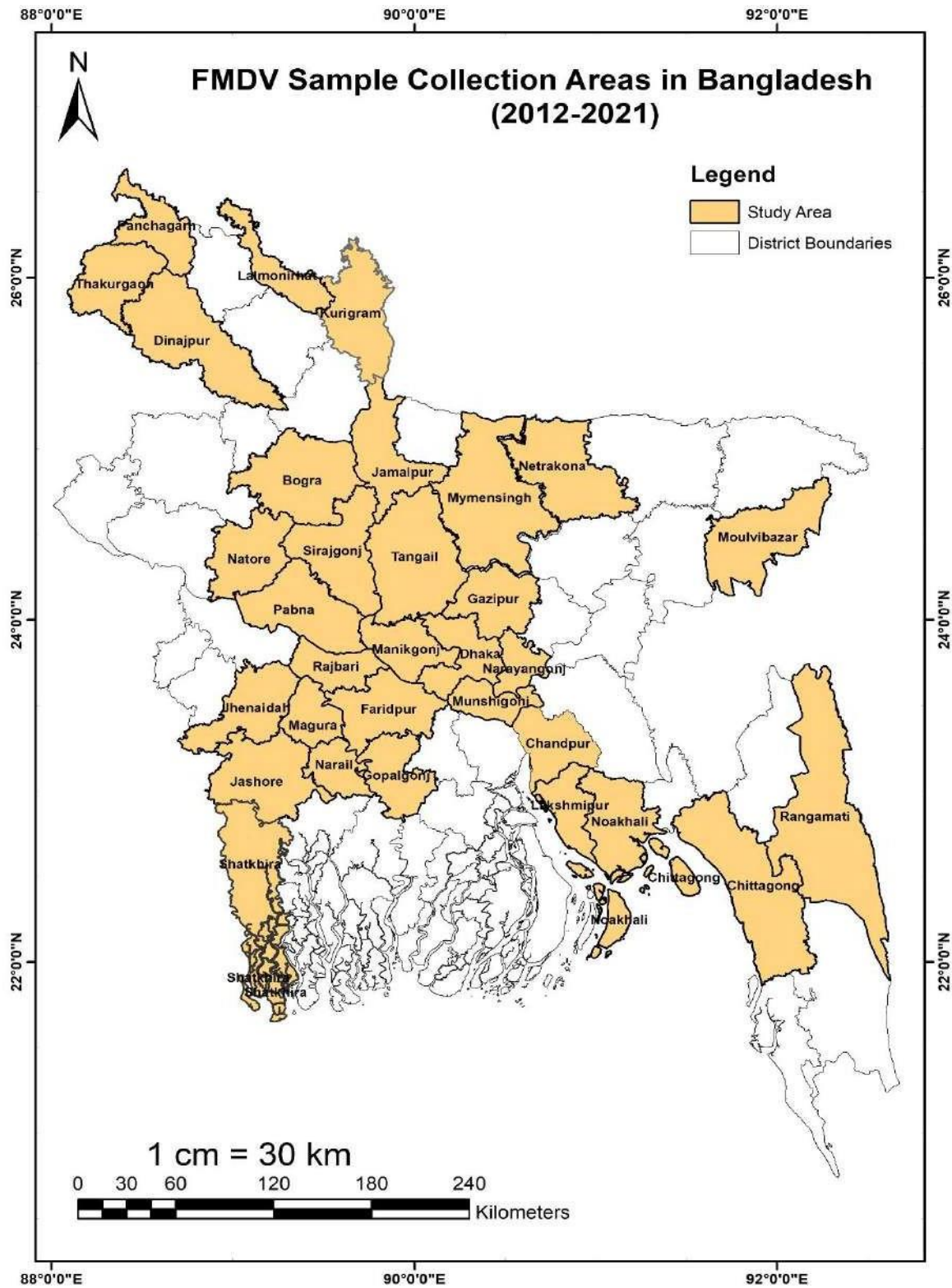
Figure 2.2: FMD sample collection areas.

2.3 Epidemiological research of Foot and Mouth Disease virus

To create approaches to FMD identification and prevention, it is imperative to have a complete knowledge of the type and amount of alterations in the FMDV genome that are associated to epidemiologic variables. The methodology for the demographic epidemiology study is detailed below.

2.3.1 Demographic Epidemiology Research

A questionnaire format was created (Appendix-IX) for the initial investigation of cattle in FMDV-affected regions during gathering of samples. The purpose of this questionnaire is to identify the FMD risk variables for cattle in the Bangladeshi prefecture between May 2012 and December 2021. The investigation of outbreaks in this study was based on the sample collected during 2012-2021 in the Microbial Genetics and Bioinformatics Laboratory, University of Dhaka. It should be noted that the collection of the sample and reporting of the outbreak were interrupted by the pandemic situation of COVID-19 in Bangladesh during 2020-21.

2.3.2 Prevalence study

Based on the population size (n=3580) used in this investigation, morbidity, mortality, and case fatality rates were computed. The percentage of morbidity is displayed. It is computed by dividing the total population for a given time period by the number of cases of an illness, accident, or disability, as shown below:

$$\text{Morbidity Rate} = \frac{\text{Number of Disease Cases}}{\text{Population}} \times 100$$

The mortality rate is calculated as the number of animals that died in a specific location and time frame, divided by the local population.

$$\text{Mortality rate} = \frac{\text{No. of death by disease}}{\text{Population}} \times 100$$

The case fatality rate is the proportion of cases with a certain cause of death to all cases with the same cause of death. The formula is as follows:

$$\text{Case fatality rate} = \frac{\text{number of deaths attributable to a specific disease}}{\text{Total cases involving the same condition}} \times 100$$

2.3.3 Risk factor analysis

The Statistical Package for Social Science, SPSS 26.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to explore and statistically verify the impact of several risk factors, including season, age, gender, breed, and farming system, on the occurrence about FMD cases. The findings of this study will offer preliminary data for epidemiological research, and on the basis of this, control and eradication programs will be put up to stop the spread of FMD outbreaks in the future.

2.4 Viral RNA Extraction from Tissue Sample

To isolate intact RNA from tissue samples following four steps were maintained carefully

I. Tissue or cells must be rupturing successfully

II. Inactivation of endogenous ribonucleases (RNases)

III. Elimination of contaminated DNA and protein.

Tongue or foot epithelial tissue weighing about 60 mg was measured and put in an elution tube. An automated MaxwellR 16 Instrument (Promega, USA) was used to homogenise the sample with 396 l of lysis buffer (66 µl /10 mg tissue) until the sample was thoroughly lysed. Guanidine thiocyanate (GTC) is a component of lysis buffer, which is used to break up materials, dissolved nucleoprotein structures, and deactivate ribonucleases. The Maxwell® 16 Total RNA Purification Kit's lysis buffer combines guanidine thiocyanate's disruptive and protecting qualities. 500 µl of lysate were transferred to a 2 ml Extra Gene microcentrifuge tube (USA). Lysates were mixed with 835 µl of blue RNA dilution buffer (included in the kit's Appendix I). As much as possible, the combination was maintained chilled. The samples were mixed with 100 l of clarifying agent, which was included in the package. The Clearing Agent is used to extract genomic DNA from the sample lysate in a targeted manner. The resin was thoroughly resuspended by shaking or vortexing before a cleaning agent was added. After that, the vortex thoroughly combined the sample and the cleansing agents for 30 seconds. The mixture was cooked for 3 minutes in a heat block set to 70˚C. After heating, the combination went through the vortex process once more for 30 seconds. For optimal cooling, the sample was subjected to incubation for 5 minutes at room temperature. One clearing column was inserted into a collection tube to complete the clearing column assembly. A clearing column and a centrifuge at 12000 x g for 2 minutes were both used to centrifuge about 700 µl of the material. Without disturbing the pellet of the clearing agent, flow-through from the collection tube was transported to well#1 of the Maxwell® 16

| a) Layout of Cartridge | b) Sample loading | c) Maxwell 16 Extraction System |
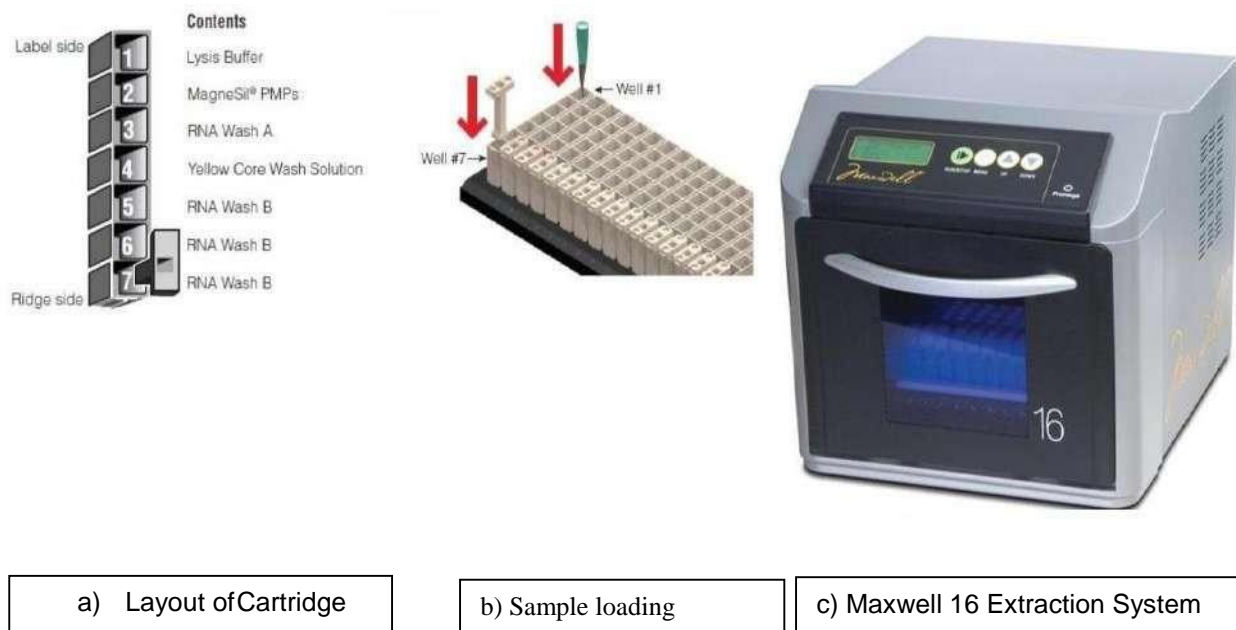
Figure2.3: (a-c) RNA Extraction system.

 LEV RNA Cartridge. If the sample volume exceeds 700 µl, the centrifugation and sample loading steps were repeated. Plunger was placed in Maxwell® 16 LEV RNA Cartridge well #7. A variety of chamber facilities for RNA extraction are included in the Maxwell® 16 RNA Cartridge (Figure 2.3(a-c)). 300 µl of water free of nucleases were poured into the elution tube. The Maxwell® 16 RNA Cartridge and elution tube were then inserted into the instrument's designated chamber. After establishing the RNA extraction programme, the device took around 34 minutes to collect the RNA yield from the elution tube. The isolated RNA was kept at -80° until PCR and reverse transcription.

2.5   Complementary DNA (cDNA) Generation from Extracted RNA (Tissue)

According to the manufacturer's instructions, the isolated RNA was reverse transcribed into complementary DNA (cDNA) using the ImProm-IITM Reverse Transcription System (Promega, USA;) (Appendix-I). The RNA was reverse transcribed using oligo(dT) and hexameric random primers. An aliquot of the positive control RNA was mixed with the oligo (dT) primer, and experimental RNA was combined with the random and oligo (dT) primers to create cDNA. In order to rule for unwelcome contamination, a negative control (No RNA Template) was established. Thermally denatured at 70 °C for 5 minutes, the primer/template mixture was then refrigerated on ice. This stage was carried out using a heat block (Veriti 96 well Thermal cycler, Applied Biosystem, USA) (Figure 2.4). ImProm-IITM reverse transcriptase, 5X reaction buffer, magnesium

chloride, dNTPs, and ribonuclease inhibitor were added to a reverse transcription reaction mixture that has been arranged on ice. It was suggested but not required to include 1 unit/l of Recombinant RNasin® Ribonuclease Inhibitor in experimental setups. The final step involved adding the reaction mixture on ice with the template-primer combination and starting a heat cycler to convert the RNA to cDNA. The cDNA synthesis mixture was introduced straight to amplification processes without any cleanup or dilution.

Figure 2.4: Veriti 96 well Thermal cycler, Applied Biosystem, USA.

2.5.1   Combining target RNA, Primer, and the denaturation process

1. Dilution tubes with sterile, thin walls from ExtraGene, USA and reaction tubes from Eppendorf, USA were used.

2. Thawed on ice were the isolated RNA and the 1.2 kb Kanamycin Positive Control RNA.

3. For each reverse transcription procedure, up to 1 g of experimental RNA and 10 l of nuclease-free water were mixed with the cDNA primer on the ice. When more than one reaction was intended to be carried out using a single RNA: primer combination, the quantities were doubled to accommodate the additional reactions. Table 2-1 lists the target RNA/Primer combination, the positive control, and the negative control.

4. After being tightly closed, each RNA tube was put into a heat block that had been prepared to 70°C for five minutes. The heat block was set up to maintain a temperature of 4 degrees Celsius after heating for 5 minutes so that a chilling environment could be generated to incubate the primer/RNA mixture. To collect the condensate and preserve the original volume. These tubes were subsequently turned in a minicentrifuge (ExtraGene, USA) for 10 seconds. Prior to adding the reverse transcription reaction mix, the tubes were kept covered and chilled.

Table 2.1: RNA/Primer Mixture for cDNA Preparation

Positive control	
1.2kb Kanamycin Positive Control RNA(1µg)	5.0 µl
Oligo(dT) Primer (0.5µg/reaction)	2.0 µl
Nuclease-Free Water	3.0 µl
Final Volume	**10 µl**

Negative (No RNA Template) Control	
Random primer (Hexameric primer)	2.0 µl
Oligo (dT) Primer (0.5 µg/reaction)	2.0 µl
Nuclease-Free Water	6.0 µl
Final Volume	**10 µl**

Experimental Reaction	
Experimental RNA	5.0 µl
Random primer (Hexameric primer)	2.0 µl
Oligo (dT) Primer (0.5 µg/reaction)	2.0 µl
Nuclease-Free Water	1.0 µl
Final Volume	**10 µl**

2.5.2 Reverse Transcription

The following ImProm-IITM Reverse Transcription System components were combined in a sterile 1.5 ml microcentrifuge tube (Eppendorf, USA) and kept on ice to create the reverse transcription reaction mix. Each cDNA synthesis reaction required 30 µl of reaction mix to be prepared. Before being poured into the reaction tubes, the reaction mixture had been gently vortexed to mix and stored on ice. Table 2.2 lists the volumes required for each component.

Table 2.2: **Reaction Mixture for making cDNA**

Positive Control	
RNase Free H_2O	13.2 µl
5X Reaction Buffer	8.0 µl
$MgCl_2$ (6mM)	4.8 µl
dNTP Mix (final concentration 1.0 mM each dNTP)	2.0 µl
ImPro Reverse Transcriptase	2.0 µl
Final Volume	**30 µl**
Negative (No RNA Template) Control	
RNase Free H_2O	13.2 µl
5X Reaction Buffer	8.0 µl
$MgCl_2$ (6mM)	4.8 µl
dNTP Mix (final concentration 1.0 mM each dNTP)	2.0 µl
ImPro Reverse Transcriptase	2.0 µl
Final Volume	**30 µl**

Experimental reaction	
RNase Free H$_2$O	9.6 µl
5x Reaction Buffer	8.0 µl
MgCl2 (8mM)	6.4 µl
dNTP Mix (final concentration 1.0 mMeach dNTP)	2.0 µl
Recombinant RNasin® RibonucleaseInhibitor	2.0 µl
ImPro Reverse Transcriptase	2.0µl
Final Volume	**30 µl**

Table 2.3: **Perfect reaction condition for Reverse Transcription reaction**

Step	Temperature (°C)	Time (minute)
Annealing	25	5
Extension	42	60
Inactivation of Reverse Transcriptase	70	15

The reaction mix had been hold on ice and gently vortexed to combine it. Each reaction tube was then filled with a reverse transcription reaction mix (30 µl aliquots) that was kept on ice. That phase required careful handling to avoid cross-contamination. Each reaction received 10 µl of the RNA and primer mix, for a total reaction volume of 40 µl per tube. The tubes were then kept in a temperature-controlled heat block that was set to equilibrate at 25°C and 5 minutes incubation. The tubes were then kept at 42°C for up to an hour in a controlled-temperature heat block to allow the cDNA product to extend. According to the manufacturer's recommendations, the expantion temperature may be best between 37°C and 55°C. Following the extension stage, the reaction tubes were incubated at 70°C for 15 minutes in a controlled-temperature heat block (Table 2.3).

2.6   PCR Amplification
2.6.1 Screening for FMDV

The heat-inactivated reverse transcription reaction's byproducts were added to the PCR mixture, followed by thermal cycling, to directly amplify the cDNA. For PCR amplification, GoTaq® Hot Start Colorless Master Mix (Promega, USA) has been employed. Tables 2.4 provide information on the ingredients in the GoTaq® Hot Start Colorless Master Mix.

Table 2.4: Ingradients 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)
MgCl2 (4 mM)

Table 2.5: formulation of the PCR for the detection of FMDV

PCR Mixture Components	PositiveControl (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(10µM)	2 µl (400nM)	2 µl (400nM)	2 µl (400nM)
Template	Variable (< 500 ng)	No Template	Variable (< 500 ng)

Primer pairs were used to test each sample's RT product for the presence of FMDV. Primers were used for first screening like (Ullah *et al.,* 2014) VP1UF-NK61; 16F-16R (Nandi *et al.,* 2015); 16F-NK61; (Reid et al., 2000; Samuel & Knowles, 2001). To guarantee that all DNA templates were denaturized, all of the PCR tubes containing the proper mixes were heated in the thermal cycler (Applied Biosystem, USA) at 94°C for 5 minutes. These phases were repeated thirty-five (35) times, with a final expantion of ten minutes at 72 degrees. Following this, PCR tubes were kept at -20°C until further examination. The cycle profile for every primer-target pair was changed in accordance with this **(Table 2.6).**

Table 2.6: The FMDV screening primers

FMDV Serotypes	Primers	Sequence (5'-3')	Annealing Temperature (∘C)	Location	Reference
All	VP1UF	GCRCAGTACTA CRCSCAGTAC	55	VP1	Ullah *et al.,* 2014
All	16F	GAGAACTACGG WGGWGAGAC	55	VP1	Nandi *et al.,*2013
All	16R	GCACCGWAGTT GAAGGAGGT	55	VP1	
All	NK61	GACATGTCCTC CTGCATCTG	55	2B	Reid *et al.*,2000; Samuel & Knowles, 2001

2.6.2 The amplifying process of the VP1 Region by PCR

In order to detect the presence of FMDV, cDNA made from extracted RNA (Cell Culture Supernatant) was employed. VP1UF and NK61 primer pairs were used in PCR as per section 2.4.1's instructions. A sample that had previously been determined to be FMDV positive was utilised as a positive control, and the addition of a PCR negative control helped to confirm the outcome. The cell culture negative control underwent the same PCR procedure as the positive control. The following brief **Table 2.7** discusses the cyclic nature of PCR.

Table 2.7: Cyclic condition of PCR

Primer Pair	Denaturation (ºC)	Annealing (ºC)	Extension (ºC)
VP1UF/NK61	94	55	72
16F/NK61	94	55	72

According to the procedure outlined in section 2.6, the PCR result was first seen, and then Agarose gel electrophoresis was performed. Following the centrifugation technique, Utilising Promega's Wizard® SV Gel and PCR Clean-Up System, PCR products were purified. Section 2.7 deciphered the specifics of the technique, and Section 2.7.2 determined the concentration of the PCR-purified result.

2.7   Agarose Gel Electrophoresis

For the purpose of seeing the amplified products, the amplified products were run on a 1.0% agarose gel with a 100 bp-DNA ladder (Promega, USA). In 1x TAE buffer, 60 ml of 1.0% agarose were made (Appendix II). The mixture was melted after being cooked in the microwave for 4-5 minutes on medium. 3 µl of ethidium bromide (stock 10 mg/ml) was then added when the liquid had cooled to roughly 45°C from the boiling point. The well former (comb) was inserted after the gel had been put onto the gel case. After that, the casing was left to rest for roughly 15 minutes on a flat surface. The comb was then taken out of the gel and buffer 1x TAE was added to the tank. Samples (1 µl of loading buffer **(Table 2.8)** and 5 µl of PCR product) were prepared on parafilm. A 6 µl molecular weight marker and a 1 µl loading buffer were used to generate the molecular weight marker (Appendix II). Samples were added to the wells that had been cut into the gel. For 35 minutes, electrophoresis was conducted at 100 volts. The AlphaImager HP Gel-documentation system (Cell Bioscience, USA) was used to view the gel.

Table 2.8: 6x Orange Loading Dye Composition

Reagent	Final concentration	Amount
Glycerol	30%	300.0 μl
10% Bromophenol blue	0.25%	25.0 μl
10% Xylene cyanol	0.25%	25.0 μl
Deionized water	-	650.0 μl
Total Volume		**1000.0Ml**

2.8   Purification of PCR products
2.8.1   Cleaning of Amplicons

Following Agarose gel electrophoresis, PCR-positive samples were cleaned using The Wizard® SV Gel and PCR Clean-Up System (Promega, USA; Appendix I). A centrifugation-based method was used to purify the PCR products. The Wizard® SV Gel and PCR Clean-Up System was created in response to the ability of DNA to bind to silica membranes in the presence of chaotropic salts (guanidine isothiocyanate). Following amplification, a portion of the PCR is mixed with the guanidine isothiocyanate-containing Membrane Binding Solution (MBS) and promptly filtered. MBS was added to the PCR amplification in an identical amount. A collection tube was used to convey the mixture to the minicolumn assembly, which had been previously set up. The SV minicolumn was centrifuged at 16,000 g (14,000 rpm) for 1 minute following a brief (2 minutes) incubation at room temperature. After being discarded, the flow-through SV minicolumn was subjected to two washings with the kit's included membrane wash solution with ethanol added. The SV minicolumn was cleaned, and then DNA was eliminated in Nuclease Free Water (supplied in the kit). The volume of the elution depends on the post-purification objective, such as the sequencing reaction. Prior to further processing, the purified PCR product had been kept at -20°C.

2.8.2   Determination of amplicon amount

A NanoDropTM spectrophotometer from Thermo Fisher Scientific Inc., Wilmington, DE, USA, has been used to calculate the product's volume. The PCR product was quantified in ng/ μl. The ratio's value (OD 260 / OD 280) fell between 260 and 280 nm. The nucleic acid purity (DNA) can be estimated using the OD 260/280 ratio, which yields a value of 1.8.

2.9   Molecular conformation of Foot and Mouth Disease virus
2.9.1   Sequencing of PCR products (VP1 Coding Region)

After the PCR products had been purified, they were delivered to Macrogen, Korea, together with the appropriate primers, for single-pass DNA sequencing using the Sanger method.

2.9.2 Study of Evolutionary History
2.9.2.1 Homology Search and Serotype Identification

Each of the forward and reverse primers were utilized in the sequencing experiment (Table 2.6). By using sequence viewer software like chromas, the sequences (tracer files) were inspected and cut based on the sequences' quality. Seq Man version 7.0.0 (Lasergene, DNASTAR, USA) was used to combine the forward and reverse sequences into a single contig. The identity of the isolated virus and its serotypes were discovered by comparing the sequences with other sequences from the Gen Bank using the basic local alignment search tool BLAST (Altschul et al. 1990; Morgulis et al. 2008).

2.9.2.2 The evolutionary history and Topotype, Lineage, and Sub-lineage of FMDV Isolates Identification

From 2012 to 2021, our team sequenced 122 typical VP1-specific PCR products representing 71 outbreaks and submitted them to the NCBI database (https://www.ncbi.nlm.nih.gov/genbank/). (Ullah *et al.*, 2014, 2015; Sultana *et al.*, 2014; Ali *et al.*, 2016; Nandi *et al.*, 2015; Siddique *et al.*, 2018). In order to identify the genotype based on the clade formation in MEGA11, representative VP1 sequences of Bangladeshi isolates from 2012 to 2021 from our laboratory as well as from reported sequences by other researchers in Bangladesh were included in the phylogenetic study (Tamura *et al.*, 2021). The Appendix V lists the VP1 sequences of the Bangladeshi isolates used in this study.

Using the ClustalW tool (Thompson *et al.*, 1994), the consensus VP1 coding sequences (full 1D area) of local FMDV isolates were aligned with the relevant gene sequences from GenBank. Based on the Kimura-2 parameter model (Kimura, 1980), phylogenetic Neighbor-Joining (Saitou & Nei, 1987) trees were created (bootstrap replicates 1000). To represent variations in evolutionary rates between locations, a discrete Gamma distribution with a value of 1 was utilized. Due to the fact that not all of the sequences were perfectly matched across the whole range, less than 5% alignment gaps, missing information, and ambiguous bases were permitted at any place.

2.9.2.3 Phylogenetic analysis

From 2012 to 2021, our team sequenced 122 typical VP1-specific PCR products representing 71 outbreaks and submitted them to the NCBI database (https://www.ncbi.nlm.nih.gov/genbank/)(Ullah *et al.*, 2014, 2015b; Sultana *et al.,* 2014; M. R. Ali *et al.*, 2016; Nandi *et al.,* 2015b; Siddique *et al.*, 2018). In order to identify the genotype based on the clade formation in MEGA11, representative VP1 sequences of Bangladeshi isolates

from 2012 to 2021 from our laboratory as well as from reported sequences by other researchers in Bangladesh were included in the phylogenetic study (Tamura et al., 2021). The Appendix V lists the VP1 sequences of the Bangladeshi isolates used in this study. Using the Clustal W tool (Thompson *et al.,* 1994), the consensus VP1 coding sequences (full 1D area) of local FMDV isolates were aligned with the relevant gene sequences from Gen Bank. Based on the Kimura-2 parameter model (Kimura, 1980), phylogenetic Neighbor-Joining (Saitou & Nei, 1987) trees were created (bootstrap replicates 1000). To represent variations in evolutionary rates between locations, a discrete Gamma distribution with a value of 1 was utilized. Due to the fact that not all of the sequences were perfectly matched across the whole range, less than 5% alignment gaps, missing information, and ambiguous bases were permitted at any place.

2.9.2.4   Mutational analysis

Changes in nucleotide sequence and amino acid sequence of VP1 of a representative of the novel sub-lineage group was compared against an NCBI reference sequence and a current vaccine strain using a global alignment tool based on the Needleman-Wunsch algorithm in NCBI. The percentage of identity between the sequences was calculated using this method.

2.9.2.5   Analysis of the substitution of different VP1 amino acids

The overall variances across all FMDV serotype O, A, and Asia1 viruses were examined using the VP1 coding sequences of FMDV isolates reported in Bangladesh. After using MEGA11 software to align codons, which the amino acid was translated using the genetic code as a basis (*Tamura et al*., 2021). Sequences from the Ind-2001BD2 sublineage and the MYMBD21 sublineage were removed from the mutational pattern analysis since none of the sequences given for these two sublineages had any documented amino acid changes.

2.9.2.6   Prediction of the VP1 Region's 3-D Structure

The SWISS Model was used to upload the amino acid sequence of VP1, and appropriate templates were chosen. The model was then built up after that. Molprobity v4.4's Ramachandran plot analysis was used to validate the model. PyMOL was used to download and display the PDB file.

2.10   Whole genome analysis
2.10.1 Virus isolation
2.10.1.1 creating the media for cell culture

For the creation of cell lines and virus isolation, DMEM (Dulbecco's Modified Eagles' Medium) was utilized. In this experiment, liquid DMEM (Biochrome, Germany) with stable glutamine was employed. According to Dulbecco & Freeman (1959), the DMEM was formulated for commercial

use. 3.7 g/l NaHCO3, 1.0 g/l glucose (standard formulation), and 58.0 mg/l glutamine are notable components. Penicillin and streptomycin (10000 g/ml) and Gentamycin (10 mg/ml) were added to DMEM as a supplement to prevent contamination caused by bacteria and mycoplasma. Only 0.05 mg/l of riboflavin was added compared to the original formulation in order to prevent harmful photo-oxidative effects. For the goals of virus isolation and cell culture, two different types of formulation were created. Those were:

Growth Media	10% FBS (Fetal Bovine Serum)
Maintenance Media	2% FBS (Fetal Bovine Serum)

2.10.1.2 Preparation of Cell Line

The Baby Hamster Kidney cell line(BHK-21) was employed for virus isolation and infection. The BHK-21 cell line was maintained and passed through passages before infection. Liquid nitrogen was used to keep the cell safe. From the storage tank, a cryovial holding 1.5 ml of BHK-21 cell line-containing medium was taken out. The cell-containing vial was defrosted in a water bath set at 37° C. Before cell passaging, DMSO (Dimethyl Sulfoxide), which was added during storage, had to be removed. Using a sterile serological pipette, the entire contents of the vial were transferred to a 15 ml sterile falcon tube. To the falcon tube was put an additional 3.5 ml of brand-new DMEM. Using the right balance, a falcon tube holding 5 ml of medium and cells was centrifuged at 1000 rpm for 2.30 min. The DMSO-containing supernatant was discarded before the pellet was suspended in 5 ml of fresh DMEM. Fresh DMEM was gradually raised and added in drops. A pipette was used to apply resuspended medium to one corner of the 25 cm2 cell culture flask. After that, media was gently dispersed around the flask's inside surface. The flask was sealed, then incubated at 37 °C in a humidified atmosphere with 5% CO2 (Nuaire, USA). Flask was incubated to check for appropriate saturation and cell development. The experiment was conducted using aseptic technique.

2.10.1.3 Passaging of Cell

Confluency of the monolayer of the cell was observed under an inverted microscope (Olympus, USA). BHK-21, an adherent cell needs to passage before reaching confluency. Media (spent) was decanted. After that, 5 ml of PBS (Phosphate Buffered Saline) was used to wash the cell's monolayer in order to get rid of any undesired leftovers. To prevent the cells from drying out, this phase was carried out as soon as feasible. The flask was then filled with 800 l of cold trypsin, and it was incubated at 37 °C for 2–3 minutes after that. To totally separate cells, the flask's side was lightly tapped. The flask was then filled with 3.2 ml of brand-new 10% DMEM (growth medium)

media. To assist break up the cell clumps, the cells were pipetted up and down many times. The cell suspension was promptly disseminated in fresh flasks in the necessary volume (0.5 to 1.0 mL) using the same pipette. The final volume was adjusted to 5 ml by adding new growing media. The flask was sealed and then incubated at 37° C in an incubator with a humidified environment containing 5% CO2. The cell line was passaged 5-6 times to maintain the cell line and finally, a cell line having proper growth conditions (log phase; 80-90% confluent) was selected to inoculate the virus.

2.10.1.4 Treatment of Sample

Representative tissue samples were selected to infect the cell line which showed positive results during FMDV screening. Grinding of the tissue was done. For precession, a blank control was done in parallel. By using a mortar and pestle to grind sterile sand with fresh DMEM, 0.22 m millipore (Millipore Sterivex - GS 0.22 m disposable filter units) was used to filter the filtrate, which was then employed as a negative control. In the same mortar and pestle, a sample of tissue was pulverized as a negative control. The BHK-21 cell line was infected using the filtrate.

2.10.1.5 Sample Inoculation and Virus Culture

For virus infection, a cell line with ideal growth conditions (log phase; 80–90% confluent) was used. After decanting the used medium, PBS wash was used. The cell line was inoculated using 200 µl of the sample's filtrate and 1.8 ml of fresh 2% DMEM (Maintenance medium). In the negative control, filtrate from blank media was used in place of sample filtrate. The flasks were correctly labeled and then incubated for 60 minutes at 37 °C in a humid atmosphere with 5% CO2. The final volume of the flasks was then elevated to 5 ml by the addition of fresh 2% DMEM. The following 24 to 48 hours were spent incubating cell culture flasks. The presence of the cytopathic effect (CPE) was periodically evaluated in cell culture flasks.

2.10.1.6 Harvesting of viruses

The virus was extracted after exhibiting considerable CPE in comparison to the negative control. Adherent cells were separated from the flask's inner surface using a tiny scrapper. A sterile 15 ml falcon tube was used to collect the whole contents of the flask. Cell culture supernatant was obtained using centrifugation (1000 rpm for 2.30 min). A new, 15 ml falcon tube was used to decant the majority of the supernatant. To recover intracellular viruses, the residual cell pellet and supernatant underwent 2-3 cycles of freezing and thawing. Following this procedure, a cell pellet containing a falcon tube was combined with the remaining cell culture supernatant. To gather cell culture supernatant, centrifugation (1000 rpm for 2.30 min) was completed. The same steps were taken for the negative control. The positive sample's cell culture supernatant was saved for later use and used for another passage.

2.10.1.7 Preservation of Virus

1.5 ml aliquot of cell culture supernatant (positive sample) was done in sterile 2 ml cryovials. Vials were stored at -80º C freezer (Nuaire, USA).

2.10.2 Extraction of Viral RNA from Cell Culture Supernatant, cDNA preparation, and PCR amplification followed by agarose gel electrophoresis and product purification

Similar to the process outlined in section 2.3, 450 l of cell culture supernatant was combined with 50 l of lysis buffer in a 2 ml micro- centrifuge tube to extract RNA from the supernatant. To get the cells to lyse, the mixture was vortexed. To facilitate full lysing, the lysate was kept as much as possible on ice. From extracted RNA, complementary DNA (cDNA) was created using the method outlined in section 2.4.

2.10.3 Amplification of the VP1 Region by PCR

cDNA was subjected to PCR amplification using the primer pairs VP1UF and NK61 **(Table 2.7)** in accordance with the process outlined in sub-section **2.5**. In order to confirm the presence of FMDV in cell culture supernatant, PCR product was evaluated by combining 5 µl of the reaction mix with 1 µl of 6x orange loading dye solution and resolving the sample by agarose gel electrophoresis alongside a DNA size marker as per the methodology stated in sub-section **2.6**.

2.10.4 Amplification of the Whole Genome by PCR

The produced RT product (cDNA) was submitted to an amplify of the whole genome for the investigation of comparative genomics employing the following techniques in the positive case of the presence of FMDV in cell supernatant.

2.10.4.1 Designing PCR Primers to Amplification of Entire Genome

Designing oligo nucleotide primers is essential for ensuring accurate DNA amplification. On the internet, there are many specialized programs for designing primers. But in this study, two fundamental strategies are taken into account when designing primers.

2.10.4.1.1   Manual Primer Design

For the successful construction of primers, the flanking DNA sequence of the target area was manually evaluated, and primers were chosen based on fundamental criteria such GC content, primer length, PCR product length, and the lack of known polymorphisms.

2.10.4.1.2   Using Specific Primer Design Software for Primer Design

The primers were made using the programme Primer-3 Plus (Rozen and Skaletsky, 1999). The serotype of the isolated virus was identified by BLAST. The closest hit of the isolate virus used as

the standard for primer design. 14 primers were designed to amplify the complete structural region of the genome. The FASTA file was uploaded into the Primer-3 Plus software window for the 5UTR to VP1 region standard isolates. No of the method used to make the primers, a variety of parameters were taken into account to ensure high PCR success rates. Primer specificity and length (typically 18 to 25 nt), melting temperature (Tm) (similar values for forward and reverse primers and ideally within 10C), annealing temperature (T), complementary primer sequences (less than 3 bp of intra-primer homology), GC content (between 45 and 55%), repeat stretches of poly pyrimidines (T and C) or poly purines (A and G), and repeat stretches of poly pyrimidines were the most. Some of the primers included degenerate bases to increase their adaptability to particular FMDV serotypes. Using MEGA 5.2 software, initial primers were matched with sequences that were closer to an isolated virus in order to introduce degeneracy. Degenerate bases were inserted in the alignment file where variance at the nucleotide level was seen using the BioEdit program (Hall, 2008). By using BLAST search, the chosen primers were checked for cross matching. Using the online instrument IDT Oligo Analyzer 3.1 edition, the caliber of the chosen primers was examined. Analysis was done on the changes in free energy that occur when various secondary structures, such as self-dimers and heterodimers, develop. To order oligo synthesizer (IDT- Integrated DNA Technology, USA), the finest primers were chosen.

2.10.5 Amplification of the whole genome by PCR

Using several internal primer pairs, PCR was used to synthesize 16 overlapping fragments from the full FMDV serotype O genome that had been extracted (**Table 2.9**). The placement of the serotype O overlapping primer pair positions is shown schematically in Figure 2.5. 400nM of each primer pair, 2X GoTaq® Hot Start Colorless Master Mix (Promega, USA), and nuclease-free water
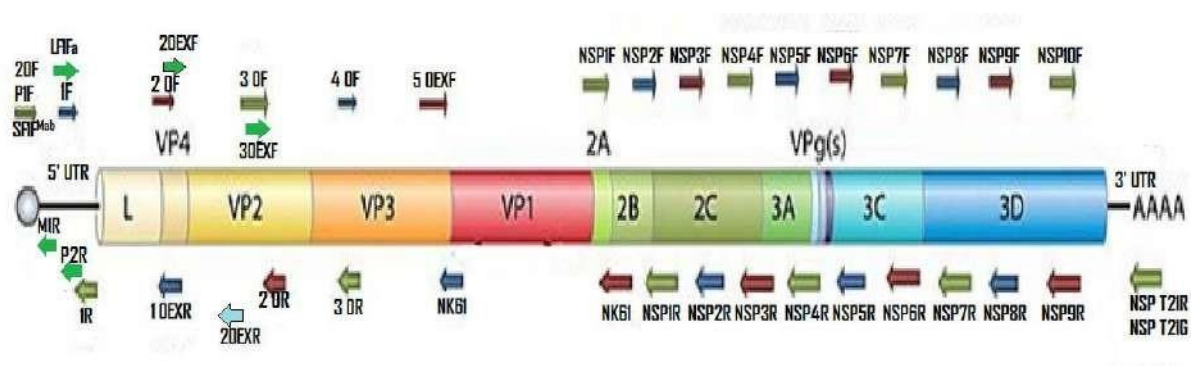
were added to create the PCR reaction mix. Along with the test

Figure 5: Primer pairs were utilised to amplify the full FMDV serotype O genome.

Table 2.9: **Primer pairs used to amplify the complete genome of FMDV serotype O**

Primers	5'-3' Sequence	Location	Position	Amplicon size (bp)	Reference
20F	TTGAAAGGGGGCRCTAGGGT	5'UTR	1-20		**Designedinthis study**
1R	CCAGTCCCCTTCTCAGATC	5'UTR	948-965	965	**Reid *et al.*, 2000**
1F	GCCTGGTCTTTCCAGGTCT	5'UTR	640–658		**Reid *et al.*, 2000**
1OEXR	CCCTCGTGYAGYTCAAGACC	VP4	1329–1348	708	
2OF	CCMTTCYTCGAMTGGGTCTA	VP4	1254–1273	1006	
2OR	TGGTTWCCCACTGCRGTGAC	VP2	2241–2260		
3OF	ARGACTTYGTGAGYGGGCC	VP2	2035–2053	861	
3OR	AAGTGCAGGTTRATGGTGCC	VP3	2877–2896		**Designed in this study**
4OF	CAAGGTSTATGCCAACATCG	VP3	2507–2526	890	
4OR	RTYTGCATCAGGTCCAACAC	VP1	3378–3397		
5OEXF	GAGAACTACGGTGGTGAGAC	VP1	3276–3295		
NK61	GACATGTCCTCCTGCATCTG	2B	3971–3994	718	**Samuel and Knowles, 2001**
NSP1F	GAGACGTYGAGTCCAACCC	2B	3939-3958	596	
NSP1R	CTTCTGAGGCGATCCATG	2C	4517-4535		
NSP2F	CAGCTCARAGCACGTGACAT	2C	4423-4443	566	
NSP2R	GCCATRGGCGGGATRAA	2C	4972-4989		
NSP3F	TGACCACTTYGACGGTTA	2C	4860-4878	609	
NSP3R	ACCATCCCCTCRAAGAAYTC	3A	5449-5469		
NSP4F	CGRAGGTTYCACTTTGAC	3A	5098-5116	507	
NSP4R	CATRATCACTATGTTTGCCA	3A	5585-5605		
NSP5F	GAATTCTTTGAGGGGATGGT	3A	5449-5469	576	**Abdul-Hamid, Firat-Sarac *et al.*, 2011**
NSP5R	CACTTTCAAAGCGACAGG	3C	6007-6025		
NSP6F	CRAGCTGAAGGACCCTAC	3B	5831-5849	564	
NSP6R	GGGGGTKCCYTTCTTCAT	3C	6377-6395		
NSP7F	GGACAGGACATGCTCTCAG	3C	6283-6302	656	
NSP7R	GGACAGGACATGCTCTCAG	3D	6922-6939		
NSP8F	ATGCGCAAAACCAAGCT	3D	6736-6753	588	
NSP8R	AATTTGCGGTCCGTTGT	3D	7307-7324		
NSP9F	RACCTTCCTGAAGGACGAR	3D	7170-7189	508	
NSP9R	GTCCAGCTCRACTCCCTC	3D	7660-7678		
NSP10F	AACGTGTGGGATGTGGA	3D	7393-7410	832	
T21G	TTTTTTTTTTTTTTTTTTTTG	3'UTR	8205-8225		

samples, a positive control was used to optimize reactions and remove the variable of bad DNA quality. A no-DNA-template negative control was also used to guarantee that there was no contamination during PCR operation. An initial denaturation at 940C for 5 minutes is followed by 35 cycles of denaturation at 940C for 1 minute, annealing at 550C for 1 minute, extension at 720C for 1 minute 30 seconds, and a final extension at 720C for 7 minutes in PCR for cDNA amplification. To get the necessary amplicon (832 bp), 4 Rapid Amplification of cDNA Ends (RACE) primers were optimized against NSP 10F primer for the amplification of the 3' Untranslated Region (UTR). This study contained the best optimized primer for the desired amplicon. By combining 5 µl of the reaction mix with 1 µl of the 6x orange loading dye solution and resolving the sample by agarose gel electrophoresis alongside a DNA size marker in accordance with the sub-section **2.6** methodology, the PCR result was evaluated.

2.10.6 PCR Product Purification

Decryption of the PCR product concentration and purification of the PCR product using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) following centrifugation-based methodology.

2.10.7 Complete genome Amplification and Sequencing

PCR amplification method using 17 sets of primer pairs **(Table-2.9)** spanning the entire genome of the isolated virus was employed to amplify 17 overlapping fragments of the virus genome. Purified amplicons were sequenced from Macrogen, Inc. Seoul, South Korea.

2.10.8 Complete Genome Assembly and Annotation

Using SeqMan version 7.0.0 (Lasergene, DNASTAR, USA), overlapping PCR amplicons of the isolate's whole genome (BAN/MY/My-466/2021) were combined into a comprehensive consensus sequence. The assembly project's parameters were all left at their default settings. Subsequent BLAST searches corrected degenerate traces that were depicted in the consensus (Altschul *et al*., 1990). The National Center for Biotechnology Information (NCBI) BLAST search was used to identify the serotype, and the genome was then annotated by comparing it to the NCBI Ref Seq (Reference sequence for Foot-and-Mouth Disease Virus, accession no. NC_004004.1). The entire genome of BAN/MY/My-466/2021 was annotated using pairwise and multiple alignments of the Ref Seq and the genome in ClustalW (Thompson *et al.,* 1994) of MEGA11 (Tamura *et al.,* 2021).

2.10.9 Phylogeny Analysis

For phylogenetic analysis of the complete genome of BAN/MY/My-466/2021, a total of 20 complete genomes of FMDV reference sequences were retrieved from the GenBank database of

NCBI. A total of 85 sequences were taken into consideration for VP1 based phylogeny for determining the subtype of the isolated virus (BAN/MY/My-466/2021). Multiple sequence alignment was performed in ClustalW program of MEGA 11 software (Tamura *et al*., 2021) and On the basis of the Kimura-2 parameter model (Kimura, 1980), a tree was created using the Neighbor-Joining approach (Saitou & Nei, 1987). To mimic variations in evolutionary rates among sites, a discrete Gamma distribution with a value of 1 was utilized, and a bootstrap value of 1000 was used to assess the branching point's credibility. As not all of the sequences were perfectly matched across the whole range, alignment gaps, missing data, and ambiguous bases of slightly fewer than 5% were permitted at any place.

2.10.10 Comparative Sequence Analysis

Nucleotide and amino acid sequence identity of BAN/MY/My-466/2021 against other reference strain or vaccine strains was calculated using a global alignment tool based on the Needleman-Wunsch algorithm (Needleman & Wunsch, 1970) in NCBI. Genetic distance between group was calculated in MEGA11 (Tamura *et al*., 2021) using Kimura-2 parameter method (Kimura, 1980).

2.10.11   Protein Variability Analyis

Protein variability of capsid proteins based on Wu-Kabat method (Kabat *et al.*, 1977) was calculated in Protein Variability Server (Garcia-Boronat *et al*., 2008) using FASTA protein alignment as input where BAN/MY/My-466/2021 capsid proteins were selected as reference.

2.11 Research on structural genomics
2.11.1 Anticipation of the Secondary Structure of UTR

Mfold Web Server was used to computationally predict the secondary structure of the S-fragment, pseudoknots, and Internal Ribosomal Entry Site (IRES) of the 5' UTR of the local isolates and NCBI reference sequence (RefSeq). The Vienna RNA Package, University of Vienna, used the RNAfold web server to estimate the secondary structure of the 3' UTR.

2.11.2 Prediction of the 3-D Structure of capsid proteins

Homology modelling of capsid proteins comprising the antigenic region (VP1, VP2, VP3, VP4) of the isolate BAN/MY/My-466/2021 and VP1 of vaccine strains was performed using the SWISS-MODEL server (Biasini *et al*., 2013; Waterhouse *et al.,* 2018). Using the Ramachandran plot (Ramachandran *et al*., 1963) analysis in Molprobityv4.4 (Chen *et al.,* 2010), the quality of three-dimensional structures was verified. The 3-D models were visualized using PyMOL (Schrödinger, LLC, 2015) software.

2.12 Sequence submission to NCBI GenBank

Through the online submission tool BankIt, the VP1 sequences of the FMD positive tissue samples (Appendix V) and the whole genome sequence of the local FMDV isolate (Appendix VIII) were uploaded to the NCBI GenBank database. The BAN/MY/My-466/2021 or My-466 FMDV full genome has accession number OP957418.   .

3   RESULTS

The most contagious disease that affects animals with cloven hooves, foot-and-mouth disease (FMD), has the power to completely devastate already insecure animal industries. Bangladesh must develop a risk-based management strategy based on in-depth epidemiological research by the years 2012 to 2021 in accordance with the Food and Agriculture Organization's (FAO) Progressive Management Pathway for Foot and Mouth Disease (PCP-FMD). This study focused on the epidemiological assessment of ongoing outbreaks and determining the role of significant risk variables behind the FMD outbreak pattern using the sample gathered between 2012 and 2021. It was conducted as a part of the control program. It is significant to highlight that the COVID-19 pandemic had an impact on the epidemiological research for this study. In-depth genome amplification, thorough molecular characterization, an analysis of the evolutionary divergence between FMDV isolates and vaccine strains, the emergence of novel FMDV strains over a long period of time (2012–2021), an analysis of the VP1–amino acid substitution of FMDV isolates, and an analysis of the recently emerged PanAsia-2 sublineage in Bangladesh were also covered in this study.

The following sections can be used to highlight the study's findings:

- A Brief History of the Sample Collection

- Collection of Epidemiological data with the help of questionnaires and interviews.

- Epidemiological investigation of FMD outbreaks during 2012–202111 in Bangladesh.

- Risk factors and serotype-based prevalence analyses of FMD cases in Bangladesh from 2012 to 2021.

- Construction of the phylogenetic tree and identification of circulating FMDV subtypes during 2012–2021.

- Phylogenetic Analysis of FMDV Serotypes

- Evolutionary divergence analysis of FMDV isolates with the available vaccine strain.

- (Emergence of novel strains of FMDV over a period of many years, 2012–2021).

- Analysis of VP1 Amino Acid substitution during the period 2012–20211.

- Occurrence of the first cases of FMD outbreak by the PanAsia-2 lineage with the emergence of a novel, distinct sub-lineage in Bangladesh

- Comparative VP1-based mutational and structural analyses of representative FMDV PanAsia-2 isolate BAN-21 with reference strains and vaccine strains.

- Isolation and genome amplification of the representative novel BAN-21 isolate.

3.1 Brief History of Sample collection

The epithelial tissue, in particular the linings of the gums, dental pads, tongue, and interdigital space (Figure 3.1), is the optimal tissue for the identification and evaluation of the FMD virus since the virus replicates in the epithelial lining. The infection was initially diagnosed based on the clinical picture, which included a high body temperature and blisters on the foot and mouth. For the purpose of characterizing and isolating FMDVs that were circulating in Bangladesh, as well as for research on the molecular epidemiology of FMD in Bangladesh and complete genome analysis with the aim of selecting the most suitable representative of the vaccine strain(s), samples (including the total number) were collected based on clinical symptoms. A total of 481 epithelial tissue samples **(Figure 3.2)** representing 3580 animal populations were gathered between 2012 and 2021 from 32 districts, including 71 outbreaks **(Figure 3.3)**, and 230 of these samples tested positive with FMDV in a VP1-based PCR assay, which corresponds to the 1960 population. But from August 2019 to December 2021, I obtained 156 tongue or foot epithelial tissue samples from various districts. The epidemiology research comprised this entire animal population (3580).

Figure3.1: Clinical indications and manifestations of FMD outbreaks in cattle were suspected

3.2 Collection of Epidemiological data with the help of questionnaires and Interviews

Herdsmen are fully aware of FMD, also known locally as Khura rog, and are familiar with its clinical symptoms, seasonality, duration, and mechanism of transmission, according to information acquired through a questionnaire. The data set contained all the information needed for epidemiological study, including gender, age, breed, farming system, animal movement, collection date and location, etc. A predetermined questionnaire was used to collect this information.
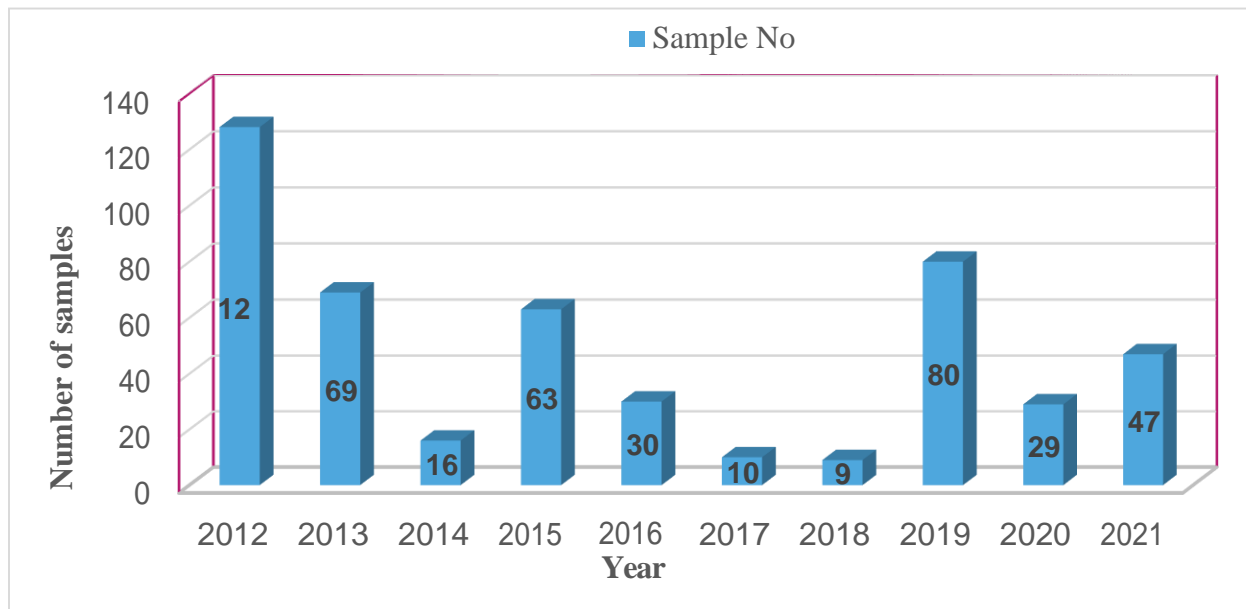
Figure3.2: Year wise sample collection in 2012-2021from different district
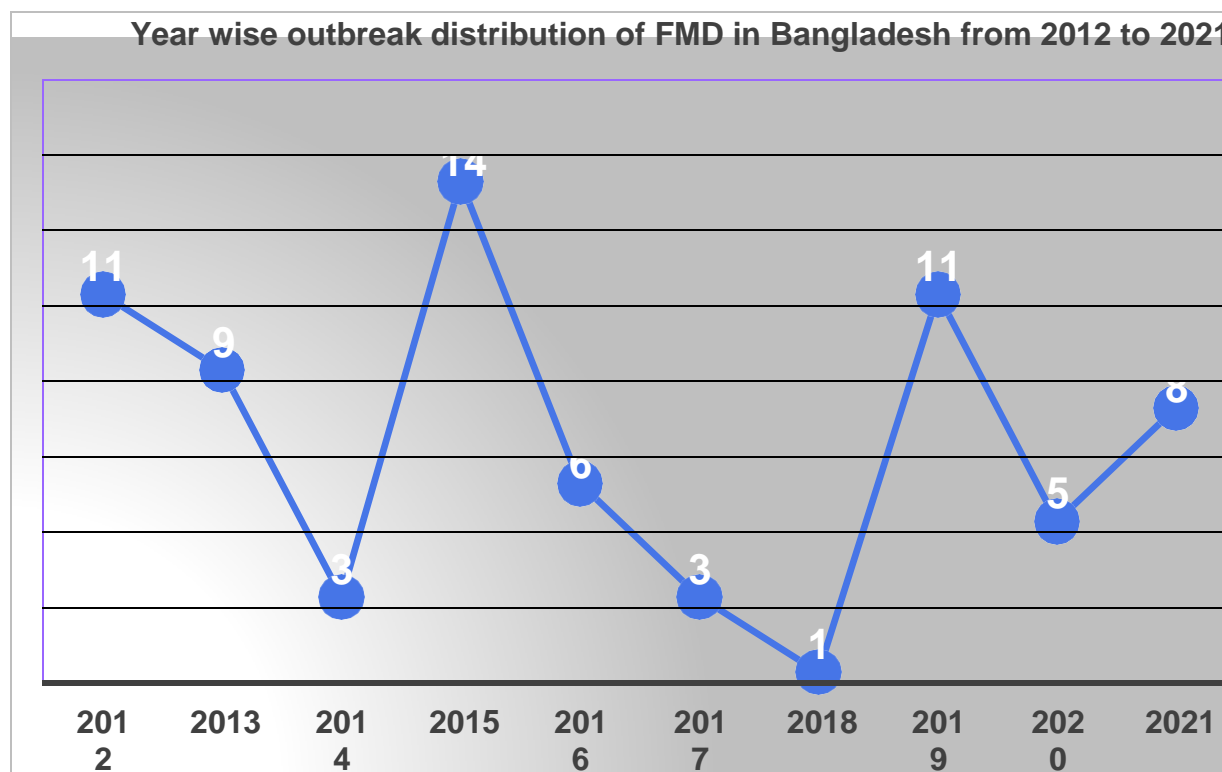
Figure3.3: Distribution of FMD outbreaks from 2012 to 2021

Herdsmen to treat mouth ulcers and minimize the consequences of the condition, combine fried borax powder with honey or molasses and massage the mixture onto the tongues of affected animals. Herdsmen apply turmeric powder, glycerin, and antibiotics to the damaged regions after washing them with potassium permanganate (ppm 0.001%) solution or 4% sodium bicarbonate solution to prevent secondary bacterial infection in ravaged animals.

3.3 Epidemiological investigation of FMD outbreaks during 2012–2021 in Bangladesh

In total, 481 epithelial tissue samples from 3580 animal populations were obtained between 2012 and 2021 from 32 districts, including 71 outbreaks **(Figure 3.3).** Of these, 222 samples tested positive for FMDV in a VP1-based PCR assay, which represents the 1960 population. The epidemiology investigations included several animal populations. In Dhaka and Gazipur, all three serotypes have been identified. Each of the 32 districts contained Serotype O. In the districts of Dhaka, Gazipur, Chittagong, and Chandpur, serotype A was identified. In the districts of Jessore, Dhaka, and Gazipur, serotype Asia 1 was discovered. The locations of the various outbreaks and FMDV serotypes in existence are shown by different colour **(Figure 3.6)**. FMD had morbidity, mortality, and case fatality rates of 54.7% (1960/3580), 10.4%(372/1960), and 19% (372/1960), respectively, in the entire animal population of 3580 **(Figure:3.5).**
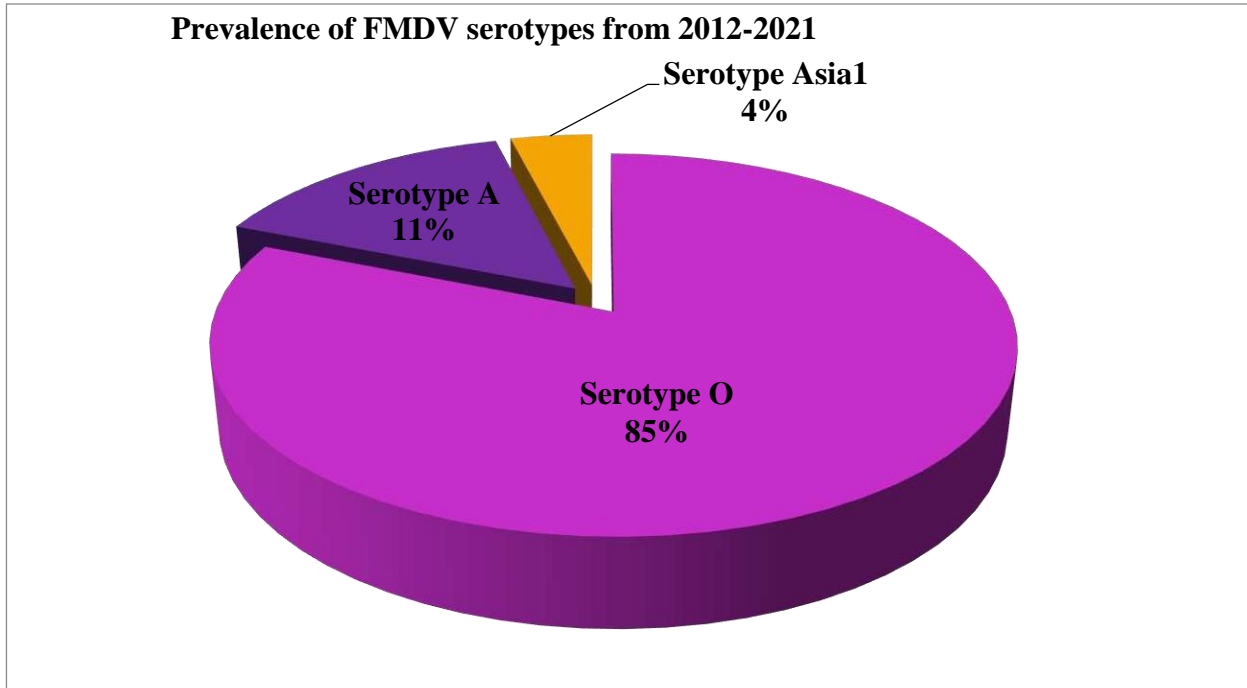
Figure3.4: Prevalence of FMDV serotypes from 2012-2021.
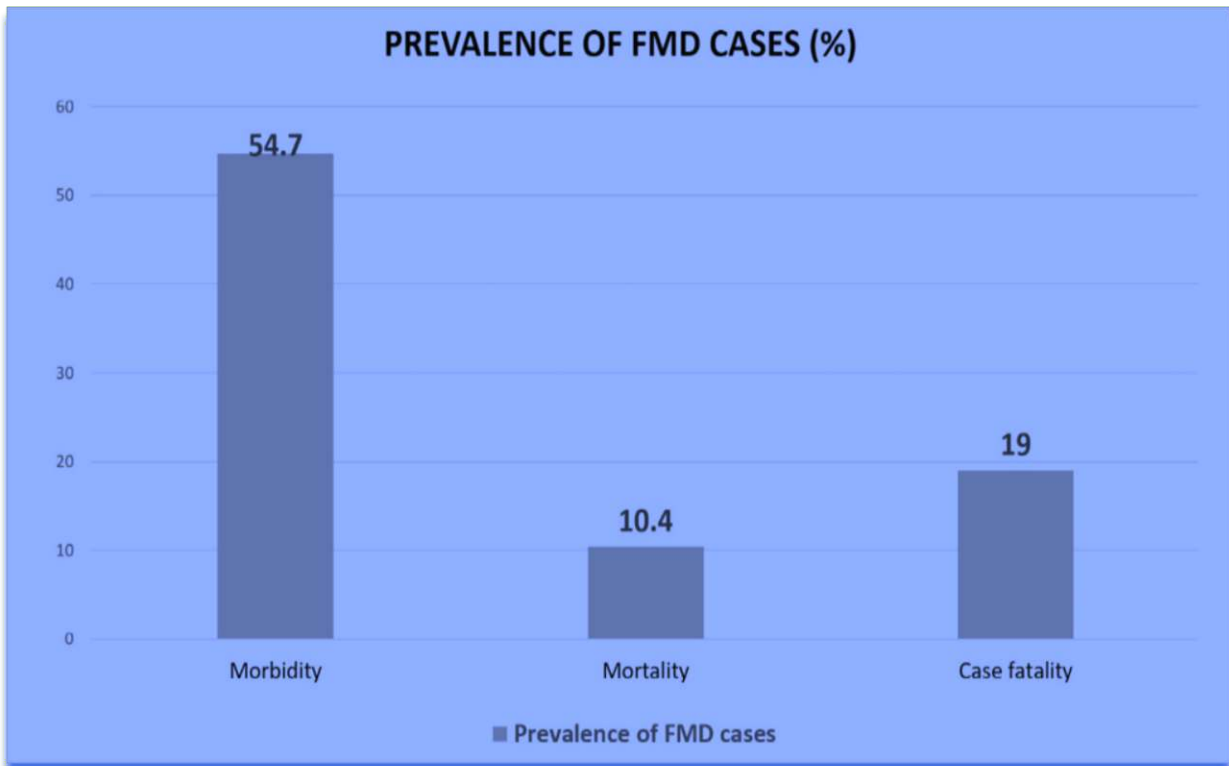
Figure3.5: Prevalence of FMD cases (%)
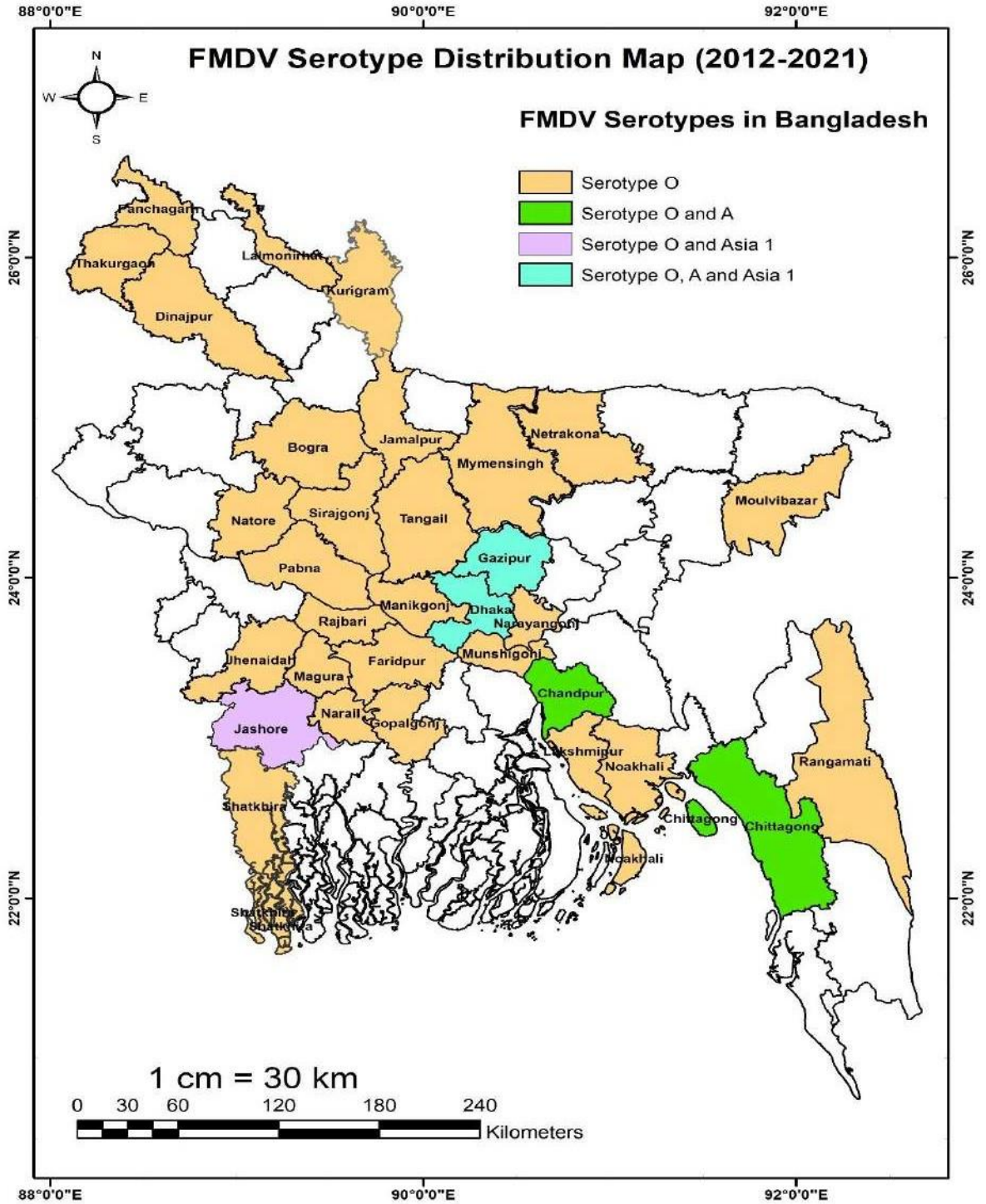
Figure3.6: Geographical distribution of circulating FMDV serotypes in Bangladesh during 2012-2021. Where the different colours indicate the different serotypes outbreaking areas.

3.4 Risk factor analysis

Seasonal variations in FMD epidemic frequency were observed. It was shown that 51% (36/71) of the outbreaks took place during the rainy season (July to October), 28% (20/71) of the outbreaks occurred during the winter (November to February), and 21% (15/71) of the outbreaks took place during the summer (March to June) (**Figure 3.7**). At the 0.05 significance level, when the p-value was 0.00, it was found that there was a substantial seasonal influence on the
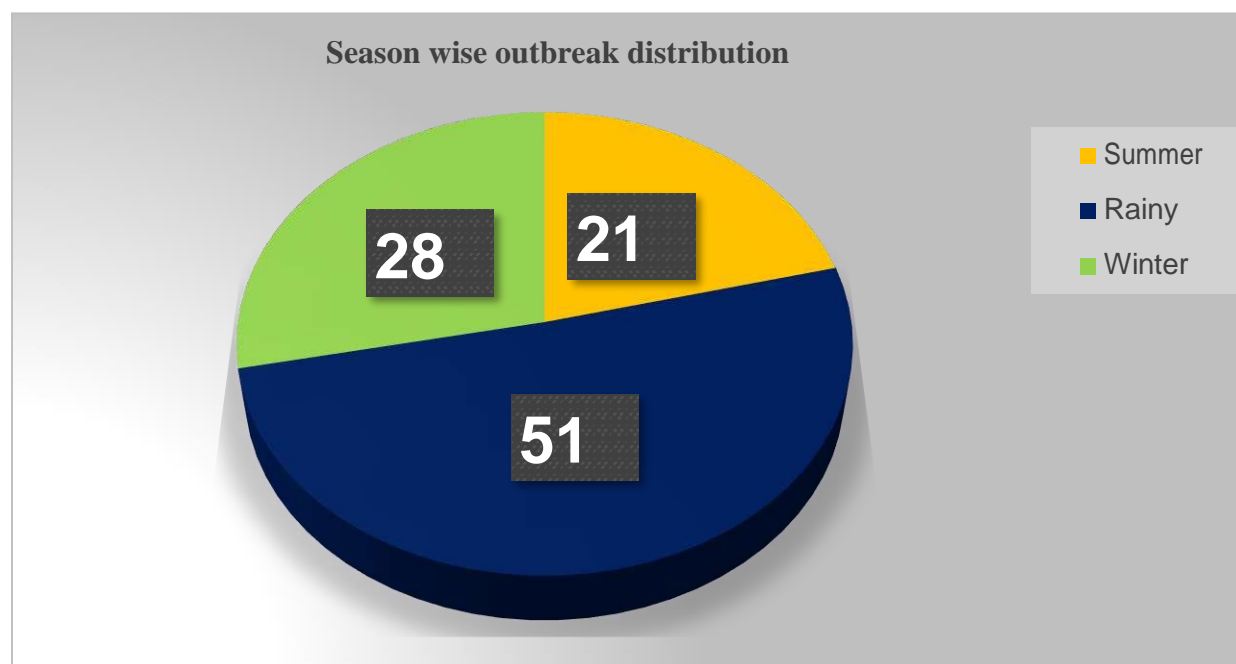
frequency of FMD outbreaks.

Figure3.7: Percentage of FMD outbreaks in different seasons.

In this study, a number of risk factors, including age, gender, breed, farming system, and vaccination status, were taken into account to determine their impact on the FMD morbidity rate. Morbidity, mortality, and case fatality rates were 51.9% (709), 8.4% (114), and 16.1%, respectively, in young cattle (>1 year before breeding) out of 1365, whereas they were 64.2% (1109/1728), 11.1% (192/1728), and 17.3% (192/1109), respectively, in the adult population. The morbidity rate was 29.2% (142 out of 487), the mortality rate was 13.6% (66 out of 66), and the case fatality rate was 46.5% in the other age group (calves up to 1 year) (Figure 3.8). Adult populations had greater rates of morbidity whereas calf groups had higher rates of mortality and fatality. The chi-square test's P value was 0.00, indicating that there is a substantial correlation between age and the prevalence of FMD. In the chi-square test, the FMD morbidity rate in male

animals was higher than that in female animals, with a difference of 59.4% (970/1632) compared to 50.8% (990/1948) (Appendix III). Cross-bred cattle were 53% (693/1307) susceptible to illness, while local breed cattle had a 55.7% (1267/2273) morbidity rate. According to the chi-square test, the breed of animal did not significantly correlate with FMD instances (P-value: 0.062> 0.05). Cattle raised in an intense farming system had a susceptibility rate of 50.9% (288/566), which was lower than the susceptibility rate of animals raised in a semi-intensive farming system, which was 55.5% (1672/3014). The farming system and FMD were significantly associated, as indicated by the P-value of 0.025>0.05. A total of 59.2% (1503/2539) of the non-vaccinated animals and 43.9% (457/1041) of the vaccinated cattle had FMD (Figure 3.9). The chi-square test, which showed that vaccination. reduced FMD cases by 1.3 times (P = 0.000.05) supported this claim (**Appendix III).**
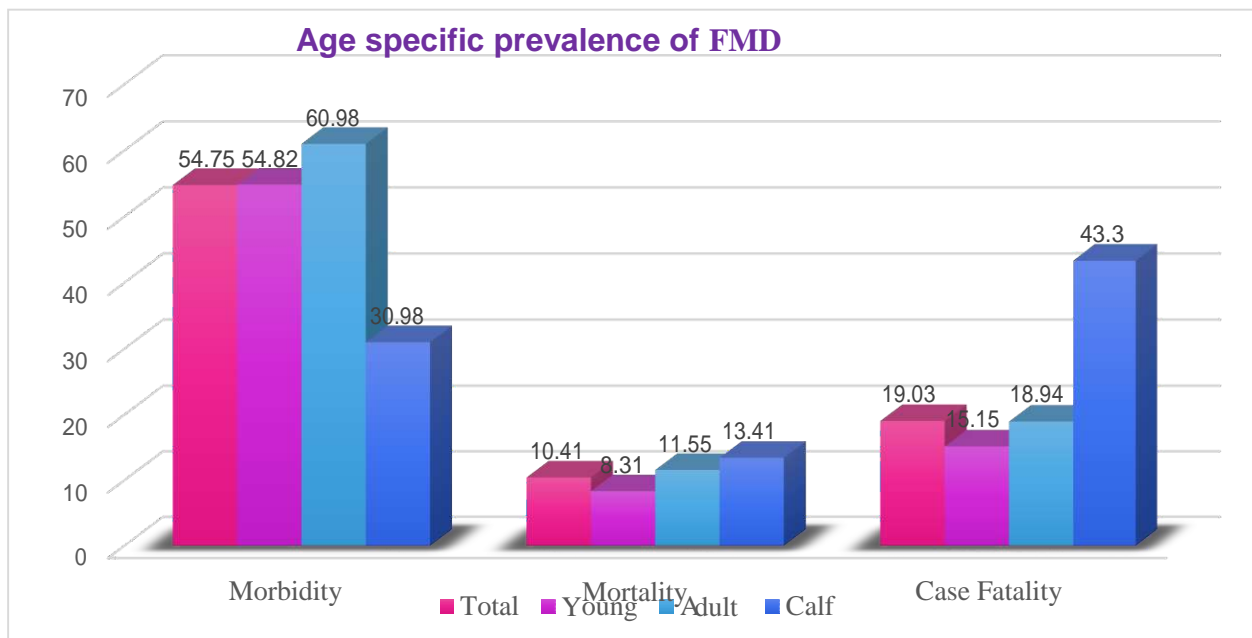
Figure 3.8: Age-specific prevalence of FMD cases.
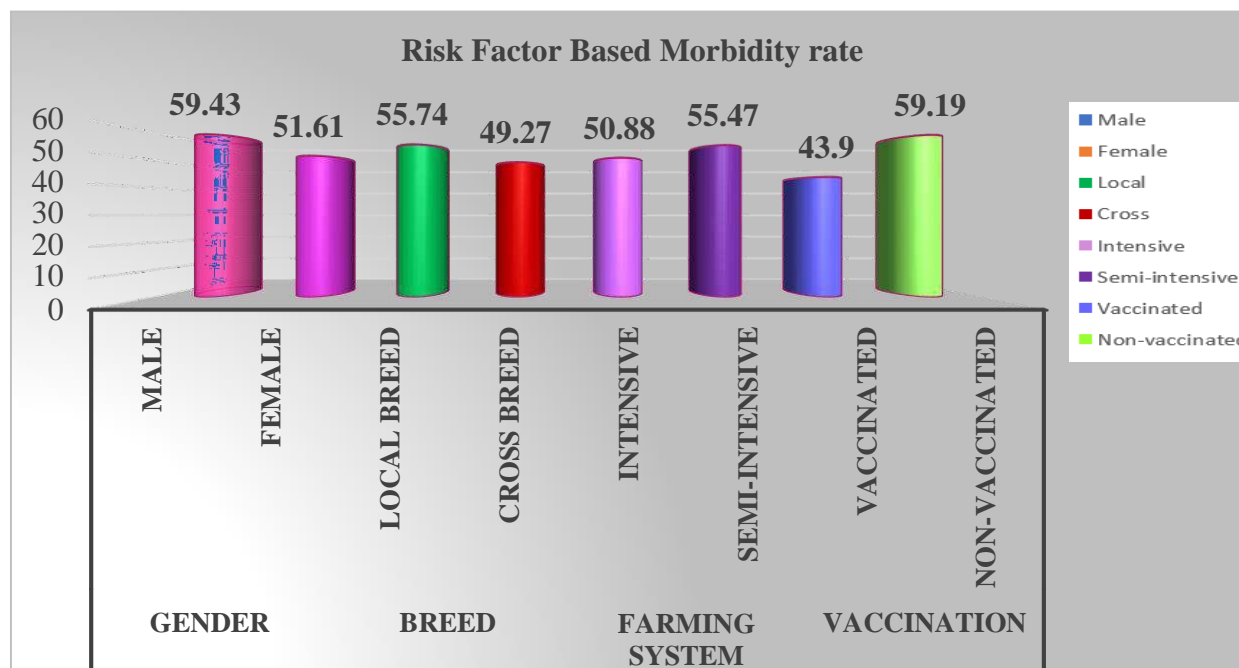
Figure 3.9: Risk factor (gender, breed, farming system and vaccination) specific morbidity rates of FMD.

3.5   FMDV Testing in Clinically Suspected Animals

A total of 481 epithelial tissue samples representing 3580 animal populations were obtained between 2012 and 2021 from 32 districts, including 71 outbreaks **(Figure 3.2),** and 222 of these samples tested positive with FMDV in a VP1-based PCR assay, which corresponds to the 1960 population. The epidemiology investigations included several animal populations. FMD had morbidity, mortality, and case fatality rates of 54.7% (1960/3580), 10.4% (372/1960), and 19% (372/1960), respectively, in the entire animal population of 3580 **(Figure 3.5).** Reverse transcription was done on the tissue sample RNA that had been isolated. Utilizing three sets of universal primer pairs (VP1UF/NK61, 16F/NK61, and 16F/16R), the PCR products were used for a VP1-based PCR assay to identify the presence of the Foot and Mouth Disease viruses. A partial VP1 region of the FMDV genome is targeted by the primer pair 16F/16R in this instance, amplifying the region by about 426 bp; a partial VP1 region is also targeted by the primer pair 16F/NK61, amplifying the region by about 745 bp; and the entire FMDV VP1 region is targeted by the primer pair VP1UF/NK61, amplifying the region by about 1141 bp **(Figure 3.10).**
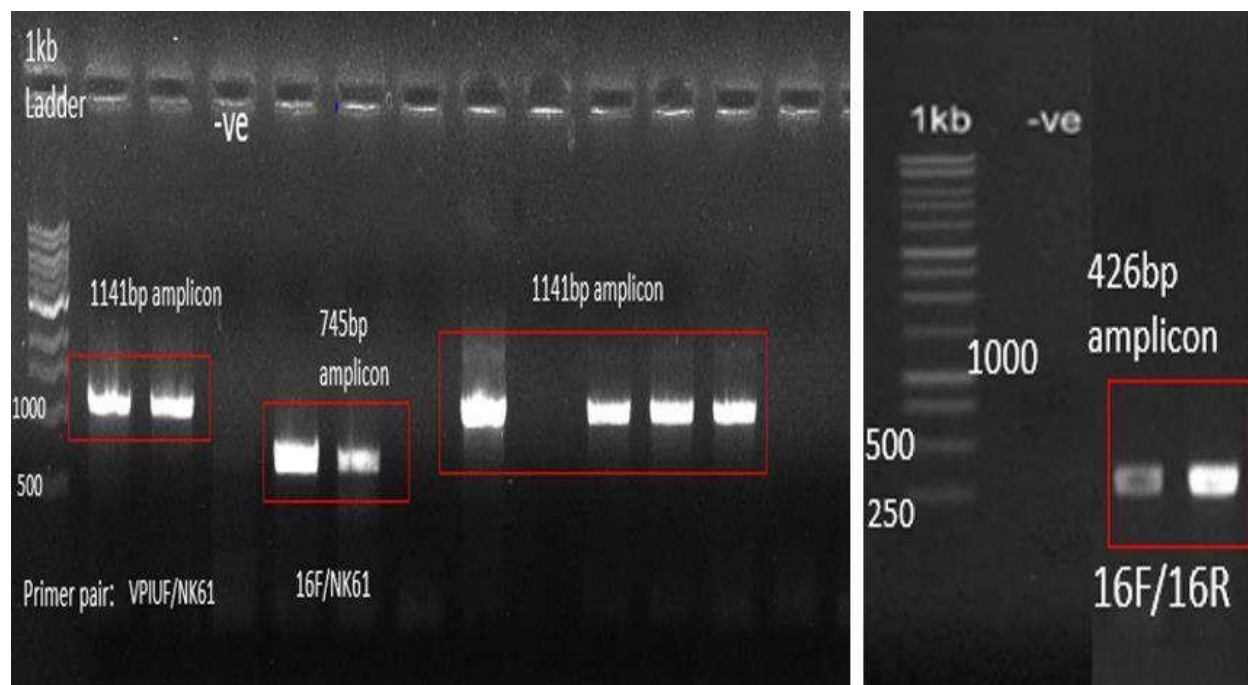
Figure 3.10: **VP1-specific PCR amplification products (Representative). Amplicon sizes are around 1141, 745, and 426 bp. Here two different markers were used: 1kb (Biolab, USA) and 1kb (Promega, USA) marker.**

3.6 Distribution of FMD serotypes over ten years (2012–2021)

Serotype O, the most common serotype, was to blame for 85% (60 out of 71) of the outbreaks, while serotypes A and Asia1 were responsible for 11% (8 out of 71) and 4% (4 out of 71) of the outbreaks, respectively. According to FMDV VP1 coding sequences found in Bangladesh, serotypes O, A, and Asia1 were active in 2021–2022. From 2012 to 2021, serotype O was discovered every year (Hossain *et al*., 2022; Siddique *et al.,* 2018). In the previous ten years, serotype A was also present, but it was not in use in 2015, 2018, or 2021 (Nandi *et al.,* 2015a; Ullah *et al*., 2014). In 2012–2013 and in 2018 (M. R. Ali *et al.,* 2020b; Hossen *et al*., 2020; Ullah *et al*., 2015a), Asia1 was recorded. No cases of serotype C were found in Bangladesh at that time **(Figure-3.11).**
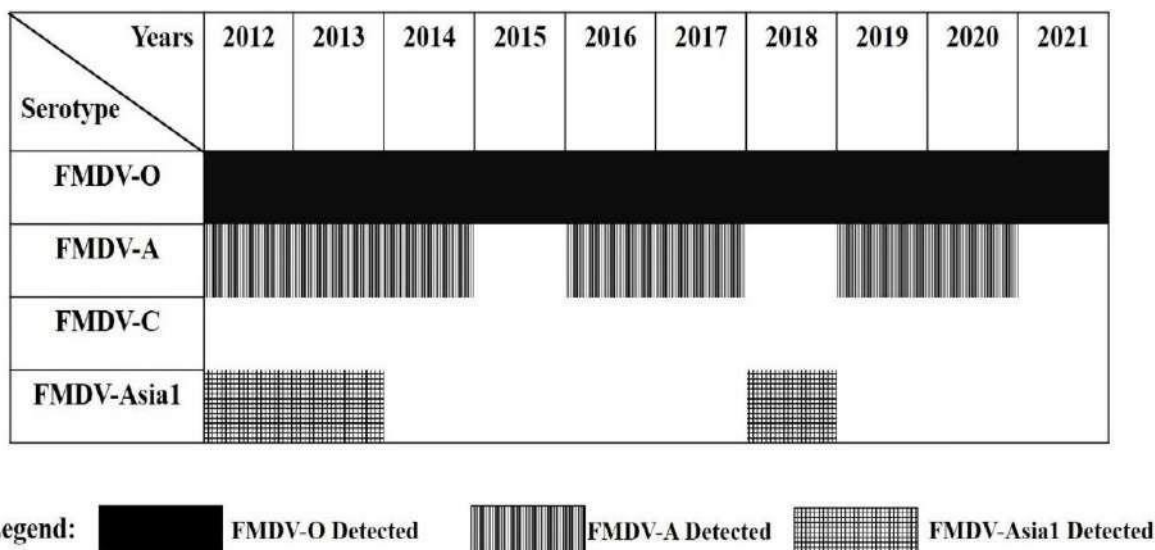
Figure3.11: Occurrence of FMDV serotypes in Bangladesh (2012-2021).

3.7 Study of Evolutionary History
3.7.1 Homology Search and Serotype Identification

For thorough genetic characterisation of the isolates, FMDV samples collected between 2012 and 2021 underwent VP1 nucleotide-based phylogenetic analysis. VP1 reference sequences from regional isolates and bordering nations were obtained from the NCBI gene library **(Appendix V)** in order to determine phylogenetic relationships. The VP1 coding area was used to create the phylogenetic trees in MEGA11 using the neighbor-joining method. The evolutionary distances between sites, which are expressed in base substitutions per site, were calculated using the Kimura 2-parameter technique (Kimura M., 1980). The gamma distribution (shape parameter = 1) was used to model the rate variance between sites. For ease of data presentation from sequences reported from our laboratory as well as from other researchers in Bangladesh, only representative FMDV VP1 sequences were taken into account in the phylogenetic investigation. In this section, the FMDV samples of 2012–21 were characterized based on their serotypes. The phylogenetic tree below **(Figure 3.12)** shows the phylogenetic inference on the serotype of FMDV samples based on 230 VP1 sample sequences.
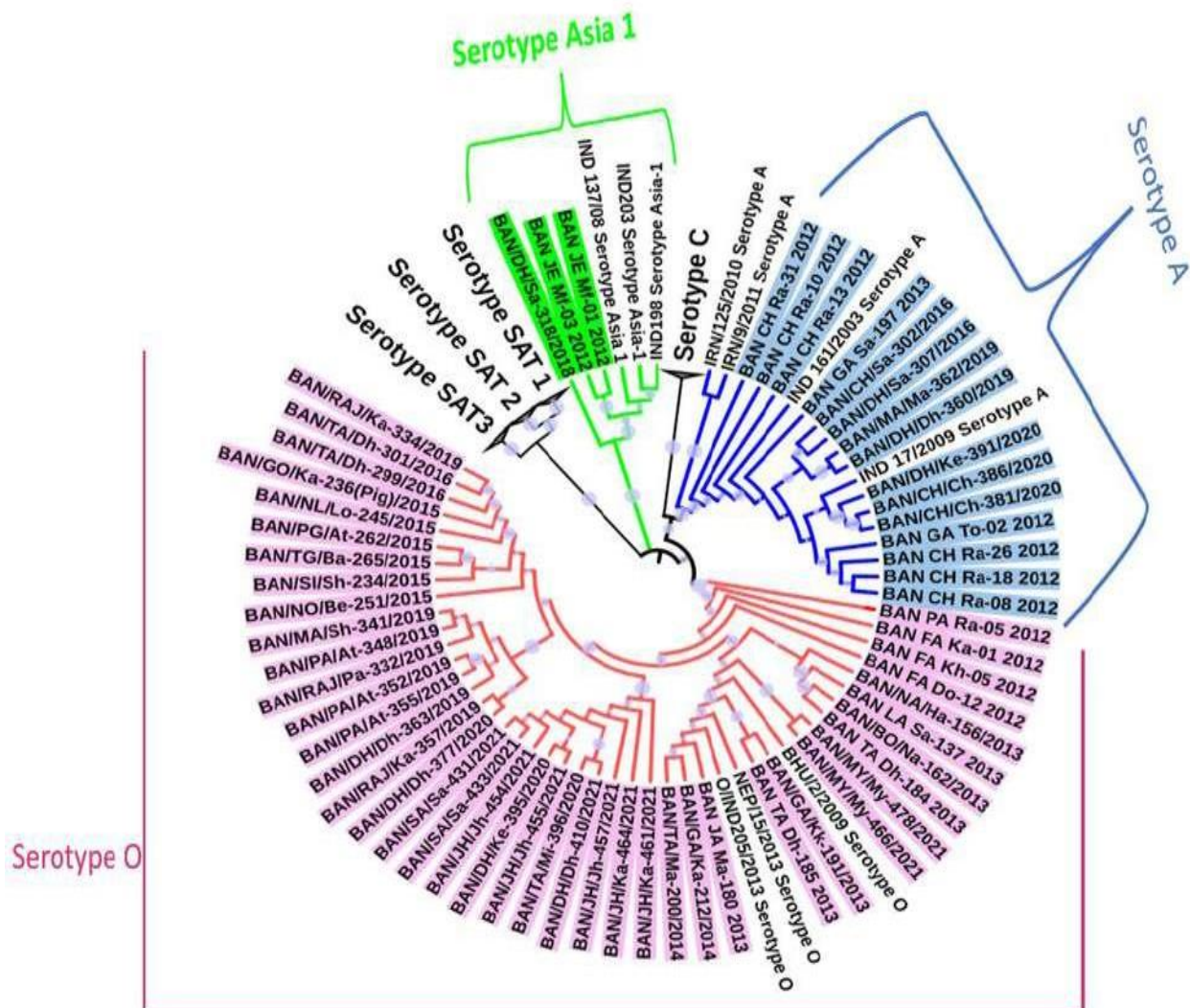
Figure 3.12: Phylogenetic reconstruction based on the Neighbour-joining method showing circulating serotypes of FMDV in Bangladesh during 2012-2021.

3.7.1.1   FMDV Serotype O

Two distinct lineages, the Ind-2001 and SA-2018 lineages under serotype O, were shown by phylogenetic analysis to circulate in Bangladesh between 2012 and 2021 (Figure 3.13). Only the Ind-2001BD1 sublineage was discovered in the past five years out of the Ind-2001d, Ind-2001BD1, and Ind-2001BD2 sublineages that were detected under the Ind-2001 lineage. The World Reference Laboratory for Foot-and-Mouth Disease, or WRLFMD, designated Ind-2001BD1 as Ind-2001e (Bachanek-Bankowska *et al.,* 2018; WRLFMD, 2022).

In 2021, the SA-2018 lineage was first reported. These isolates (OP320455.1-OP320458.1), also known as MYMBD21, displayed a unique clade in the phylogenetic tree with likely emergence from Indian SA-2018 isolates (Figure 3.13). The phylogenetic tree showed that only three serotypes (O, A, and Asia1) were flowing in our country between the years of 2012 and 2021, when FMDV samples were sequenced in this lab. The most common serotype in our nation out of these three is serotype O. Other serotypes were not discovered. The isolates reported in Bangladesh are indicated with dot symbols and unnamed isolates with square symbols. Some branches are compressed for better visualization.
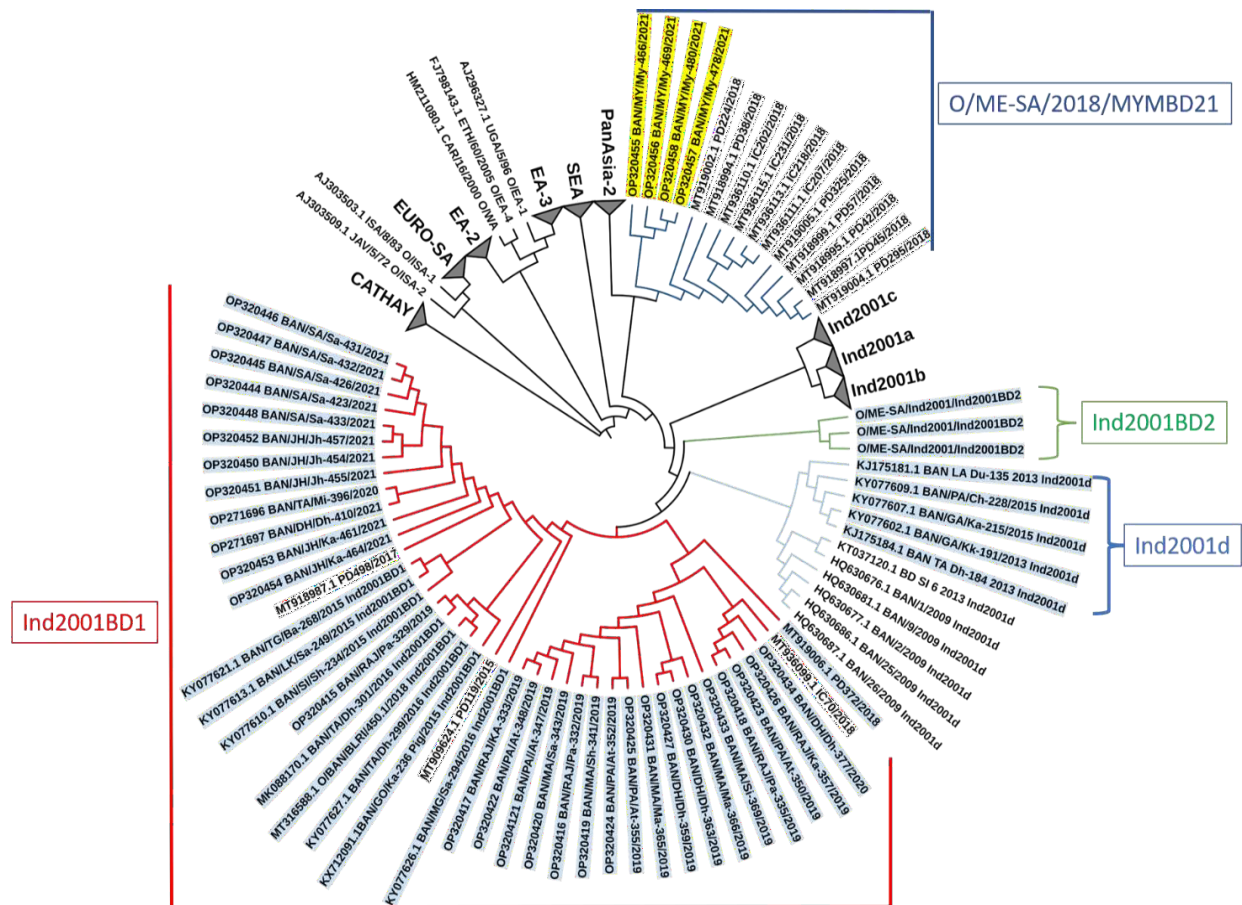
Figure 3.13: **Phylogenetic reconstruction based on Neighbour-Joining method and Kimura-2 parameter model in MEGA11 showing subtypes of circulating serotype O in Bangladesh during 2012-2021.**

3.7.1.2 FMDV Serotype A

Throughout the period of 2012 to 2020, all serotype A isolates grouped together under the ASIA topotype. Only the G-VII lineage, which falls under this topotype, was circulating. In **Figure 3.14.** Under this lineage, no known established sub-lineage was documented.
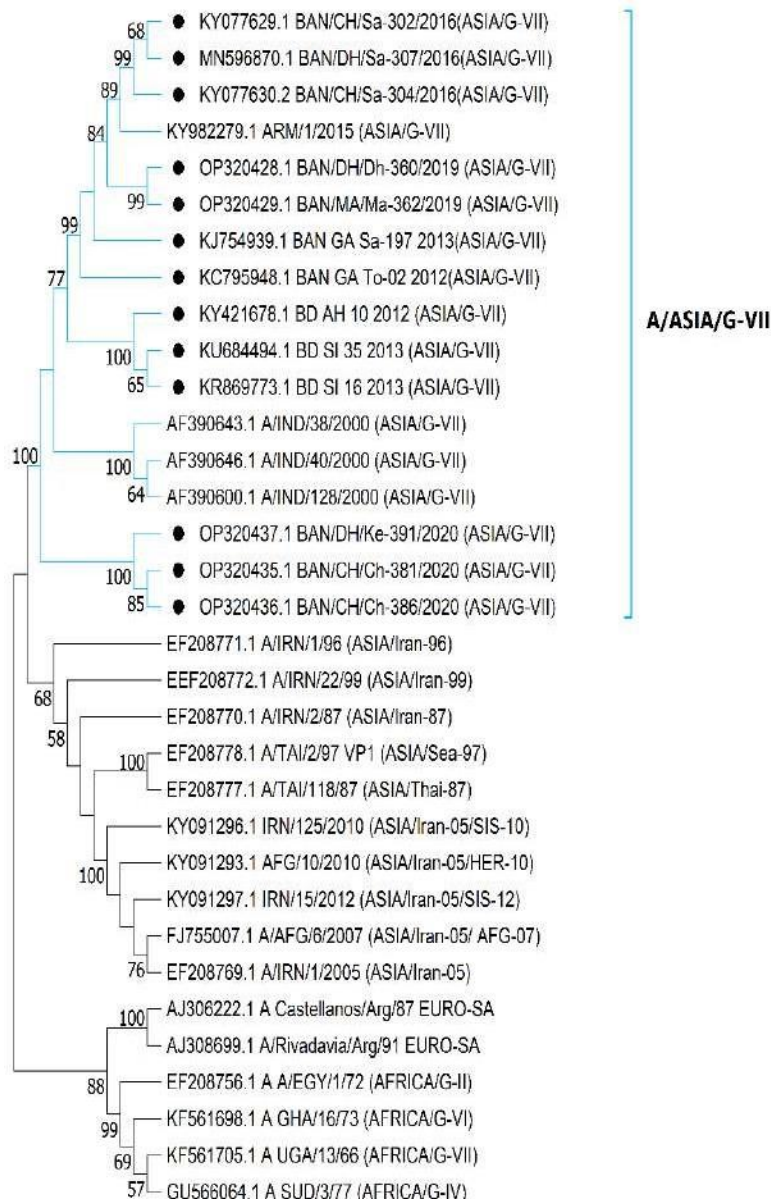
Figure3.14: Phylogenetic reconstruction based on Neighbour-Joining method and Kimura-2 parameter model in MEGA11 showing topotype and lineage of FMDV serotype A circulating during 2012-21. The sequences reported in Bangladesh are marked with black dots

3.7.1.3   FMDV Serotype Asia 1

A phylogenetic tree was created using the whole VP1 expressing regions of the field isolates generated in this study using previously discovered sequences of serotype Asia-1 viruses from diverse lineages retrieved from the NCBI GenBank database (Figure 3.15). According to
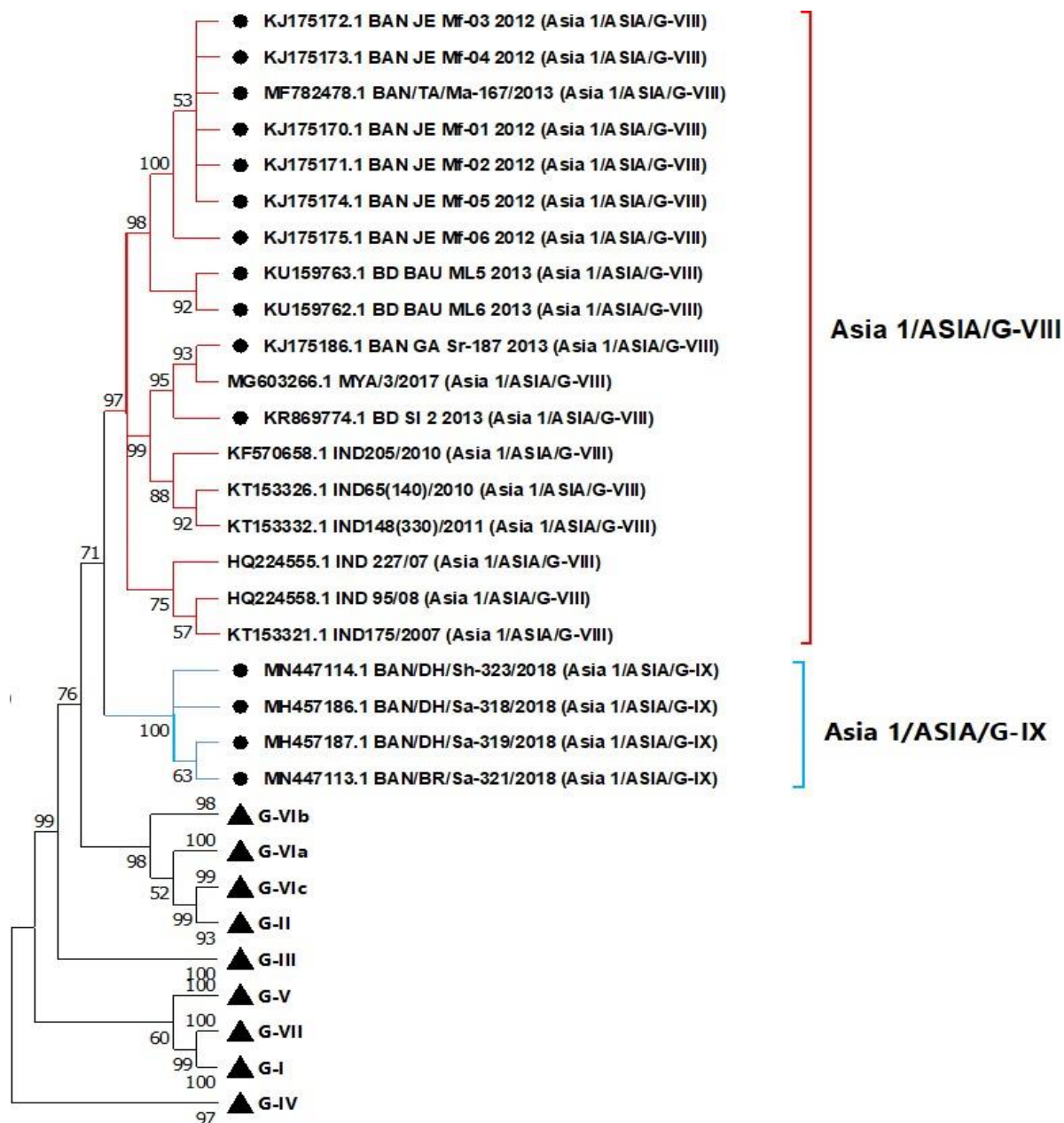
Figure 3.15: Phylogenetic reconstruction based on Neighbour-Joining method and Kimura-2 parameter model in MEGA11 showing lineages of serotype Asia 1 circulating in Bangladesh during 2012-2021.

phylogenetic reconstruction, the G-VIII lineage circulated between 2012 and 2013, and Asia1 remerged in Bangladesh in 2018 as the unique lineage G-IX (BD-18) (M. R. Ali *et al.,* 2020) Figure 3.15). Since 2018, no Asia1 serotype has been discovered. The sequences reported in Bangladesh are marked with black dots. Some branches are compressed for better visualization. During 2012-21, few novel strains were introduced in FMDV circulation of Bangladesh which is illustrated in **Figure 3.15**. According to the VP1 phylogeny, the genetic branch C, which had been significantly circulating in India from 1993 to 2001 before reemerging in 2005, was responsible for this., was home to the sequences of the regionally dominant serotype Asia-1. All of Bangladesh's Asia-1 outbreaks since 2012 have been brought on by Lineage C. Throughout the time, outbreaks of the Asia-1 serotype were seen in Bangladesh's Jessore and Gazipur districts. The VP1 coding region of the FMDV Asia-1 isolates shared 94.42–100% nucleotide similarity after being sequenced for molecular epidemiological investigation. These viruses are related to those that have been going across India since 2008.

3.8 Major events over the period of 2012–2021

In 2012, Ind2001BD1 and, in 2013, the Ind2001BD2 sublineage emerged. A novel lineage of Asia1 emerged in 2018. In 2021, a novel sublineage, MYMBD21, of the O/ME-SA/2018 lineage has been identified. In Bangladesh, within ten years, several novel strains emerged, which is considered an epidemiologically significant event **(Figure 3.16).**
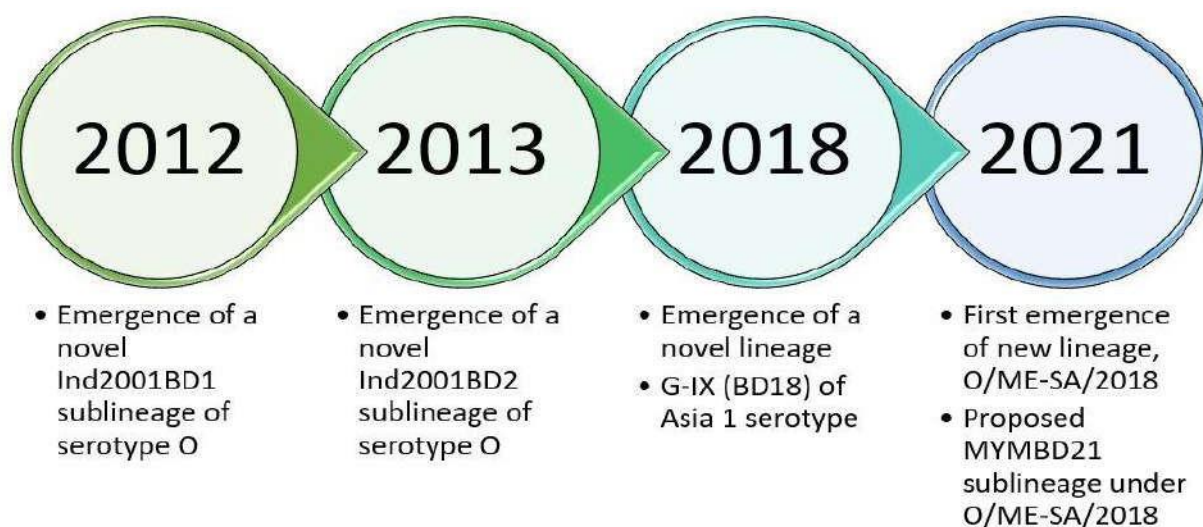
Figure 3.16: Emergence of novel strains of FMDV over the period of ten years (2012-21).

3.9 Mutational Trends Analysis of VP1 Amino Acids during 2012–21

The amino acid changes that happened in FMDV VP1's highly variable antigenic domains (B-C loop, G-H loop, and C-terminal) between 2012 and 2021 (**Table 3.1**). **Appendix VI** is a list of all the mutations discovered in VP1 between 2012 and 2021. solely a few sequences of the serotype O sublineages Ind-2001BD2 (3) and SA-2018/MYMBD21 (4) that circulated solely in a specific year were available. Therefore, the mutational analysis was not conducted on these lineages. Only for the serotype O sequences were mutations detected at the 52nd residue of the B-C loop, positions 135, 138, 139, 140, 155, 156, and 158 of the G-H loop, and in the C-terminal 197, 200, 201, 204, 207, and 212 locations. For serotype A, specific alterations were found at locations 134, 143, 148, 190, 194, 196, and 209. N47S, T50V, M146L, and M211L were exclusive to Asia 1. All three serotypes, O, A, and Asia1, shared mutations at residues 43 and 48. The mutational pattern for serotypes O, A, and Asia1 seen between 2012 and 2021 is shown in **Table 3.2.** In this case, colored boxes (red for serotype O, blue for serotype A, and gray for serotype Asia1) denoted the existence of a certain mutation in a given year, while a white box denoted the lack of the same mutation.

Table 3.1: Amino acid substitutions at major antigenic regions of VP1 of FMDV serotypes during 2012-21. Amino acid exchanges in different serotypes are separated with commas. Multiple exchanges for a single position are separated with forward slashes.

Region	FMDV Serotype	Amino Acid Substitutions	Unique Substitutions
B-C Loop (43-59) (Kitson et al., 1990; Siddique et al., 2018)	O, A, Asia1	T43A/I, G43V, T43N	
	A, Asia1	N44D, A44T/E	A44E for Asia1 (G-IX lineage)
	O, A	[Q45K; K45Q], V45A/T/L	K45Q for Ind2001d sub-lineage
	O, A	N46D, S46G/N	
	Asia1	N47S	For G-IX lineage
	O, A, Asia1	I48V, T48I, I48T	I48T for Asia1 (G-IX lineage)
	Asia1	T50V	For Asia1 (G-IX lineage)
	O	D52K	
	O	T60N	
G-H Loop (130-160) (Logan et al., 1993; Siddique et al., 2018)	A	N134S	
	O	K135R	
	O	E138G/A/K	
	O	S139G	For Ind2001d sub-lineage
	O	[N140G/A/D; A140T]	N140G/A/D for Ind2001d sub-lineage
	O, A	[P142T/A; T142A], R142H	P142T/A for Ind2001d sub-lineage
	A	V143T/A	
	Asia1	M146L	For Asia1 (G-IX lineage)
	A	G148E	
	O	A155V	
	O	A156T	
	O	P158T	For Ind2001d sub-lineage
C-Terminal (190-213) (Momtaz et al., 2014; Siddique et al., 2018)	A	M190L	
	A	E194K/D	
	A	S196L	
	O	D197E/G/S	For Ind2001BD1 sub-lineage
	O, A	E198Q/D, Q198R	E198Q/D for Ind2001d sub-lineage
	O	R200T	
	O	H201P/R	
	O, Asia1	K202Q, E202K	E202K for Asia1 (G-IX lineage)
	O	K204R/N	
	O	A207P	
	A	A209T	
	O, Asia1	K210N, V210M	
	Asia1	M211L	Asia1 (G-IX lineage)
	O	L212F	
	O, A	L213F, L213P	

Table3.2: Yea r wise distribution of amino acid substitutions in VP1 antigenic regions of serotype O (yellow box), serotype A (blue box) and serotype Asia1 (grey box).

Antigenic Region	Amino acid substitutions	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
B-C Loop	K41I (Ind-2001e)								O		
	I42L	A	A	A					A		
	G43V					A			A		
	T43I (Ind-2001e)					O		O	O		
	T43A (Ind-2001d)		O								
	T43N (G-IX)							Asia1			
	N44D									A	
	A44T (G-VIII)		Asia1								
	A44E (G-IX)							Asia1			
	K45Q		O		O						
	V45A		A								
	V45L									A	
	V45T	A	A	A					A		
	Q45K (Ind-2001e)		O		O	O		O	O	O	O
	N46D (Ind-2001e)		O		O	O		O	O	O	O
	S46G					A					
	S46N									A	
	N47S (G-IX)							Asia1			
	I48V (Ind-2001d)		O								
	T50V (G-IX)							Asia1			
	D52K (Ind-2001e)								O		
G-H Loop	N134S									A	
	K135R (Ind-2001e)									O	O
	K135R (Ind-2001d)				O						
	E138A (Ind-2001e)	O									
	E138K (Ind-					O			O		

Region	Mutation	1	2	3	4	5	6	7	8	9	10
	2001e)					🟧			🟧		
	E138G (Ind-2001d)				🟧						
	S139G (Ind-2001d)		🟧								
	A140T (Ind-2001e)								🟧		
	N140G (Ind-2001d)		🟧		🟧						
	N140A (Ind-2001d)		🟧								
	N140D (Ind-2001d)		🟧		🟧						
	T142A (Ind-2001e)								🟧		
	P142T (Ind-2001d)		🟧		🟧						
	R142H								🟦	🟦	
	V143T	🟦	🟦	🟦					🟦	🟦	
	V143A					🟦					
	G148E	🟦									
	M146L (G-IX)							⬜			
	A155V (Ind-2001d)		🟧								
	A156T (Ind-2001e)				🟧						
	A156T (Ind-2001d)		🟧								
	P158T (Ind-2001d)		🟧								
C-Terminal	**M190L**	🟦	🟦	🟦						🟦	
	E194D	🟦									
	E194K								🟦		
	S196L		🟦							🟦	
	D197E (Ind-2001e)		🟧		🟧	🟧		🟧	🟧	🟧	🟧
	D197G (Ind-2001e)					🟧					
	E198Q (Ind-2001d)		🟧		🟧						
	E198D (Ind-2001d)		🟧	🟧							
	Q198R								🟦		
	R200T (Ind-2001d)		🟧								

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
H201R (Ind-2001e)				orange							
H201P (Ind-2001d)		orange									
K202Q (Ind-2001d)		orange									
E202K (G-VIII)		gray									
K204R (Ind-2001e)										orange	
K204R (Ind-2001d)				orange							
K204N (Ind-2001d)		orange									
A207P (Ind-2001d)		orange									
A209T	blue										
V210M (G-VIII)		gray									
K210N (Ind-2001d)		orange									
M211L (G-IX)							gray				
L212F (Ind-2001d)		orange									
L213F (Ind-2001e)										orange	
L213P		blue									

Positions 43, 138, 140, 142, 197, 198, 201, and 204 were shown to have more than two amino acid alterations in serotype O isolates, with the 140th residue being the most variable. Three mutations remained in the sublineage Ind-2001e (or Ind-2001BD1) sequences under serotype O from 2013 to 2021. Q45K, N46D, and D197E are these amino acid swaps. It's possible that those mutations stabilized inside the Ind-2001e sublineage. From 2012 to 2018, the C-termini and B-C loop of Ind-2001e (or Ind-2001BD1) under serotype O altered the most. 2019 saw the detection of numerous distinct substitutions (K41I, D52K, A140T, T142A, K204R, and L213F). In 2020-21 isolates of Ind-2001e, K135R was found. In 2013, Ind-2001d showed a great deal of variation, with C-termini showing the highest frequency of mutation. Ind-2001d displayed a variety of mutations over time. From 2012 to 2017, Serotype A exhibited mutations primarily in the B-C loop and G-H loop. The most recent 2019–20 samples had a higher frequency of mutations and more mutations in the C–terminal region. Some of the alterations from the viruses of 2012–14 persisted in the serotype A virus of 2019–20. I42L and V45T were kept in 2019

sequences, while M190L and S196L were kept in 2020 sequences. Both the viral sequences from 2019 and 2020 had V143T. Numerous previously undiscovered mutations (N44D, V45L, S46N, N134S, R142H, E194K, and Q198R) were present in the sequences from 2019–20. Residues 45, 46, 143, and 194 all showed more than two amino acid swaps. Only the 44th residue had three amino acid changes among the isolates of serotype Asia1. A44T, E202K, and V210M were G-VIII lineage-specific within the Asia1 serotype. The sequences of the G-IX lineage of serotype Asia1 contained the following distinct mutations: T43N, A44E, N47S, T50V, M146L, and M211L.

3.10 Key Mutational Features in Ten Years (2012-2021)
3.10.1 Serotype O

- o Most of the mutations observed in VP1
- o Stable mutations: Q45K, N46D, and D197E (found in all sequences from 2012–17)
- o Ind2001d showed diverse mutations throughout the years, with C-termini having the highest frequency of mutations.
- o Mutations in VP1 that possess three major antigenic sites were responsible for the frequent emergence of novel strains within serotype O.

3.10.2 Serotype A

- o 2012–2017: mutations occurred mostly in the B–C loop and G–H loop.
- o 2019-20: introduction of unique mutations: N44D, V45L, S46N, N134S, R142H, E194R, and Q198R
- o I42L, V45T, V143T, and M190L mutations recurred that were previously seen in 2012–14 sequences.
- o A higher frequency of mutations was detected, but no lineage turnover or effect on antigenicity was evident.

3.10.3 Serotype Asia1

- o The G-IX lineage emerged from the G-VIII lineage and exhibited unique mutations: T43N, A44E, N47S, T50V, M146L, and M211L.
- o A44E: uncharged alanine (A) conversion to negatively charged glutamic acid (E)
- o T50V: polar threonine (T) was substituted by non-polar valine (V).
- o Changes in charge and polarity in the antigenic regions caused lineage turnover.

3.11 Molecular Identification of Viruses

3.11.1 Establishment of Cell Line

To create cells that were appropriate for virus injection, the stock BHK-21 cell line was sub-passaged five to six times. A cell line that would receive the virus inoculation was chosen after six rounds of cell sub-passaging. The following image (Figure 3.17) shows the cell line in passage 6.
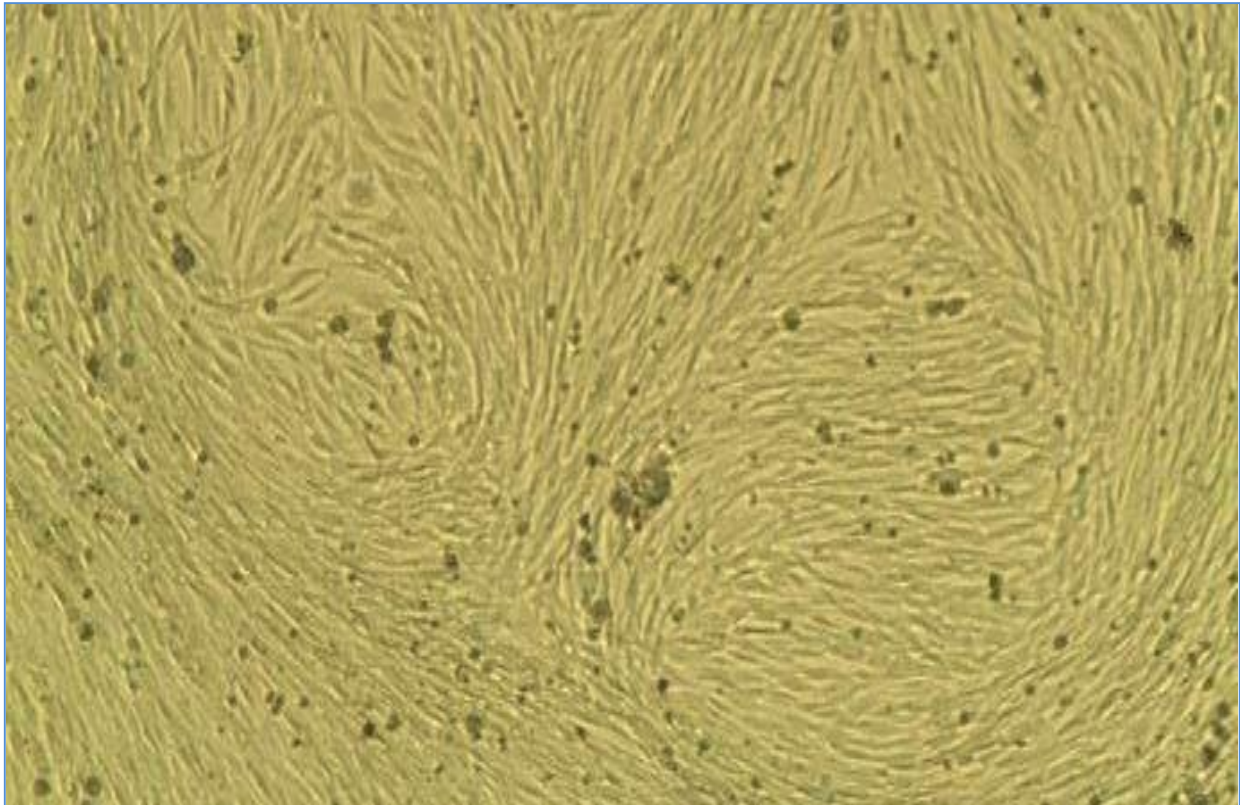
Figure 3.17 BHK-21 cell monolayer having a recognizable elongated form.

3.11.2 Isolation of Viruses

From 230 positive samples, for viral separation in BHK-21 cell culture, 29 samples were used. After the first passage or after 2-3 blind passages for virus adjustment, cytotoxic effect (CPE), which were represented by rapid killing of BHK-21 monolayer cells and virus-infected cells that were spherical and established separately **(Figure 3.18(b)),** developed. Each of the 29 chosen tissue samples was utilized to produce CPE using the BHK-21 cell line, as shown in **Figure 3.18(b).** the modified viral suspension for BHK-21 Flasks were freeze-thawed, collected in a 2 ml cryogenic vial, and kept at -80 C until next research.
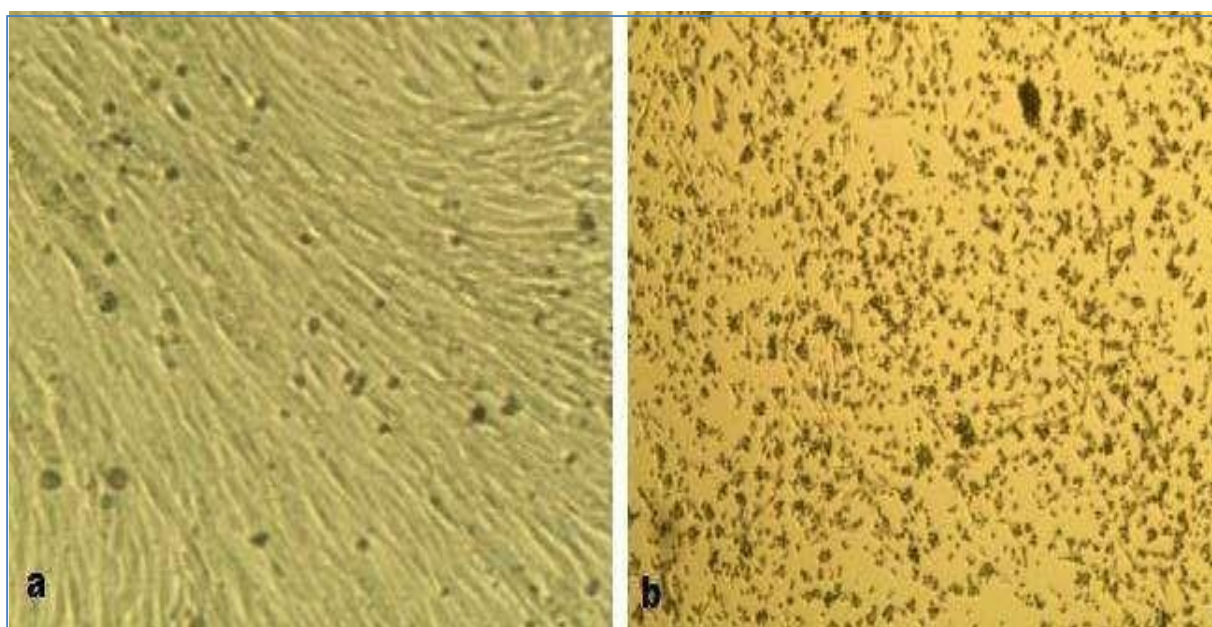
Figure3.18: (a) A monolayer of the distinctively flattened BHK-21 cell line. (b) Cytopathic effects were noticed during the second stage of virus injection.

3.11.3 Samples Screening and Serotype Detection
3.11.3.1 Sequencing of VP1 Region

The VP1-coding regions of all 29 isolated FMD viruses were effectively amplified by RT-PCR applying at the minimum 1 of the 3 stated common primer sets. This was done in order to identify the viruses. Direct sequencing of the amplicons produced the full VP1 sequences. The VP1 gene for each of these isolates had a length of 633 nucleotides and coded for 211 amino acids. The VP1 sequences of outlying viruses were detected as 23 FMDV serotype O, 5 FMDV

serotype A, and 1 FMDV serotype Asia 1 by aligning the DNA sequences with the public database on the NCBI site using the BLAST program (Table 3.3).

Table 3:.3 **Molecular detection of FMDV with VP1 Sequencing**

S. No.	Sample Pinpointing No.	Sequencing Region	Recognition process	Serotypes	Location of Sample collection
1	BAN/NA/Ha-156/2013	VP1 region	BLAST search	Type O	Natore
2	BAN/JA/Me- 180/2013	VP1 region	BLAST search	Type O	Jamalpur
3	BAN/GA/Sa- 197/2013	VP1 region	BLAST search	Type A	Gazipur
4	BAN/TA/Ma- 200/2014	VP1 region	BLAST search	Type O	Tangail
5	BAN/GA/Ka- 212/2014	VP1 region	BLAST search	Type O	Gazipur
6	BAN/CO/Ti- 218/2015	VP1 region	BLAST search	Type O	Comilla
7	BAN/GO/Ka- 236/2015	VP1 region	BLAST search	Type O	Gopalgonj
8	BAN/NL/Lo- 241/2015	VP1 region	BLAST search	Type O	Narail
9	BAN/DI/Sa- 254/2015	VP1 region	BLAST search	Type O	Dinajpur
10	BAN/TA/Dh-301/2016	VP1 region	BLAST search	Type O	Thakurgonj
11	BAN/CH/Sa-304/2016	VP1 region	BLAST search	Type A	Chandpur
12	BAN/DH/Sa-318/2018	VP1 region	BLAST search	Type Asia 1	Dhaka
13	BAN/DH/Dh-327/2019	VP1 region	BLAST search	Type O	Dhaka
14	BAN/RAJ/Pa-329/2019	VP1 region	BLAST search	Type O	Rajbari
15	BAN/RAJ/Ka-334/2019	VP1 region	BLAST search	Type O	Rajbari
16	BAN/RAJ/Pa-337/2019	VP1 region	BLAST search	Type O	Rajbari
17	BAN/PA/At-348/2019	VP1 region	BLAST search	Type O	Pabna
18	BAN/PA/At-351/2019	VP1 region	BLAST search	Type O	Pabna
19	BAN/PA/At-352/2019	VP1 region	BLAST search	Type O	Pabna
20	BAN/DH/Dh-359/2019	VP1 region	BLAST search	Type O	Dhaka
21	BAN/DH/Dh-360/2019	VP1 region	BLAST search	Type A	Dhaka
22	BAN/DH/Dh-361/2019	VP1 region	BLAST search	Type A	Dhaka
23	BAN/DH/Dh-362/2019	VP1 region	BLAST search	Type A	Dhaka
24	BAN/MA/Ma-366/2019	VP1 region	BLAST search	Type O	Manikgonj
25	BAN/DH/Dh-367/2019	VP1 region	BLAST search	Type O	Manikgonj
26	BAN/SA/Sa-431/2021	VP1 region	BLAST search	Type O	Satkhira
27	BAN/SA/Sa-432/2021	VP1 region	BLAST search	Type O	Satkhira
28	BAN/SA/Sa-440/2021	VP1 region	BLAST search	Type O	Satkhira
29	BAN/MY/My466/2021	VP1 region	BLAST search	Type O	Mymensingh

3.11.4 VP1-based phylogeny

In a prior investigation conducted by our lab, in-depth studies of the isolate's VP1 sequencing demonstrated the presence of the O/ME-SA/2018 lineage in Bangladesh along with an emerging unique sublineage called MYMBD21 (unpublished observations). The existence of My-466 as a MYMBD21 sublineage under the O/ME-SA/2018 lineage was further verified in this work by VP1-based phylogeny (Figure 3.19), and an evolutionary divergence of 6% between Indian isolates of the O/ME-SA/2018 lineage and My-466, computed in MEGA11. The isolate in Figure 3.19 suggested a probable transition from the PanAsia-2 lineage to the O/ME-SA/2018 lineage.
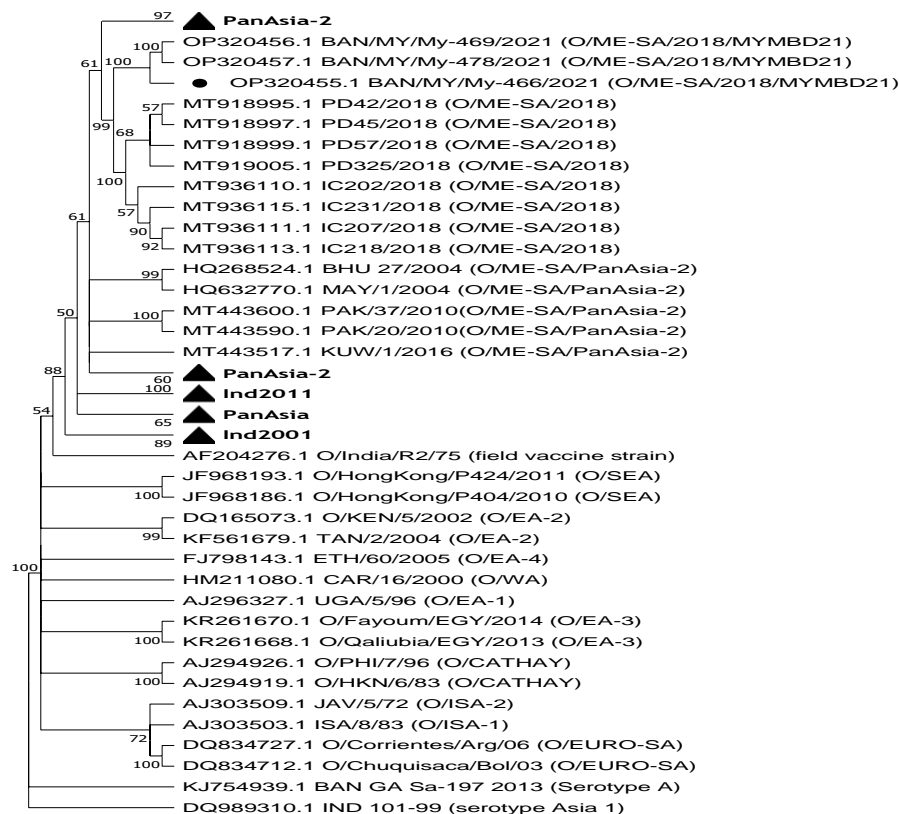
Figure 3.19 Based on the Neighbor-Joining method and the Kimura-2 parameter model, VP1-based phylogenetic reconstruction was performed in MEGA11. The tree showed clustering of MYMBD21 isolates (OP320455-OP320457) forming a distinct clade with other isolates of O/ME-SA/2018 lineage. This lineage showed possible evolution from PanAsia-2 lineage. The isolate under this study was labelled with bullet shape

3.11.5 Whole genome expansion

Of the 29 identified FMD viruses, total nine FMDV isolates (table 3.4) were whole genome amplified. Of them, five were serotype O, two were serotype A, and the rest were serotype Asia 1. During my study period, we amplified a representative serotype O virus (BAN/MY/My-466/2021) found in Bangladeshi cattle populations with a view to finishing genome sequencing and conduct a thorough genome-wide analysis in accordance.

Table 3.4: Whole genome characterization of FMDV strains circulating in last ten years in Bangladesh

Sequence ID	Serotype	Subtype	Accession no.	Reference
BAN/NA/Ha-156/2013	O	Ind2001d	KF985189.1	(Sultana et al., 2014)
BAN/BO/Na-161/2013	O	Ind2001BD2	MK071699	(Alam et al., 2019)
BAN/TA/Ma-167/2013	Asia1	G-VIII	MF782478.1	(Ali et al., 2017)
BAN/GA/Sa-197/2013	A	G-VII	HM854025	(Ullah et al., 2014)
BAN/GO/Ka-236(Pig)/2015	O	Ind2001BD1	KX712091.1	(Ali et al., 2016)
BAN/TA/Dh-301/2016	O	Ind2001BD1	MK088170.1	(Al Amin et al., 2020)
BAN/CH/Sa-304/2016	A	G-VII	MK088171.1	(Amin et al., 2019)
BAN/DH/Sa-318/2018	Asia1	G-IX	MN366244.	(Rubayet Ul Alam et al., 2019)
BAN/MY/My-466/2021	O	O/ME-SA/2018/MYMBD21	OP957418.1	

3.11.6 Complete Genome Annotation

Under the accession number OP957418.1, the assembled whole genome sequence of BAN/MY/My-466/2021 was added to the NCBI GenBank database. The isolate's entire genome annotation is shown in Table 3.5. A polyprotein with a length of 2,332 amino acids is represented by the 6,996 nt open reading frame (ORF) in the 8,216 nucleotides (nt) of the BAN/MY/My-466/2021 genome. An ORF is surrounded by a 120-nt 3 UTR and a 1100-nt 5 Un- Translated Region (5' UTR). A, T (U), G, and C make up 24.8%, 21.3%, 25.8%, and 28.2% of the sequence, respectively. S-fragment (1-370 nucleotides), poly (C) tract (371-386 nucleotides), pseudoknots (387-543 nucleotides), and intra-ribosomal entry site (IRES) (544-1100 nucleotides) are all found in the 5' UTR. There is a 25-nt poly (A) tail in the 3' UTR. The structural section

(Lpro, 2A, 2B, 2C, 3A, 3B, and 3D) is 4,788 nt long and encodes 1596 amino acids, while the non-structural part is 2208 nt long and encodes structural proteins with a total of 736 amino acids.

Table 3.5: Complete Genome Annotation of BAN/MY/My-466/2021

Gene fragments	Nucleotide range	Nucleotide length (bp)	Amino acid residue
5′UTR	1-1100	1100	-
Lab	1101-1703	603	201
VP4	1704-1958	255	85
VP2	1959-2612	654	218
VP3	2613-3272	660	220
VP1	3273-3911	639	213
2A	3912-3959	48	16
2B	3960-4421	462	154
2C	4422-5375	954	318
3A	5376-5834	459	153
3B	5835-6047	213	71
3C	6048-6686	639	213
3D	6687-8096	1410	470
3′UTR	8097-8216	120	-

3.11.7 Complete genome analysis

3.11.7.1 Local Alignment of the Complete Genome

In the BLAST search, the closest hit of the BAN/MY/My-466/2021 complete genome was FMDV type O isolate BHU_27/2004, complete genome (accession no. HQ268524.1) with 92.85% identity covering 99% of the query sequence, whereas the max score of the hit against the query was 11900 and the expected value (E-value) of getting another hit in the database was 0. The next closest hit was the complete genome of TUR/12/2013 (accession no. KM268895.1), which had 100% query coverage and 92.59% identity. 92.44% identity was found for another hit, May 1, 2004 (accession no. HQ632770.1). The immediate next hits were FMDV type O isolates BAN/BO/Na-161/2013 (accession no. MK071699.1), Tibet/CHA/99 (accession no. AJ539138.1), BAN/GO/Ka-236(Pig)/2015 (accession no. KX712091.1), TUR/18/2010

(accession no. JX040491.1), and PAK/14/2017 (accession no. MH784405.1) sharing about 92% identity **(Figure 3.20)**. A nucleotide divergence of 7-8% suggests that the isolate could belong to a different lineage, which is further investigated in the following phylogenetic analysis.
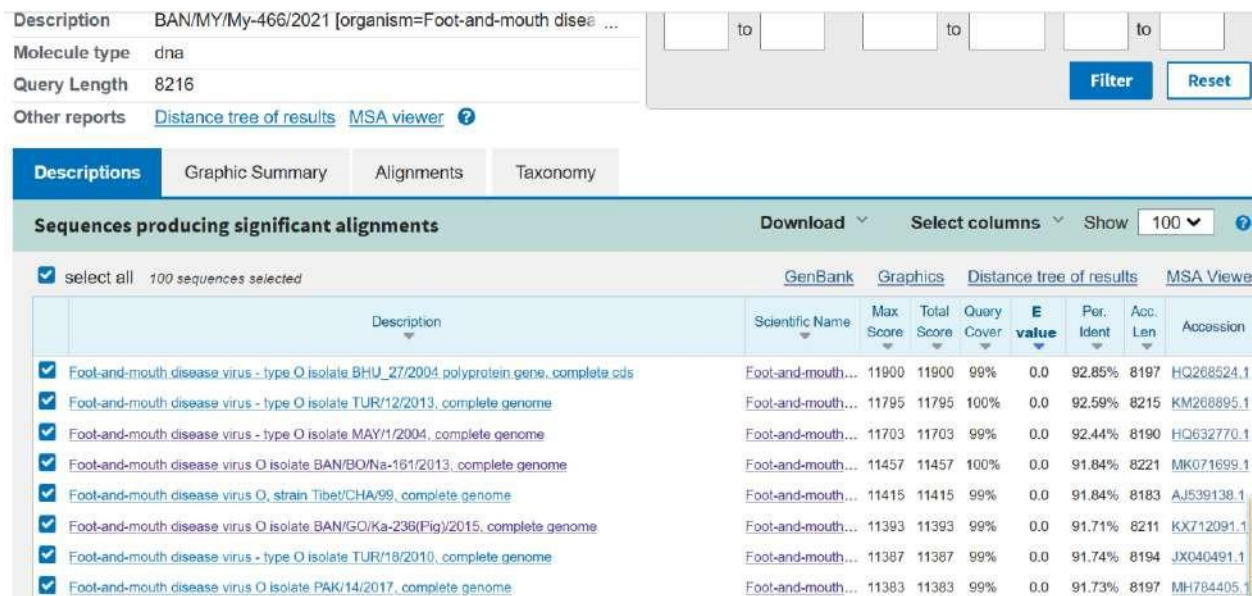
Figure 3.20: BLAST Search Results BAN/MY/My-466/2021 complete genome

3.11.8 Phylogenetic Analysis

A complete genome-based phylogeny **(Figure 3.21)** revealed the formation of a distinct clade by BAN/MY/My-466/2021 from other lineages (Ind2001, PanAsia, PanAsia-2) of the ME-SA topotype under serotype O but showed possible evolution from the PanAsia-2 lineage. While in VP1-based phylogeny, BAN/MY/My-466/2021 clustered more closely, forming a separate clade with isolates of the O/ME-SA/2018 lineage, a novel lineage that was first discovered in India in 2018 (Dahiya *et al*., 2021), than PanAsia-2. VP1 of BAN/MY/My-469/2021 and BAN/MY/My-478/2021 isolates belonged to the same type of virus as BAN/MY/My-466/2021 isolated in our laboratory. Evolutionary divergence calculated in MEGA11 between Indian isolates of the O/ME-SA/2018 lineage and BAN/MY/My-466/2021 was about 6% **(Appendix IX).**
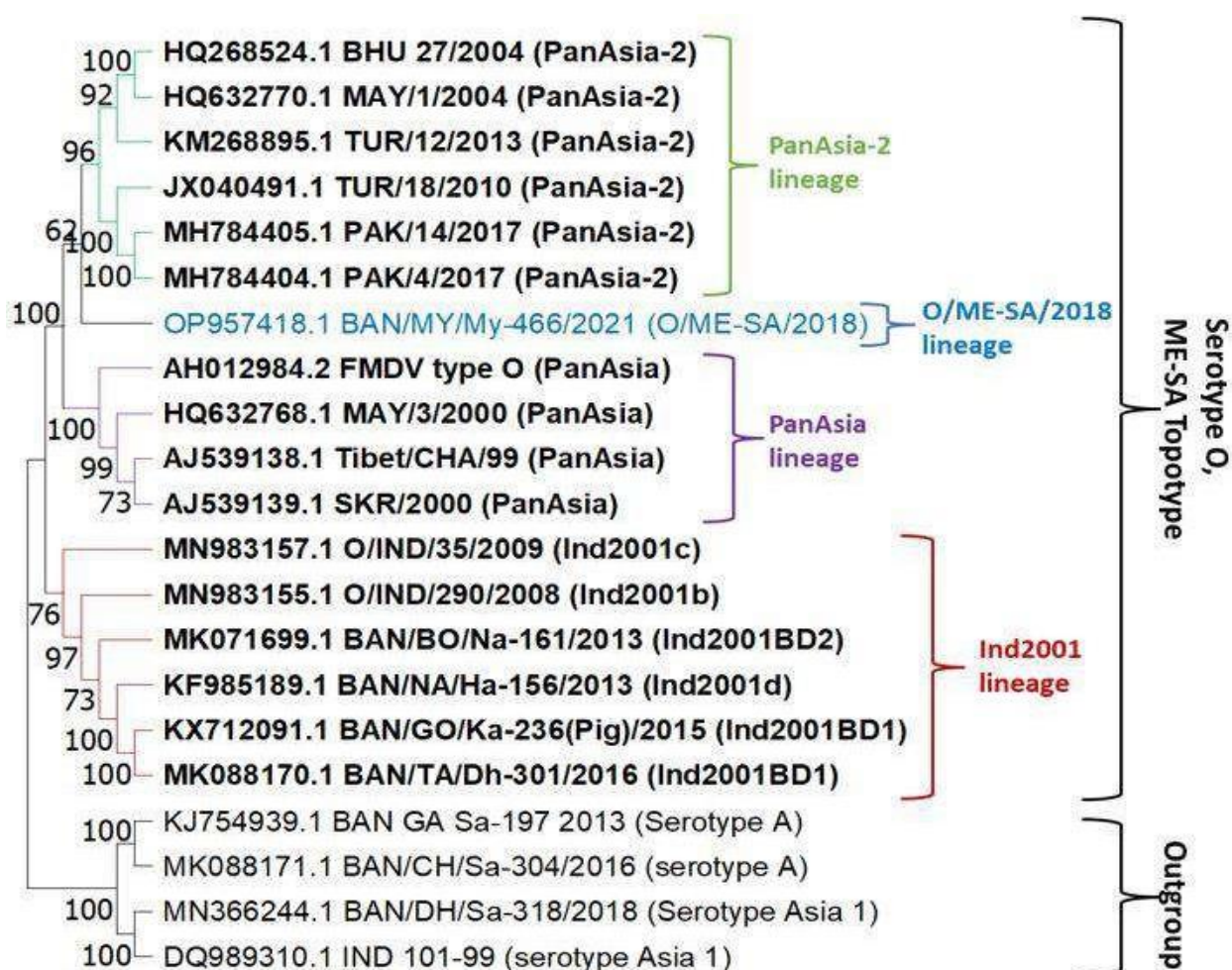
Figure 3.21: Full genome-wide phylogenetic reconstruction in MEGA11 based on the Neighbor-Joining method and the Kimura-2 parameter model reveals the formation of a unique branch by BAN/MY/My-466/2021 from the PanAsia-2 lineage. Branches of PanAsia-2 isolates were indicated with green color, PanAsia isolates with purple and Ind2001 lineage with dark red color. The isolate under this study was labelled with blue color. Serotype A and Asia 1 isolates were taken as outgroup.

3.11.9 Sequence Comparison

It was evident from the VP1-based phylogeny that the BAN/MY/My-466/2021 isolate belonged to the O/ME-SA/2018 lineage, with possible evolution from the PanAsia-2 lineage (Figure 3.20). For comparative complete genome analysis, an isolate, BHU_27/2004 (accession no. HQ268524.1) under the PanAsia-2 lineage, was considered a reference sequence because BHU_27/2004 exhibited the highest identity with the isolate in a BLAST search **(Figure 3.19)** and no complete genome for the O/ME-SA/2018 lineage was available.

3.11.9.1 Sequence comparison of BAN/MY/My-466/2021 against the reference sequence

The nucleotide and protein identities of the BAN/MY/My-466/2021 sequences against the reference strain (BHU_27/2004) are presented in **Table 3.6. 5**⊠UTR of BAN/MY/My-466/2021 demonstrated 91% nucleotide identity with 95 substitutions, including 12 gaps **(Appendix X)**. Within 5´ UTR, the S-fragment, poly (C) tract, pseudoknot, and IRES regions shared 91%, 44%, 89%, and 94% identity, respectively (Supplementary Figures S4-S7). The 3´ UTR region was 17% (99/120) divergent from the reference strain. Among the structural parts (VP4-VP1), VP2 and VP1 showed more nucleotide divergence (8%) whereas VP3 showed more amino acid changes (3%). Lpro and 3A regions among the non-structural regions (Lpro, 2A, 2B, 2C, 3A, 3B, 3C, and 3D) showed more nucleotide variation with only 89% and 91% nucleotide identity, respectively. About 97% protein identity was observed in the 2B and Lpro regions, and 2% alterations in amino acids were found in the 3A region.

Table 3.6: Percentage of nucleotide and protein identity between BAN/MY/My-466/2021 and reference sequence (BHU_27/2004)

Gene Segments	Nucleotide Identity (%)	Protein Identity (%)
5´UTR	91%	--
Lpro	89%	97%
VP4	96%	99%
VP2	92%	99%
VP3	94%	97%
VP1	92%	98%
2A	92%	100%
2B	95%	97%
2C	94%	100%
3A	91%	98%
3B	92%	100%
3C	93%	99%
3D	94%	99%
3 UTR	83%	--

3.12 Mutational Analysis against Vaccine Strains

Mutational analysis of My-466 capsid protein was performed against the current field vaccine strain, O/India/R2/75 (accession no. AF204276.1) (Mahapatra, M. *et al*., 2015), and the proposed local vaccine strain, BAN/TA/DH-301/2016 (accession no. MK088170.1) (Al Amin, Md. *et al.,* 2020). Capsid proteins diverged 4% (30 substitutions out of 736 residues) from the field vaccine strain, and 3% divergence (23 substitutions out of 736 residues) was observed from the proposed local vaccine strain **(Appendix IX, S12, S13).** Amino acid changes in each of the four capsid proteins are listed in Tables 3.7 and 3.8. Against the field vaccine strain, 5% protein variability was observed in both VP1 and VP3, but VP1 exhibited the majority of the mutations within the critical antigenic site, the G-H loop (130–160) **(Table 3.7, Figure 3.22).** Comparison with the local vaccine strain detected VP1 as the most variable region with 5% divergence **(Table 3.8, Figure 2.23).** VP4 was found to be the more conserved capsid region against both vaccine strains **(Tables 3.7 and 3.8).**
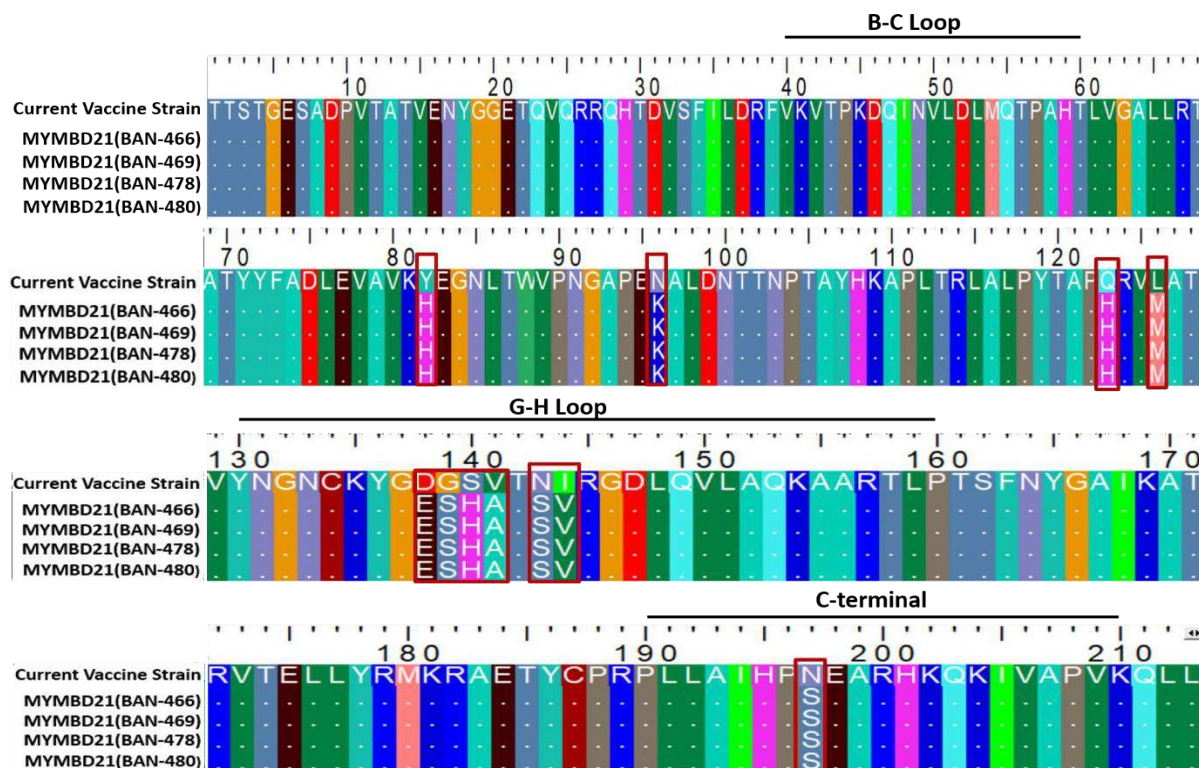
Figure 3.22: Amino acid substitutions in the VP1 sequences between the current vaccine strain and emerging MYMBD21 isolates

Table 3.7: Amino acid changes in BAN/MY/My-466/2021 against field vaccine strain (O/India/R2/75)

Capsid Proteins	Protein Identity (%)	Regions	Amino acid residue	Field vaccine strain (O/India/R2/75)	BAN/MY/My-466/2021
VP4	99%		80	I	F
VP2	97%	B-C loop (70-80) [35,36]	70	S	P
			74	A	V
			79	H	Y
		E-F loop (131-134 or 132-135) [35,36]	133	Q	T
			134	N	K
			154	V	M
VP3	95%		8	S	G
			14	L	F
			25	V	A
			44	F	L
			56	R	H
		B-B knob (58-61) [35,37]	60	G	D
			86	I	M
			96	H	Q
			195	E	D
			215	V	I
			219	R	T
			220	D	E
VP1	95%		82	Y	H
			96	N	K
			123	Q	H
			126	L	M
		G-H loop (130-160) [2,38]	138	D	E
			139	G	S
			140	S	H
			141	V	A
			143	N	S
			144	I	V
		C-terminal (190-213) [2,39]	197	N	S

Table 3.8:Amino acid changes in BAN/MY/My-466/2021 against proposed local vaccine strain (BAN/TA/Dh-301/2016)

Capsid Proteins	Protein Identity (%)	Regions	Amino acid residue	Proposed Local vaccine strain (BAN/TA/Dh-301/2016)	BAN/MY/My-466/2021
VP4	100%	--	--	--	--
VP2	98%		23	I	T
		B-C loop (70-80) [35,36]	79	H	Y
			93	G	S
		E-F loop (131-134 or 132-135) [35,36]	133	Q	T
			191	N	T
VP3	97%		8	S	G
			14	L	F
			44	F	L
			131	K	E
			174	A	T
			215	V	I
			220	Q	E
VP1	95%		13	T	A
		B-C loop (43-59) [2,8]	43	I	T
			96	A	K
			126	L	M
		G-H loop (130-160) [2,38]	138	K	E
			139	G	S
			140	A	H
			141	V	A
			143	N	S
		C-terminal (190-213) [2,39]	197	E	S
			198	Q	E
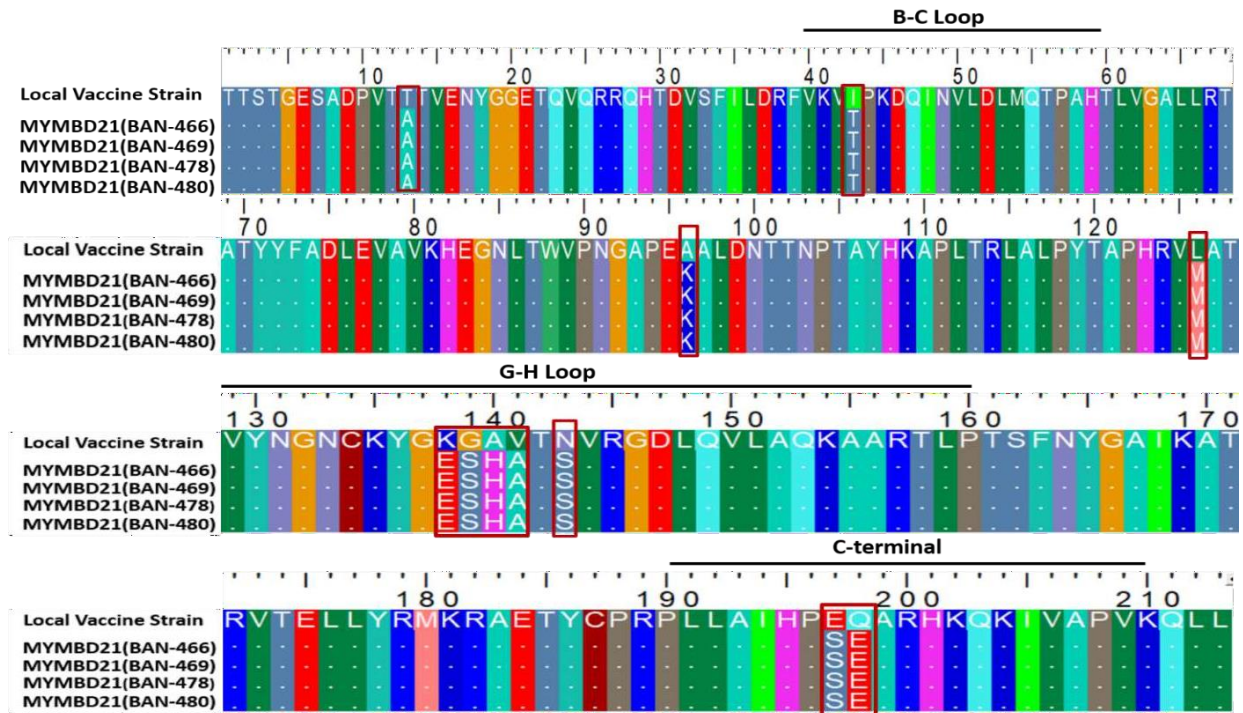
Figure 3.23: **Amino acid substitutions in the VP1 between local vaccine strain (BAN/TA/Dh-301/2016) and emerging MYMBD21 isolates**

Amino acid variability calculation using the Wu-Kabat protein variability coefficient also confirmed that VP1 is the most variable region and VP4 is the most conserved region among the capsid proteins. Within the VP1 region, the most variability was found in the amino acid sequence between positions 138 and 144, which is the G-H loop (Figure 3.24).
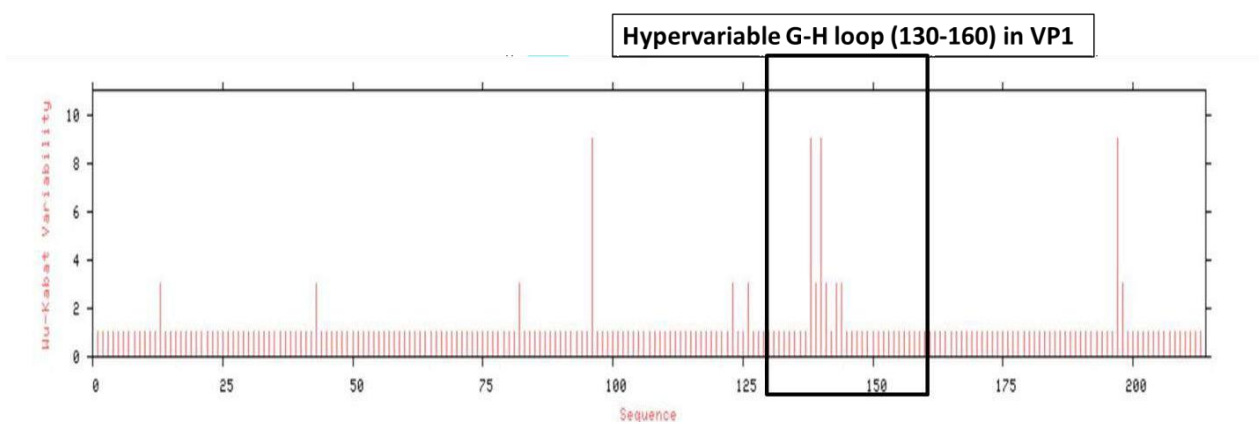
Figure 3.24: VP1 Protein Variability plot of BAN/MY/My-466/2021 against field and proposed local vaccine strains.

3.13 Three-dimensional (3D) Modeling of Capsid Proteins

3D models for capsid proteins of BAN/MY/My-466/2021 and vaccine strains were Capsid proteins VP1, VP2, and VP3 possess major antigenic regions of the virus, which are indicated in 3D models of the capsid proteins in **Figure 3.25 (a, b, c)**. A superimposed capsid protein model against vaccine strains was also generated to detect whether any structural change is associated with mutations in major antigenic sites. More than 92% of the residues were located in the favoured area according to the Ramachandran plot for all structures. The result of the quality assessment can be found in **Figure 3.24.**
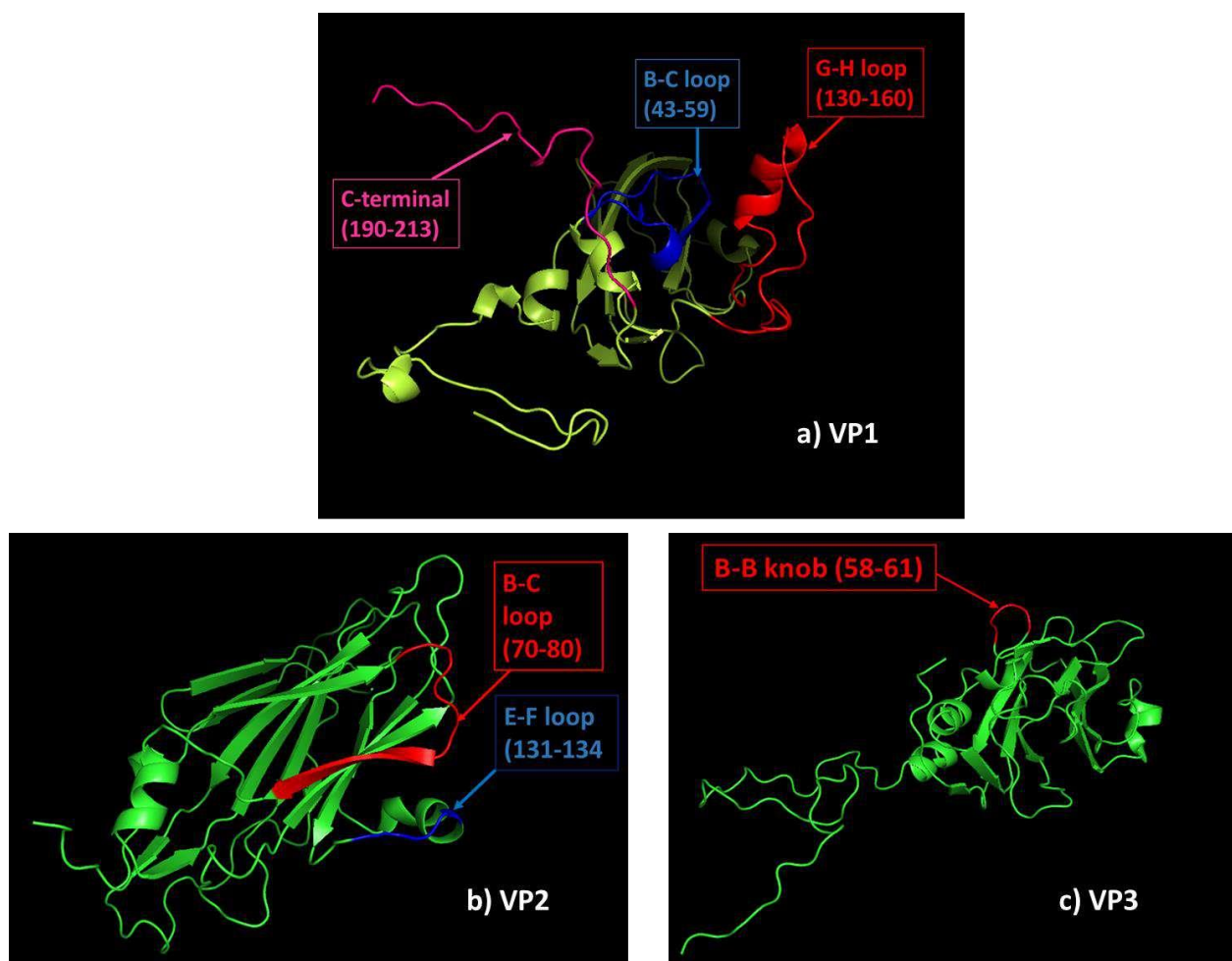
Figure 3.25: Three-dimensional model of capsid proteins (VP1, VP2, VP3) indicating antigenic regions of BAN/MY/My-466/2021. (a) VP1 model: B-C loop (red), G-H (blue) loop and C-terminal (pink). (b) VP2 model: B-C loop (red), E-F loop (blue). (c) VP3 model: B-B knob (red).

3.14 Superimposition of the VP1 model of BAN/MY/My-466/2021 against Vaccine Strains

The critical antigenic location, the G-H loop, was shown to have undergone a significant structural displacement, when VP1 of the model of BAN/MY/My-466/2021 was superimposed on both VP1 models of field (O/India/R2/75) and proposed local vaccine (BAN/TA/Dh-301/2016) strains, which indicated antigenic heterogeneity in BAN/MY/My-466/2021 against both vaccine strains (Figure-3.26).
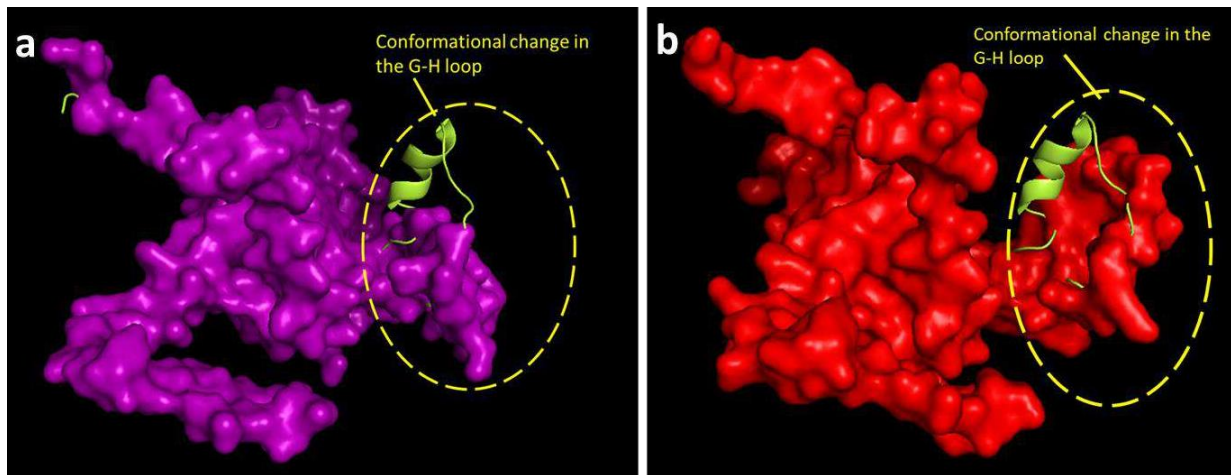
Figure 3.26: Superimposed 3D model of VP1 showing conformational change in the G-H loop. VP1 of BAN/MY/My-466/2021 was presented in cartoon style (yellow), VP1 of available field vaccine strain (O/India/R2/75) (purple) and proposed local vaccine strain (BAN/TA/Dh-301/2016) (red) was presented in surface style. (a) VP1 of BAN/MY/My-466/2021 superimposed on VP1 of available field vaccine strain (O/India/R2/75) (b) VP1 of BAN/MY/My-466/2021 superimposed on VP1 of proposed local vaccine strain (BAN/TA/Dh-301/2016*)***

4 DISCUSSION

In poor nations like Bangladesh, where FMD is endemic. One of the main obstacle to the improvement of the growth of the veterinary sector is foot-and-mouth disease (FMD). Foot-and-Mouth disease virus (FMDV) is considered the most destructive infectious agent with a multiplicity of serotypes for livestock and has caused severe damage to the agro-based economy. In the Establishment of the FMD effective control program according to the OIE/FAO 5-step Progressive Control Pathway (PCP), evolution, molecular epidemiology studies, and depiction of circulating viruses in Bangladesh are of utmost importance. This study looked at the outbreaks reported from 2012 to 21 and recorded the prevalence and distribution of serotypes in outbreak areas in order to discover the associated risk factors.

4.1 Epidemiological Investigation and risk factor analysis

The period of 2012–2021 was epidemiologically critical, showing changes in the circulation pattern of the FMD virus (FMDV) in Bangladesh with the emergence of multiple FMDV variants. Between 2012 and 2021, 481 epithelial tissue samples representing 3580 animal populations were collected from 32 districts, including 71 outbreaks **(Figure 3.3),** of which 230 samples were detected as FMDV-positive in a VP1-based PCR assay representing the 1960 population. The FMD cases included in the study had 54.7% morbidity, 19% fatality, and 10.4% mortality **(Figure 3.5)**.

The occurrence of the highest percentage (51%) of FMD outbreaks during the rainy season might be due to relatively higher humidity and lower temperatures that facilitated the survival of the virus, while higher temperatures during the summer might be responsible for the lower number of outbreaks. The effect of climate was also found in a previous study by Rahman *et al*. (2020).

The morbidity rate was higher in adults, as older animals are more likely to get exposed to the virus than calves and young cattle. But mortality and fatality rates were higher in calves, which might be due to the lower immunity in calves than in adults **(Figure 3.8)**. A study lead by Chowdhury *et al.* (2020) also found that the frequency of FMD cases in older cattle was higher than in younger cattle. Other studies also found similar results (Mostary *et al.*, 2018; Biswal *et al.*, 2019). The reason behind more susceptibility in male animals (59.4%) than females (50.8%)

might be due to more exposure of male cattle in field settings for agricultural purposes, where they might come into close contact with other infected cattle. A higher susceptibility to cases was observed in local cattle (55.7%) than in crossbred cattle (53%), as crossbred cattle are reared following more safety measures on farms than local ones in household settings. Under the intensive farming system, animals are kept in closed settings following proper animal health guidelines, whereas, in semi-intensive farming, animals are allowed to graze on the field sometimes, which might have resulted in a higher prevalence of FMDV in semi-intensive farms. Similar results in the cases of gender, breed, and farming system were also found in earlier investigations (Chowdhury *et al*., 2020; Mostary *et al.,* 2018). From the chi-square test at the p 0.05 level, it was settled that season, age, gender, and farming system were significant factors for the occurrence of FMD (P-value <0.05). But the association of breed with FMD cases was not found to be significant in the statistical (chi-square) test (P-value > 0.05). Another important factor confirmed by the chi-square test was the vaccination status of the animal (P value<0.05). Vaccinated cattle had a lower risk of infection than non-vaccinated cattle (**Appendix III**).

4.2   Evolution and molecular epidemiology of etiological agents of FMDVs in Bangladesh from 2012 to 2021

VP1-based sequencing analysis indicated that serotype O was most dominant (85%) over serotype A (11%) and Asia-1 (4%) (**Figure 3.4**). From FMDV VP1 coding sequences reported in Bangladesh, it was revealed that serotypes O, A, and Asia1 were circulating during 2021–21. Serotype O was detected each year from 2012 to 2021 (Hossain K.A. *et al.,* 2022; Siddique *et al*., 2018). Serotype A was also present in the past ten years but was not in circulation in 2015, 2018, or 2021 (Nandi *et al.,* 2015a; Ullah *et al*., 2014). Asia1 was reported from 2012–2013 and in 2018 (M. R. Ali *et al*., 2020b; Hossen *et al*., 2020; Ullah *et al*., 2015a). No serotype C case was detected in Bangladesh (**Figure 3.11**) during the period.

4.3   The emergence of novel sub-lineages during the past ten years (2012–2021)

The emergence of novel strains of FMDV within the period of ten years (2012–2021) is an epidemiologically significant event (**Figure 3.16**). In 2012, Ind-2001d was prevalent, but the Ind-2001BD1 or Ind-2001e sublineage emerged in the same year (Siddique et al., 2018), which replaced the Ind-2001d sublineage and later in 2016 became the predominant FMDV sublineage

in Bangladesh. The circulation of Ind-2001d was not detected after 2015. In the last 5 years, Ind-2001e (Ind-2001BD1) has become the only circulating sublineage under the Ind-2001 lineage of FMDV in Bangladesh. In 2013, another novel Ind-2001BD2 sublineage was detected, but its circulation did not continue in the subsequent years (Siddique *et al.,* 2018) **(Figure 3.13)**. Under serotype Asia1, the G-VIII lineage was circulating during 2012–13, and in 2018, the Asia1 serotype emerged as a novel lineage, G-IX (BD-18), in Bangladesh **(Figure 3.15)** (M. R. Ali *et al*., 2020b). In 2021, a new lineage, SA-2018, invaded Bangladesh and was first detected in our laboratory. The earliest mention of this lineage was made in India in 2018 (Dahiya *et al.,* 2021). In VP1-based phylogeny, the isolates under the SA-2018 lineage (OP320455.1–OP320458.1) formed a separate clade emerging from the Indian isolates, indicating an evolutionary relationship with those Indian isolates **(Figure 3.13)**. In our previous study, these isolates were proposed as a novel sublineage, MYMBD21, under the SA-2018 lineage, showing a 5-6% nucleotide distance from the Indian isolates (Hossain K.A. *et al*., 2022). The circulation of this lineage had not been reported before in Bangladesh. According to Subramaniam *et al.* (2012) and later found to have spread in India in 2011 (Yuvaraj *et al.,* 2013), PanAsia-2 FMD outbreaks predominated in India between 2006 and 2008 (Subramaniam *et al.,* 2012). PanAsia-2 is not predominant in the Indian subcontinent. The ANT-10 sub-lineage of the PanAsia-2 lineage was reported in 2019, however the PanAsia-2 lineage is frequently reported in Pakistan. According to Bachanek-Bankowska et al. (2019), PanAsia-2 viruses have already been discovered in Afghanistan, Bulgaria, Israel, Iran, Pakistan, Saudi Arabia, and Turkey. The lineage has a history of long cross-border movements from the Middle East to the Southeast Asian region, but there was no report of PanAsia-2 from Bangladesh before. There was a report of two isolates in 2013 in Bangladesh named the PanAsia-02 sub-lineage (Hossen *et al*., 2020). The isolates, however, exhibited 98–99% similarity with certain uncharacterized Indian isolates from 2011 (Subramaniam *et al.,* 2013). As a result, little precise information was given regarding the lineage of these two isolates. Also, there was no report of outbreaks by the PanAsia-02 sub-lineage after 2013. BAN-21 shared only 92% nucleotide identity with the Bangladeshi PanAsia-02 sub-lineage, and the amino acid sequence of VP1 of these two isolates was 5% divergent. This indicates that a novel sub-lineage of PanAsia-2 under the ME-SA topotype has emerged in Bangladesh for the first time. The divergence of BAN-21 from Indian isolates and isolates from other established sub-lineages indicates that PanAsia-2 might have undergone a long cross-border movement, evolved through the process, and finally appeared as a BAN-21 sub-lineage in

Bangladesh. The result of this study confirmed the creation of a novel sub-lineage under PanAsia-2 in Bangladesh. This indicates that a novel sub-lineage of PanAsia-2 under the ME-SA topotype has emerged in Bangladesh for the first time. In the case of serotype A, only one lineage, G-VII, under the Asia topotype was detected in Bangladesh **(Figure 3.14)**.

4.4   Mutational analysis

From the amino acid substitution analyses, it was evident that most of the changes occurred in the antigenic sites, the G-H loop, the B-C loop, and the C-terminal loop. The RGD motif containing Arg-Gly-Asp was found to be conserved in all serotypes as it is an important recognition element in integrin-dependent cell adhesion processes (Dill & Eschbaumer, 2020a; Logan *et al*., 1993; Stewart & Nemerow, 2007).

Many single amino acid substitutions were detected in the VP1 sequences of FMDV. Amino acid residues at 43–46, 48, 142, 198, 202, and 210 positions were variable in more than one serotype **(Table 3.1)**. Amino acid substitutions K45Q, T142A of serotype O, E194K of serotype A, and E202K of serotype Asia1 were also apparent in previously conducted studies (Bai *et al*., 2010a, 2014a; Dill *et al*., 2017; Mohapatra *et al*., 2015a). Frequently reported exchanges were reported in amino acid residues 142, 194, and 210 in a previous study (Dill & Eschbaumer, 2020b), and substitutions were also detected in those positions for Bangladeshi isolates included in this study (Table 1). Amino acid residue 142 is located within the immunodominant epitope's G-H loop adjacent to the RGD motif, and substitutions at this site can alter the structural conformation of the G-H loop of VP1 depending on the type of amino acid exchange (Zhao *et al*., 2003). Again, amino acid variations at positions 134, 143, and 158 within the G-H loop and positions 196, 198, 210, and 213 within the C-terminal were found in other studies (Anil *et al*., 2012; Bai *et al*., 2010b, 2014b; Dill *et al*., 2018; Gonzalez *et al*., 1991; Gullberg *et al.,* 2013, 2014; Mohapatra *et al.,* 2015b; Pandey *et al*., 2014), and these positions were also variable among Bangladeshi isolates in this study **(Table 3.1)**. Substitutions at 210 amino acid residues detected in serotype O and Asia 1 can cause failure in the VP1-2A product cleavage (Dill & Eschbaumer, 2020a; Gullberg *et al.,* 2014; Kristensen *et al.,* 2017).

Amino acid exchanges in which a negatively charged amino acid was restored by a positively charged amino acid occurred in amino acid residues 52, 138, 194, and 202. In residue 52, negatively charged aspartic acid (D) was substituted for positively charged lysine (K). Residues

138, 194, and 202 had common amino acid substitutions: negatively charged glutamic acid (E) was substituted by positively charged lysine (K). Another common case of amino acid exchange occurred in amino acid residues 44, 46, and 140, where uncharged, polar asparagine (N) was substituted by polar, negatively charged aspartic acid (D) **(Table 3.1)**. Position 194 was found as a variable site that is very close to residues 195–197 that form one of the walls of the heparan sulfate (HS) binding site. The introduction of a positive charge by the E194K mutation in serotype A can affect the HS receptor binding to the virus (Dill & Eschbaumer, 2020a; Fry, 2005).

In comparison to the other type O sublineages discovered in Bangladesh, Ind-2001d has more genetically varied viruses within serotype O (Siddique *et al.*, 2018). Significant mutations S139G, N140A, P142T, and P158T were found between the Ind-2001d and Ind-2001e sublineages, which were significant for the G-H loop displacement between these two sublineages (Siddique et al., 2018). In Table 2, Ind-2001e isolates collected from 2019 to 2021 showed more variations in the G-H loop, whereas isolates from 2012–18 mostly varied within the B-C loop and G-H loop. Special mutations in the G-H loop (K135R, A140T, and T142A) during the 2019–21 circulation of Ind-2001e were detected, which suggests a changing mutational pattern that might affect the integrin receptor binding and modulate the orientation of the G-H loop. Significant mutations at these sites might result in a drastic change in the antigenicity of the virus.

Serotype A isolates from 2019 to 2020 showed more variations in the B-C loop and C-terminus. Asparagine (N) is converted into negatively charged aspartate (D) at the 44th residue located in antigenic site 3 within the B-C loop and can affect antigenicity (Dill & Eschbaumer, 2020a; Fry, 2005). E194K (negative to positive charge conversion) and Q198R (uncharged to positive charge conversion) mutations at the C-terminal site can affect antigenic site 2 and thereby modulate the heparan binding site, as these two positions are adjacent to one of the walls of the heparan binding site (Acharya *et al.,* 1989; Dill & Eschbaumer, 2020a; Fry, 2005).

Within the G-VIII lineage of Asia1 isolates, a few mutations at positions 44, 202, and 210 were found, among which A44T demonstrated polarity change and E202K (Glutamate, E to lysine, K) referred to negative charge conversion into positive charge. Among the unique mutations of the G-IX lineage, charge and polarity shifts also occurred. Uncharged alanine (A) was converted into

negatively charged glutamic acid (E) at the 44th residue, and changes in polarity were observed at position 50, where polar threonine (T) was substituted by non-polar valine (V) in the B-C loop, affecting antigenic site 2. These mutations might be responsible for the lineage turnover from G-VIII to G-IX.

Apart from the mutational analysis presented in this study, further serological assays are required to confirm any changes in the antigenicity of the recently circulating FMDV strains.

4.5 Complete genome analysis

BAN/MY/My-466/2021 or My-466 isolate belongs to a novel sublineage, O/ME-SA/2018/MYMBD21, which was confirmed in a previous study carried out by our laboratory and also in the VP1-based phylogeny in this study **(Figure 3.19)**. The O/ME-SA/2018 lineage was first identified in India in 2018 (Dahiya, S. S. *et al.,* 2021) and later introduced in Bangladesh in 2021 as a novel MYMBD21 sublineage (unpublished observations). Complete genome information for this new lineage was not available before this study. The first full genome of an isolate from the O/ME-SA/2018 lineage is proclaimed in this work. which belongs to a novel sublineage, MYMBD21, under this lineage (BAN/MY/My-466/2021, or My-466 isolate).

In complete genome phylogeny, the BAN/MY/My-466/2021 complete genome did not cluster with any established lineages (Ind2001, PanAsia, PanAsia-2) of the O/ME-SA topotype, which indicated that the isolate might belong to a novel subtype. For further investigation on the lineage of the isolate, a VP1-based phylogeny was performed that showed clustering of BAN/MY/My-466/2021 with a new lineage called the O/ME-SA/2018 lineage, a novel lineage that was found to circulate in India in 2018 (Dahiya *et al*., 2021). But the isolate formed a different clade from the Indian O/ME-SA/2018 isolates **(Figure 3.19)**. A BLAST search and mean nucleotide distance calculation in MEGA11 showed about 6% nucleotide divergence between the O/ME-SA/2018 lineage and the BAN/MY/My-466/2021 lineage (Appendix IX). All this evidence indicated that the BAN/MY/My-466/2021 isolate evolved as a novel sublineage under the O/ME-SA/2018 lineage, and the isolate was named MYMBD21. This study reported the first complete genome of the MYMBD21 sublineage (BAN/MY/My-466/2021) under the O/ME-SA/2018 lineage.

The complete genome of BAN/MY/My-466/2021 (MYMBD21) showed 92.85% identity with the reference sequence (BHU_27/2004). The S-fragment of the 5 UTR was found to be 91% identical to the reference sequence with 30 amino acid changes, 2 insertions (279th, 358th), and 1 deletion (147th position) (Appendix IX, S4). In the poly (C) tract, 7 cytosine (C) insertions occurred (Appendix IX, S5). The pseudoknot region was 89% (139/157) identical, and 1 adenine insertion at the 50th position was detected (Appendix IX, S6). The IRES region was 6% divergent, and deletion of only one cytosine was found at position 536 (Appendix IX, S7). The 3-UTR portion was detected as highly variable, sharing only 83% identity. Two nucleotide insertions were detected, and 12 amino acid changes were found. The poly (A) tail of this region contained seven extra adenines compared to the reference strain (Appendix IX, S7). Fully conserved regions (100%) against reference were 2A, 2C, and 3B, and VP4, VP2, 3C, and 3D regions were 99% conserved. About 2% of amino acid changes were found in the VP1 and 3A regions. More amino acid changes (3%) were identified in Lpro, VP3, and 2B-encoded proteins **(Table 3.6).**

BAN/MY/My-466/2021 shared 96% (706/736) capsid protein (VP4-VP1) homology with the field vaccine strain, O/India/R2/75, and 97% (713/736) homology with the proposed local vaccine strain, BAN/TA/Dh-301/2016 **(Appendix IX, S9, S10).** VP4 was found to be 99% conserved against O/India/R2/75 and fully conserved against BAN/TA/Dh-301/2016 **(Tables 3.7 and 3.8; Appendix IX, S11).**

About 97% (212/218) of VP2 homology was found against the field vaccine strain. S70P, A74V, and H79Y mutations were detected in the antigenic region, B-C loop (70–80), of which S70P demonstrated polar serine (S) conversion into nonpolar proline (P), and H79Y showed substitution of positively charged Histidine (H) to uncharged tyrosine (Y). Two mutations, Q133T and N134K, were detected in the E-F loop (132-135 or 131-134) of VP2, in which uncharged asparagine (N) was substituted by positively charged lysine (K). The VP2 was 98% identical to the local vaccine strain, with five amino acid changes. Only H79Y (positive histidine to aromatic tyrosine) mutations occurred in the B-C loop and Q133T in the E-F loop, which were also detected against the field strain. The VP3 demonstrated a 5% divergence from the field vaccine strain.

In VP3, 12 amino acid changes were observed, but one mutation (G60D) in the antigenic region, B-B knob (58–61), was found where uncharged glycine (G) was converted into negatively charged aspartate (D). VP3 was 97% homologous (213/220) to the VP3 of the local vaccine strain, but no mutation in the B-B knob was found.

The amino acid sequence-based comparison revealed 95% VP1 homology with the VP1 of the field vaccine strain. 11 mismatches in amino acid sequences were found, among which 6 of the mutations (D138E, G139S, S140H, V141A, N143S, and I144V) were in the G-H loop. G139S showed changes in polarity. At the 140th position, uncharged serine (S) was changed into positively charged histidine (H). The critical amino acids for the origination of antigenic site 1 are 144, 148, 154, and 208 (Li et al., 2012). In BAN/MY/My-466/2021, valine (V) instead of isoleucine (I) was found at the 144th position of VP1 amino acid sequences, which can be considered a critical mutation. No mutations occurred in the B-C loop (43-59), and only asparagine (N) to serine (S) conversion at the 197th position was observed in the C-terminal (190-213) (Table 3.7). Comparison of BAN/MY/My-466/2021 VP1 with the proposed local vaccine strain (BAN/TA/Dh-301/2016) also revealed 95% identity with 11 mutations in VP1, of which 5 were observed in the G-H loop region (K138E, G139S, A140H, V141A, and N143S). Positive to negative charge conversion (K138E) occurred at position 138, and in the 140th position, uncharged alanine (A) was replaced by positively charged histidine (H). Changes in polarity occurred in G139S. The B-C loop mutation (I43T) changed polarity. Two mutations (E197S and Q198E) were observed in the C-terminal, where negatively charged glutamate (E) resolved into uncharged serine (S) at the 197th residue and uncharged glutamine (Q) was replaced by negatively charged glutamate (E) (Table 3.8). Frequent mutations at residues 138, 139, 142, 143, and 144 were also observed in other studies (Bai *et al.,* 2010, 2014; Martnez *et al.,* 1997; Mohapatra *et al*., 2015). Changes at residues 197 and 198 in the C-terminal were not reported as frequent mutation sites as at other sites (Baranowski *et al.,* 2000; Dill & Eschbaumer, 2020; Martnez *et al*., 1997; Mohapatra *et al.,* 2015).

VP1 was found to be the most variable region compared to other capsid proteins. Among the antigenic regions, the majority of the amino acid substitutions were detected in the G-H loop of VP1 against both vaccine strains. The G-H loop was found to be the most variable region of VP1, carrying 5–6 mutations at critical amino acid positions. Other studies also reported high variability in the hypervariable area of the G-H loop between 130–160 (for type O) amino acid

sites, which leads to the primary antigenic epitopes on the capsid coding areas (Dill & Eschbaumer, 2020; Momtaz *et al*., 2014). To know whether the mutations were related to structural changes in VP1, superimposition of the 3D structures was performed.

In the case of VP2 and VP3, no conformational change in BAN/MY/My-466/2021 against vaccine strains was observed in the superimposed 3D model, which indicated that the mutations in those regions did not contribute to significant structural variation in antigenic sites **(Appendix IX, S21, S22).**

Superimposition of the 3D model of VP1 of BAN/MY/My-466/2021 with that of both vaccine strains showed that the G-H loop of the isolate did not align with the G-H loops of vaccine strains (Figure 5). This delineated that amino acid substitutions were related to changes in the structure of the G-H loop. Structural changes in the G-H loop were also observed by Siddique *et al*. (2018) due to mutations in the vital region of the G-H loop of VP1. Substitutions in critical sites (S140H, A140H, V141A, N143S, and I144V) adjacent to the conserved RGD motif (145–147) were detected, which might be responsible for the structural change. As the RGD motif is crucial in viral attachment to host cell receptors and often a target sequence in vaccines, mutations adjacent to this site could alter host specificity, the antigen recognition site, and could contribute to vaccine escape, a trick of the virus to avoid recognition by host immunity. There is a possibility that the vaccine would not be effective against the novel lineage, which requires further serological analyses.

The unrestricted cross-border movement of animals from neighboring countries, the high mutational frequency of the virus, and various host factors could be attributed to the changing epidemiological pattern of FMD in Bangladesh. Several significant mutations accumulated within the circulating FMDV virus in Bangladesh from 2012–21. Some mutations at significant antigenic sites contributed to the frequent emergence of novel variants within ten years that challenged the effectiveness of the existing vaccines and available treatment options. For the effective control of the disease, regular monitoring of FMD outbreaks, risk factor analysis, and planning to imply effective measures should be given emphasis. This study reported the overall FMD epidemiological pattern and risk factor-based analysis from 2012 to 2021, providing a track of circulating and emerging FMD strains and analyzing mutations and variable regions within the VP1 capsid protein. The findings of the study would be valuable for better

understanding the current FMD situation, which would facilitate adopting effective preventive actions that would lead to the implementation of PCP-FMD in Bangladesh.

The coexistence of different strains of FMDV in a defined geographical region without cross-protective immunity leads to devastating impacts during outbreaks. The best possible way to counter the adversity is prevention through vaccination, rapid diagnosis, the fastest acknowledgment of newer strains in circulation to avoid their outbreak or prevalence, at least, and other steps. Complete genome analysis yields newer information, adds valuable data to our prior knowledge, and also predicts and prevents, or at least warns, of near-future outbreaks. Therefore, complete genome information of the newly detected lineage, O/ME-SA/2018, would be valuable to undertake the necessary steps for the control and eradication of the strain.

5   CONCLUSIONS

In conclusion, it has been discovered that FMD greatly restricts the growth of competitive cattle firms in emerging nations like Bangladesh. This study investigated FMD epidemics and reported the serotype O, A, and Asia1 circulation in 32 distinct Bangladeshi areas from 2012 to 2021. According to the results of the epidemiology study on FMD, the illness is endemic in Bangladesh and FMDV types O, A, and Asia-1 are constantly introducing widely. The FMDV serotypes A (genotype VII of the Asia topotype), O (Ind2001 lineage and O/ME-SA topotype), and Asia-1 (genetic lineage C) are all one genetic lineage and topotype in Bangladesh. According to this analysis, the serotype O was the most common from 2012 to 2021. It was also shown how risk factors such gender, age, breed, farming system, animal mobility, and season affected the frequency of FMD outbreaks. Within ten years, multiple novel strains appeared in Bangladesh, which is thought to be an epidemiologically significant development. Ind2001BD1 and Ind2001BD2 sublineages first appeared in 2012 and 2013, respectively. In 2018, a fresh lineage of Asia1 appeared. The O/ME-SA/2018 lineage's MYMBD21 unique sublineage was discovered in 2021. new sub-lineages have emerged, including BAN-21 under the O/ME-SA/PanAsia-2 lineage for the first time ever, Ind2001BD1 under the O/ME-SA/Ind2001 lineage, and the G-VII lineage of the AFRICA topotype under serotype A. Evolutionary divergence study demonstrated the superiority of a locally reported vaccination candidate over an Indian vaccine strain currently in use against circulating strains. In this work, evidence of vaccine escape mutations and structural alterations in antigenically important places were shown, suggesting that the sub-lineage may not be protected against by currently available vaccine strains. Understanding the evolutionary history, mutational frequency, and transmission pattern of the isolates would require knowledge of their entire genomes. Genome-wide analyses would also make it easier to choose vaccine candidates, assess the effectiveness of the currently available vaccinations, and develop successful preventative strategies. The findings emphasized the region's ongoing risk of FMD as a transboundary illness and showed how open the borders are between Bangladesh and its neighbors. In order to combat the destructive virus, this study's conclusion offers convincing evidence of the creation of new strains, emphasizes the need of developing new vaccine candidates, and stresses the need for risk-based planned control techniques.

Upcoming directions:

• Implementing techniques including regulating animal movements within and between adjacent nations, carefully assessing the danger of FMDV acquaintance, and repeatedly immunizing animals capable to the illness are essential for the effective management and prevention of FMD.

• To assess the pattern of outbreaks and the efficiency of the control campaign, ongoing monitoring is required.

• It is crucial to properly report diseases to the OIE/FAO office. The greatest barrier to identification is an unintended formal manifesto of the disease to the OIE/FAO, despite the fact that Bangladesh has currently passed stage 1 of the PCP-FMD road map.

• Bangladesh should move up a level on the PCP through the adoption of a national FMD control initiative. The system might be streamlined by using an epidemiological network. To stop epidemics from starting, a risk-based control program should be created.

• To stop animal movement, management, and product restrictions, a revised "Animal Disease Control Act" must be rigorously applied. In the framework of basic cooperation, it is necessary to put restrictions on the transportation of animals, animal products, and animal byproducts across international boundary.

• To successfully restrict the spreading of FMDV, the selection of vaccine candidates from local FMD strains with the aid of routine whole genome sequencing is essential.

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Appendix-I

Recipes and Preparation Notes for Media

1X TAE Buffer

First preparation of a 50X stock is as follows

Ingredients	Amount
Tris base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (P^H 8.0)	100.0 ml
Distilled water	Up to 1.0 liter

Prepared buffer stock was autoclaved at 121^0C for 20 minutes to sterilize. Then it was diluted 1:49 into deionized water for use as 1X working stock solution for agarose gel electrophoresis. The solution was stored at ambient 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 condition.

6X TAE Load Dye

Ingredients	Amount
Xylene cyanol	0.25 g (0.25%, wt/vol)
Bromophenol blue	0.25 g (0.25%, wt/vol)
Sterile 80% glycerol	37.5 ml (30% vol/vol)
50X TAE buffer	12.0 ml (6X)
Sterile distilled water	Up to final volume of 100 ml

First glycerol, buffer and water were mixed and xylene and bromophenol blue were added last. If the p^H is correct, the load dye solution should be blue (not blue-green). The solution was stored at ambient temperature.

Ethanol (70% vol/vol)

70 ml of absolute ethanol was mixed with 30 ml of deionized water. The solution was stored at 4^0C.

TE Buffer (10 mM Tris-HCl, 1 mM EDTA)

First preparation of 1 M Tris-HCl and 0.5 M EDTA stock solutions as follows

Ingredients	Amount
1 M Tris-HCl (p^H 8.0)	1.0 ml
0.5 M EDTA (p^H 8.0)	0.2 ml
Sterile distilled water	90.0 ml

The p^H was checked and adjusts each to p^H 8.0 with NaOH as necessary. The solution was autoclaved at 121^0 C for 20 minutes to sterilize. The indicated volume of sterile stock was mixed and then the sterile water was added such that the total volume was 100 ml.

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

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

ImProm-II™ Reverse Transcription System (Catalog No. A3800, Promega, USA)

Ingredients	Amount
ImProm-II™ Reverse Transcriptase	1.2 Kanamycin Positive Control RNA
ImProm-II™ 5X Reaction Buffer	Upstream Control Primers
MgCl$_2$	Downstream Control Primers
dNTP Mix	Nuclease-Free Water
Oligo(dT) primer	Recombinant RNasin® Ribonuclease Inhibitor
Random primers	

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

Ingredients	Amount
Membrane Binding Solution	Membrane Wash Solution
SV Minicolumn	Nuclease-Free Water
Collection Tube	

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

Ingredients	Conc. (mg/L)	Ingredients	Conc. (mg/L)	Ingredients	Conc. (mg/L)
Amine Acids					
Glyscine	30.0	L-Isoleucine	105.0	L-Serine	42.0
L-Arginine HCl	84.0	L-Leucine	105.0	L-Threonine	95.0
L-Cystine 2HCl	63.0	L-Lysine HCl	146.0	L-Tryptophan	16.0
L-Glutamine HCl	584.0	L-Methionine	30.0	L-Tyrosine	104.0
L-Histidine HCl	42.0	L-Phenylealanine	66.0	L-Valine	94.0
Vitamins					
Choline Chloride	4.0	Folic Acid	4.0	Riboflavin	0.4
D-Calcium Pantothenate	4.0	Niacinamide	4.0	Thiamine HCl	4.0
		Pyridoxine HCl	4.0	i-Inocitol	7.2
Inorganic Salts					
Calcium Chloride	200.0	Potassium Chloride	400.0	Sodium Chloride	6400.0
Ferric Nitrate	0.1	Sodium Bicarbonate	3700.0	Sodium Phosphate	125.0
Magnesium Sulfate	97.67				
Other Components					
D-glucole (Dextrose)	4500.0	Phenol Red	15.0		

Appendix-II

Standard Supply of Instrument and Equipment List

Instruments	Origin	Instruments	Origin
AlphaImager HP System Versatile Gel Imaging	Cell Bioscience, USA	Microwave oven, Model: D90N30 ATP	Butterfly, China
Autoclave, Model no: HL-42AE	Hirayama corp., Japan	NanoDrop 2000	Thermo Scientific, USA
Microcentrifuge (temperature controlled)	Sigma, USA	*Veriti 96-Well Thermal Cycler*	Thermo Fisher Scientific, USA
Class II Microbiological Safety Cabinet	Nuaire, USA	*ProFlex™ PCR System*	Thermo Fisher Scientific, USA
Electric balance, Scout, SC4010	Shimadzu, Japan	Power Pack	Toledo, Germany
Freezer (-30°C)	Liebherr, Germany	Refrigerator, 4^0C	Vestfrost, Denmark
Horizontal Gel Elctrophoresis Apparatus Hl-SET	CBS Scientific, UK	Water bath, Model:SUM	England
Microcentrifuge	Mikro20, Germany	-80º C Freezer	Nuaire, USA
Microcentrifuge tube	Eppendorf, Germany	Maxwell^R 16 Instrument	Promega, USA
Micropipettes	Eppendorf, Germany	Digital Camera	Germany
Inverted Microscope	Leica, Germany		

Appendix III

Chi-square test results:

Chi-Square Tests (Season)

Chi-square test results for testing the significance of the association of season with FMD cases calculated in SPSS Version 26.0

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	15.254[a]	2	.000
Likelihood Ratio	15.098	2	.001
Linear-by-Linear Association	.789	1	.375
N of Valid Cases	213		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 23.67.

Chi SquarenTests (Age)

Chi-square test results for testing the significance of the association of age with FMD cases calculated in SPSS Version 26.0

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	195.092[a]	2	.000
Likelihood Ratio	197.847	2	.000
Linear-by-Linear Association	186.968	1	.000
N of Valid Cases	3580		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 220.37.

Chi-Square Tests (Gender)

Supplementary Table S3. Chi-square test results for testing the significance of the association of gender with FMD cases calculated in SPSS Version 26.0

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	26.603[a]	1	.000		
Continuity Correction[b]	26.256	1	.000		
Likelihood Ratio	26.664	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	26.595	1	.000		
N of Valid Cases	3580				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 738.50.

b.Computed only for a 2x2 table

Chi-Square Tests (Breed)

Chi-square test results for testing the significance of the association of breed with FMD cases calculated in SPSS Version 26.0

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2.477[a]	1	.116		
Continuity Correction[b]	2.368	1	.124		
Likelihood Ratio	2.475	1	.116		
Fisher's Exact Test				.117	.062
Linear-by-Linear Association	2.476	1	.116		
N of Valid Cases	3580				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 591.44.

b. Computed only for a 2x2 table

Chi-Square Tests (Farming system)

Chi-square test results for testing the significance of the association of farming system with FMD cases calculated in SPSS Version 26.0

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.054[a]	1	.044		
Continuity Correction[b]	3.871	1	.049		
Likelihood Ratio	4.041	1	.044		
Fisher's Exact Test				.048	.025
Linear-by-Linear Association	4.053	1	.044		
N of Valid Cases	3580				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 256.12.

b. Computed only for a 2x2 table

Chi-Square Tests (Vaccination Status)

Chi-square test results for testing the significance of the association of vaccination status with FMD cases calculated in SPSS Version 26.0

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	69.728[a]	1	.000		
Continuity Correction[b]	69.112	1	.000		
Likelihood Ratio	69.580	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	69.708	1	.000		
N of Valid Cases	3580				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 471.07.

b. Computed only for a 2x2 table

Appendix-IV

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	-	-
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	-	-
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
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

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
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
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
BAN/DH/Sa-321/2018	+ve	+ve	+ve	Not done	Asia1	Not submitted
BAN/DH/Sa-323/2018	+ve	+ve	+ve	Not done	Asia1	Not submitted
Dhaka(August 2019)						
BAN/DH/Dh-327/2019	-	+	+		O	-
Rajbari(September 2019)						
BAN/RAJ/Pa-329/2019	+	+	-		O	**OP320415.1**
BAN/RAJ/Pa-332/2019	-	+	+		O	**OP320416.1**
BAN/RAJ/Pan-333/2019	-	+	Not done		O	**OP320417.1**
BAN/RAJ/Kal-334/2019	+	+	+	O		-
BAN/RAJ/Pa-335/2019	Not done	+	+		O	**OP320418.1**
Manikgonj(September 2019)						
BAN/MA/Sh-341/2019	+	+	-		O	**OP320419.1**
BAN/MA/Sa-343/2019	-	+	+		O	**OP320420.1**
Pabna (September 2019)						
BAN/PA/At-347/2019	+	+	-		O	**OP320421.1**
BAN/PA/At-348/2019	+	+	+		O	**OP320422.1**
BAN/PA/At-350/2019	+	-	-		O	**OP320423.1**
BAN/PA/At-351/2019	+	+	+		O	-
BAN/PA/At-352/2019	-	+	+		O	**OP320424.1**
BAN/PA/At-355/2019	+	+	+		O	**OP320425.1**
BAN/PA/At-357/2019	+	+	-		O	**OP320426.1**
Dhaka (October 2019)						
BAN/DH/Dh-359/2019	--	+	+		O	**OP320427.1**
BAN/DH/Dh-360/2019	-	+	+		A	**OP320428.1**
BAN/DH/Dh-362/2019	+	-	+		A	**OP320429.1**
BAN/DH/Dh-363/2019	+	+	-		O	**OP320430.1**
BAN/DH/Dh-364/2019	+	-	+			-
Manikgonj(October 2019)						
BAN/MA/Ma-365/2019	+	+	-		O	**OP320431.1**
BAN/MA/Ma-366/2019	+	+	+		O	**OP320432.1**
Dhaka (October 2019)						
BAN/DH/Dh-367/2019	+	+	+		O	-
Manikgonj(October 2019)						
BAN/MA/Si-368/2019	-	+	+			-
BAN/MA/Si-369/2019	+	-	+		O	**OP320433.1**
Dhaka(October 2019)						
BAN/DH/Dh-371/2019	+	-	+		Not	-

					done	
Dhaka (January 2020)						
BAN/DH/Dh-374/2020	+	+	+		Not done	-
BAN/DH/Dh-375/2020	+	+	-		Not done	-
BAN/DH/Dh-377/2020	-	+	+		O	**OP320434.1**
Chandpur (March 2020)						
BAN/CH/Ch-378/2020	-	+	+		Not done	-
BAN/CH/Ch-381/2020	-	+	+		A	**OP320435.1**
BAN/CH/Ch-386/2020	+	+	+		A	**OP320429.1**
BAN/CH/Ch-387/2020	+	-	+		Not done	-
Dhaka(November 2020)						
BAN/DH/Ke-388/2020	+	+	+		Not done	-
BAN/DH/Ke-389/2020	-	+	+		Not done	-
BAN/DH/Ke-390/2020	+	+	-		Not done	-
BAN/DH/Ke-391/2020	+	-	+		A	**OP320435.1**
BAN/DH/Ke-392/2020	+	-	+		Not done	-
BAN/DH/Ke-393/2020	-	+	+		O	**OP320438.1**
BAN/DH/Ke-394/2020	+	+	+		Not done	-
BAN/DH/Ke-395/2020	-	+	+		O	**OP320439.1**
Tangail (November 2020)						
BAN/TA/Mi-396/2020	+	+	+		O	**OP271696.1**
BAN/TA/Mi-397/2020	-	+	+		O	-
BAN/TA/Mi-398/2020	-	+	-		Not done	-
Dhaka(2021)						
BAN/DH/Sa-400/2020	+	+	+		O	**OP320440.1**
BAN/DH/Sa-401/2020	+	+	-		Not done	-
Manikgonj(January 2021)						
BAN/MA/Sh-402/2021	+	+	+		Not done	-
BAN/MA/Sh-403/2021	+	-	+		Not done	-
Dhaka(February)						
BAN/DH/Dh-410/2021	+	+	+		O	**OP271697.1**
BAN/DH/Dh-416/2021	-	+	+		O	**OP320441.1**
BAN/DH/Dh-417/2021	-	+	+		O	**OP320442.1**
BAN/DH/Dh-418/2021	-	+	+		O	**OP320443.1**
Satkhira(March 2021)						
BAN/SA/Sa-423/2021	+	+	-		O	**OP320444.1**
BAN/SA/Sa-426/2021	+	-	+		O	**OP320445.1**
BAN/SA/Sa-430/2021	-	-	+		Not done	-
BAN/SA/Sa-431/2021	+	+	+		O	**OP320446.1**
BAN/SA/Sa-432/2021	+	+	+		O	**OP320447.1**
BAN/SA/Sa-433/2021	-	+	+		O	**OP320448.1**

BAN/SA/Sa-440/2021	+	+	+		O	**OP320449.1**
BAN/SA/Sa-446/20021	+	+	-		Not done	-
BAN/SA/Sa-447/2021	-	+	+		Not done	-
Jhenaidah(March 2021)						
BAN/JH/Jh-452/2021	+	+	+		Not done	-
BAN/JH/Jh-453/2021	+	+	+		Not done	-
BAN/JH/Jh-454/2021	+	+	+		O	**OP320450.1**
BAN/JH/Jh-455/2021	+	+	+		O	**OP320451.1**
BAN/JH/Jh-456/2021	-	-	+		Not done	-
BAN/JH/Jh-457/2021	+	-	+		O	**OP320452.1**
BAN/JH/Jh-458/2021	-	-	+		Not done	-
BAN/JH/Jh-459/2021	+	+	-		Not done	-
BAN/JH/Ka-461/2021	+	+	-		O	**OP320453.1**
BAN/JH/Ka-462/2021	-	+	+		Not done	-
BAN/JH/Ka-463/2021	-	+	+		Not done	-
BAN/JH/Ka-464/2021	+	+	-		O	**OP320454.1**
Mymensingh(December 2021)						
BAN/MY/My466/2021	+	+	+		O	**OP320455.1**
BAN/MY/My467/2021	+	+	+		Not done	-
BAN/MY/My/469/2021	+	+	-		O	**OP320456.1**
BAN/MY/My/470/2021	+	+	+		Not done	-
BAN/MY/My/471/2021	-	+	+		Not done	-
BAN/MY/My/472/2021	-	+	+		Not done	-
BAN/MY/My/473/2021	+	+	-		Not done	-
BAN/MY/My/474/2021	+	-	+		Not done	-
BAN/MY/My/475/2021	-	+	+		Not done	-
BAN/MY/My/476/2021	-	+	+		Not done	-
BAN/MY/My/477/2021	+	+	+		Not done	-
BAN/MY/My/478/2021	-	+	+		O	**OP320457.1**
BAN/MY/My/479/2021	+	-	+		Not done	-
BAN/MY/My/480/2021	-	+	+		O	**OP320458.1**
BAN/MY/My/481/2021	-	+	+		Not done	-

<u>Appendix-V</u>

List of the FMDV VP1 sequences reported from Bangladesh during 2012-2021

Sequence ID	GenBank Accession No.	Sero type	Topo type	Line age	Sublineage	Reference	Sample source
BAN FA Ka-01 2012	KC795956.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN FA Kh-05 2012	KC795947.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN FA Do-11 2012	KJ175178.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN FA Do-12 2012	KJ175179.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN PA Ra-05 2012	KC795957.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN PA Sa-12 2012	KC795958.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN PA Kg-16 2012	KC795959.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN PA Kg-20 2012	KJ175180.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/GA/Kk-192/2013	KY077603.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/SI/Sh-234/2015	KY077610.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/GO/Ka-236(Pig)/2015	KX712091.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1128/genomeA.01150-16	MGBL lab, University of Dhaka
BAN/NL/Lo-245/2015	KY077611.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/LK/Sa-248/2015	KY077612.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/LK/Sa-249/2015	KY077613.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/NO/Be-250/2015	KY077614.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/NO/Be-251/2015	KY077615.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/DI/Sa-252/2015	KY077616.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/DI/Sa-254/2015	KY077617.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/PG/At-262/2015	KY077618.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/PG/At-264/2015	KY077619.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/TG/Ba-265/2015	KY077620.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/TG/Ba-268/2015	KY077621.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/MA/Ku-269/2015	KY077622.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/LA/Ad-278/2016	KY077623.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/KU/Fu-283/2016	KY077624.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/MG/Sa-287/2016	KY077625.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/MG/Sa-294/2016	KY077626.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/TA/Dh-299/2016	KY077627.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/TA/Dh-301/2016	MK088170.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.1111/tbed.12834	MGBL lab, University of Dhaka
BAN/RAJ/Pa-329/2019	OP320415.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/RAJ/Pa-	OP320416.1	O	ME-	Ind-	Ind-2001e	our lab -submitted	MGBL lab, University

332/2019			SA	2001	(Ind-2001BD1)	(unpublished)	of Dhaka
BAN/RAJ/Ka-333/2019	OP320417.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/RAJ/Pa-335/2019	OP320418.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MA/Sh-341/2019	OP320419.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MA/Sa-343/2019	OP320420.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/PA/At-347/2019	OP320421.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/PA/At-348/2019	OP320422.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/PA/At-350/2019	OP320423.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/PA/At-352/2019	OP320424.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/PA/At-355/2019	OP320425.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/RAJ/Ka-357/2019	OP320426.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Dh-359/2019	OP320427.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Dh-363/2019	OP320430.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MA/Ma-365/2019	OP320431.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MA/Ma-366/2019	OP320432.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MA/Si-369/2019	OP320433.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Dh-377/2020	OP320434.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Ke-393/2020	OP320438.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Ke-395/2020	OP320439.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/TA/Mi-396/2020	OP271696.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Sa-400/2020	OP320440.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Dh-410/2021	OP271697.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Dh-416/2021	OP320441.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Dh-417/2021	OP320442.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Dh-418/2021	OP320443.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/SA/Sa-423/2021	OP320444.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/SA/Sa-426/2021	OP320445.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/SA/Sa-431/2021	OP320446.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/SA/Sa-432/2021	OP320447.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/SA/Sa-433/2021	OP320448.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/SA/Sa-440/2021	OP320449.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/JH/Jh-454/2021	OP320450.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/JH/Jh-455/2021	OP320451.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka

BAN/JH/Jh-457/2021	OP320452.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/JH/Ka-461/2021	OP320453.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/JH/Ka-464/2021	OP320454.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN LA Du-135 2013	KJ175181.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/NA/Ha-156/2013	KF985189.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN JA Ma-180 2013	KJ175183.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN TA Dh-184 2013	KJ175184.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN TA Dh-185 2013	KJ175176.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN TA Dh-186 2013	KJ175185.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN RA Sa-189 2013	KJ175177.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/GA/Kk-191/2013	KY077602.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/TA/Ma-200/2014	KY077604.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/GA/Ka-212/2014	KY077605.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/GA/Ka-213/2014	KY077606.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/GA/Ka-215/2015	KY077607.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Dh-216/2015	KY077608.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/PA/Ch-228/2015	KY077609.1	O	ME-SA	Ind-2001	Ind-2001d	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN LA Sa-137 2013	KJ175182.1	O	ME-SA	Ind-2001	Ind-2001BD2	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/BO/Na-161/2013	MK071699.1	O	ME-SA	Ind-2001	Ind-2001BD2	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/BO/Na-162/2013	KY077601.1	O	ME-SA	Ind-2001	Ind-2001BD2	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MY/My-466/2021	OP320455.1	O	ME-SA	SA-2018	MYMBD21	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MY/My-469/2021	OP320456.1	O	ME-SA	SA-2018	MYMBD21	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MY/My-478/2021	OP320457.1	O	ME-SA	SA-2018	MYMBD21	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MY/My-480/2021	OP320458.1	O	ME-SA	SA-2018	MYMBD21	our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN CH Ra-02 2012	KC795960.1	A	ASIA	G-VII		https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-08 2012	KC795949.1	A	ASIA	G-VII		https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-10 2012	KC795961.1	A	ASIA	G-VII		https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-13 2012	KC795962.1	A	ASIA	G-VII		https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-14 2012	KC795950.1	A	ASIA	G-VII		https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-15 2012	KC795951.1	A	ASIA	G-VII		https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-16 2012	KC795952.1	A	ASIA	G-VII		https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-18 2012	KC795953.1	A	ASIA	G-VII		https://doi.org/10.1111/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-	KC795954.1	A	ASIA	G-		https://doi.org/10.111	MGBL lab, University

26 2012				VII		1/tbed.12113	of Dhaka
BAN CH Ra-28 2012	KC795955.1	A	ASIA	G-VII		https://doi.org/10.111 1/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-30 2012	KC795963.1	A	ASIA	G-VII		https://doi.org/10.111 1/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-31 2012	KC795964.1	A	ASIA	G-VII		https://doi.org/10.111 1/tbed.12113	MGBL lab, University of Dhaka
BAN CH Ra-39 2012	KC795965.1	A	ASIA	G-VII		https://doi.org/10.111 1/tbed.12113	MGBL lab, University of Dhaka
BAN GA To-02 2012	KC795948.1	A	ASIA	G-VII		https://doi.org/10.111 1/tbed.12113	MGBL lab, University of Dhaka
BAN GA Sa-197 2013	KJ754939.1	A	ASIA	G-VII		https://doi.org/10.112 8/genomeA.00506-14	MGBL lab, University of Dhaka
BAN/CH/Sa-302/2016	KY077629.1	A	ASIA	G-VII		https://doi.org/10.111 1/tbed.12834	MGBL lab, University of Dhaka
BAN/CH/Sa-304/2016	KY077630.2	A	ASIA	G-VII		https://doi.org/10.111 1/tbed.12834	MGBL lab, University of Dhaka
BAN/DH/Sa-307/2016	MN596870.1	A	ASIA	G-VII		our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Dh-360/2019	OP320428.1	A	ASIA	G-VII		our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/MA/Ma-362/2019	OP320429.1	A	ASIA	G-VII		our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/CH/Ch-381/2020	OP320435.1	A	ASIA	G-VII		our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/CH/Ch-386/2020	OP320436.1	A	ASIA	G-VII		our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN/DH/Ke-391/2020	OP320437.1	A	ASIA	G-VII		our lab -submitted (unpublished)	MGBL lab, University of Dhaka
BAN JE Mf-01 2012	KJ175170.1	Asia 1	ASIA	G-VIII		https://doi.org/10.111 1/lam.12354	MGBL lab, University of Dhaka
BAN JE Mf-02 2012	KJ175171.1	Asia 1	ASIA	G-VIII		https://doi.org/10.111 1/lam.12354	MGBL lab, University of Dhaka
BAN JE Mf-03 2012	KJ175172.1	Asia 1	ASIA	G-VIII		https://doi.org/10.111 1/lam.12354	MGBL lab, University of Dhaka
BAN JE Mf-04 2012	KJ175173.1	Asia 1	ASIA	G-VIII		https://doi.org/10.111 1/lam.12354	MGBL lab, University of Dhaka
BAN JE Mf-05 2012	KJ175174.1	Asia 1	ASIA	G-VIII		https://doi.org/10.111 1/lam.12354	MGBL lab, University of Dhaka
BAN JE Mf-06 2012	KJ175175.1	Asia 1	ASIA	G-VIII		https://doi.org/10.111 1/lam.12354	MGBL lab, University of Dhaka
BAN/TA/Ma-167/2013	MF782478.1	Asia 1	ASIA	G-VIII		https://doi.org/10.111 1/lam.12354	MGBL lab, University of Dhaka
BAN GA Sr-187 2013	KJ175186.1	Asia 1	ASIA	G-VIII		https://doi.org/10.111 1/lam.12354	MGBL lab, University of Dhaka
BAN/DH/Sa-318/2018	MH457186.1	Asia 1	ASIA	G-IX		https://doi.org/10.111 1/tbed.13381	MGBL lab, University of Dhaka
BAN/DH/Sa-319/2018	MH457187.1	Asia 1	ASIA	G-IX		https://doi.org/10.111 1/tbed.13381	MGBL lab, University of Dhaka
BAN/BR/Sa-321/2018	MN447113.1	Asia 1	ASIA	G-IX		https://doi.org/10.111 1/tbed.13381	MGBL lab, University of Dhaka
BAN/DH/Sh-323/2018	MN447114.1	Asia 1	ASIA	G-IX		https://doi.org/10.111 1/tbed.13381	MGBL lab, University of Dhaka
BD Gh 2 2013	KT037119.1	O	ME-SA	Ind-2001	Ind-2001d	https://doi.org/10.111 1/tbed.12834	Bangladesh Agricultural University
BD Sl 6 2013	KT037120.1	O	ME-SA	Ind-2001	Ind-2001d	https://doi.org/10.111 1/tbed.12834	Bangladesh Agricultural University
O/BAN/BLRI /450.1/2018	MT316588.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.431 4/ovj.v10i3.14	Bangladesh Livestock Research Institute (BLRI)
O/BAN/BLRI /450.3/2018	MT316589.1	O	ME-SA	Ind-2001	Ind-2001e (Ind-2001BD1)	https://doi.org/10.431 4/ovj.v10i3.14	Bangladesh Livestock Research Institute (BLRI)
BD BAU ML3 2013	KT982204.1	A	ASIA	G-VII		https://doi.org/10.545 5/javar.2020.g429	Bangladesh Agricultural University

BD BAU ML4 2014	**KT982205.1**	**A**	**ASIA**	**G-VII**		**https://doi.org/10.5455/javar.2020.g429**	**Bangladesh Agricultural University**
BD SI 16 2013	**KR869773.1**	**A**	**ASIA**	**G-VII**		**https://doi.org/10.5455/javar.2020.g429**	**Bangladesh Agricultural University**
BD AH 10 2012	**KY421678.1**	**A**	**ASIA**	**G-VII**		**GenBank database**	**Bangladesh Livestock Research Institute (BLRI)**
BD BAU ML5 2013	**KU159763.1**	**Asia 1**	**ASIA**	**G-VIII**		**https://doi.org/10.5455/javar.2020.g429**	**Bangladesh Agricultural University**
BD BAU ML6 2013	**KU159762.1**	**Asia 1**	**ASIA**	**G-VIII**		**https://doi.org/10.5455/javar.2020.g429**	**Bangladesh Agricultural University**
BD SI 2 2013	**KR869774.1**	**Asia 1**	**ASIA**	**G-VIII**		**https://doi.org/10.5455/javar.2020.g429**	**Bangladesh Agricultural University**
BD_SI_5_2013	**KT037118.1**	**O**	**ME-SA**	**unnamed**		**https://doi.org/10.1111/tbed.12834**	**Bangladesh Agricultural University**
BD_BAU_ML1_2013	**KT960948.1**	**O**	**ME-SA**	**unnamed**		**https://doi.org/10.1111/tbed.12834**	**Bangladesh Agricultural University**
BD_BAU_ML2_2013	**KT982203.1**	**O**	**ME-SA**	**unnamed**		**https://doi.org/10.1111/tbed.12834**	**Bangladesh Agricultural University**
O/BAN/BLRI/450.2/2018	**MT316587.1**	**O**	**ME-SA**	**unnamed**		**https://doi.org/10.4314/ovj.v10i3.14**	**Bangladesh Livestock Research Institute (BLRI)**

Appendix VI

Amino acid substitutions in VP1 of FMDV serotypes reported in Bangladesh during 2012-21

Year	Serotype	Amino Acid Substitution
2012	Asia1	A69V
2012	O	E138A
2012	A	G148E
2012	A	T22I
2012	A	Q23K
2012	A	V24I
2012	A	I42L
2012	A	V45T
2012	A	T48I
2012	A	E83D
2012	A	V143T
2012	A	M190L
2012	A	E194D
2012	A	A209T
2013	O	V24A
2013	O	T43A
2013	O	K45Q
2013	O	N100S
2013	O	A110E
2013	O	S139G
2013	O	N140G, D, A
2013	O	P142T
2013	O	A155V
2013	O	A156T
2013	O	P158T
2013	O	A170T
2013	O	K181T
2013	O	A183P
2013	O	T185P
2013	O	Y186H
2013	O	E198Q, D
2013	O	R200T
2013	O	H201P
2013	O	K202Q
2013	O	K204N
2013	O	A207P
2013	O	K210N
2013	O	L212F
2013	O	Q45K
2013	O	N46D
2013	O	A171T
2013	O	D197E

2013	A	V24I
2013	A	I35V
2013	A	I42L
2013	A	V45T,A
2013	A	T48I
2013	A	E83D
2013	A	V143T
2013	A	R168K
2013	A	M190L
2013	A	S196L
2013	A	L213P
2013	Asia1	T4A
2013	Asia1	V24T
2013	Asia1	A44T
2013	Asia1	L80V
2013	Asia1	D96G
2013	Asia1	H108Q
2013	Asia1	E202K
2013	Asia1	V210M
2014	A	V24I
2014	A	I42L
2014	A	V45T
2014	A	T48I
2014	A	E83D
2014	A	V143T
2014	A	M190L
2014	O	E198D
2015	O	G19R
2015	O	T30S
2015	O	Q45K
2015	O	N46D
2015	O	A156T
2015	O	A171T
2015	O	D197E
2015	O	H201R
2015	O	K45Q
2015	O	T68A
2015	O	E95V
2015	O	D99E
2015	O	N100S
2015	O	K135R
2015	O	E138G
2015	O	N140G, D
2015	O	P142T, A
2015	O	E198Q
2015	O	K204R

2016	O	T43I
2016	O	Q45K
2016	O	N46D
2016	O	V62E
2016	O	E138K
2016	O	A171T
2016	O	D197E,G
2016	A	G43V
2016	A	S46G
2016	A	A68G
2016	A	N108Y
2016	A	A110V
2016	A	V143A
2018	O	S33P
2018	O	F39I
2018	O	T43I
2018	O	Q45K
2018	O	N46D
2018	O	V62E
2018	O	A171T
2018	O	D197E
2018	Asia1	T3A
2018	Asia1	V24T
2018	Asia1	T43N
2018	Asia1	A44E
2018	Asia1	N47S
2018	Asia1	I48T
2018	Asia1	T50V
2018	Asia1	D96T
2018	Asia1	H108Q
2018	Asia1	M146L
2018	Asia1	M211L
2019	O	V15L
2019	O	E16R
2019	O	N17T
2019	O	G19R, P
2019	O	G20R
2019	O	K41I
2019	O	T43I
2019	O	Q45K
2019	O	N46D
2019	O	D52K
2019	O	T60N
2019	O	E138K
2019	O	A140T
2019	O	T142A

2019	O	A170S
2019	O	A171T
2019	O	D197E
2019	A	I42L
2019	A	G43V
2019	A	V45T
2019	A	R142H
2019	A	V143T
2019	A	E194K
2019	A	Q198R
2020	O	Q45K
2020	O	N46D
2020	O	K135R
2020	O	A171T
2020	O	V173G
2020	O	K181E
2020	O	D197E
2020	O	K204R
2020	O	L213F
2020	A	G19R
2020	A	N44D
2020	A	V45L
2020	A	S46N
2020	A	E83D
2020	A	N134S
2020	A	R142H
2020	A	V143T
2020	A	R168K
2020	A	M190L
2020	A	S196L
2021	O	Q45K
2021	O	N46D
2021	O	L76I
2021	O	K135R
2021	O	A171T
2021	O	D197E

Appendix-VII

Foot and mouth Disease Virus Serotype O Vaccine Candidate
BAN/TA/Dh-301/2016 Complete Genome

TTGAAAGGGGGCGCTAGGGTCTCACCCTAGCACACCACCGGCAACTCCCGCGTTGCACTCCA
CACTTCGCCCGTGTACTCGCGGGAACCGATGGACTTTCGTTCACCCACCTACAGCTGGACTCA
CGGCACCGCGAGGCCATCTTAGCTGGACTGTGCGGACGAACACCGCTTGCGCATCTCGCGTG
ACCGGTTAATACTCTTACCACTTTCCGCCTGCTTGGTCATTAGCGCTGTCCTGGGCACTCCTGT
TGGGGGCCGTTCGACGCTCCACGGTCTCCCCGCGTAACGGACTACGGTGATGGGGCCGCCT
CGTGCGGGTTGGTCGCTTGGTCTGCTTCGGTTGTTGCTCGAAGCCCGCCTTTCACCCCCCCCCC
CCCCCCAAGTTTTACCGTCGTTCCCGACGTTAAAGGGGTGTAACCACAAGCTTGGAACCGTCT
TGCCCGACGTTAATGGGTTGTAACCACGCGCTTGTACCGCCTTTCCCAGCGCTACCGGGATGT
AACCAAAAGATGAACCTTCACCCGGAAGTAAAACGGCAACCACACTCAGTTTTGCCCGTTTT
CATGAGAAATGGGACGTCAGCGCACGAAACGCGCCGTCGCTTGAGGAAGATTTGTACAAACA
CGATCTATGCAGGCTTCCACAACTGACACGAACCGTGCAATTTGAAGCTCCGCCTGGTCTTTC
CAGGTCTAGAGGGGCAACACTTTGTACTGTGCTTGACTCCACGCTCGGTCCACTAGCGGGTGT
TAGTAACAGCACTGTTGTTTCGTAGCGGAGCATGATGGCCGCGGGAACTCCCCCTTGGTGACA
AGGACCCGCGGGGCCGAAAGCCACGTCCTAACGGACCCATCATGTGTGCAACCCCAGCACGG
CAACTCTACTGTGAAAACCACTTTAAGGTGACACTGATACTGGTACTCAACCACTGGTGACAG
GCTAAGGATGCCCTTCAGGTACCCGAGGTAACAAGTGACACTCGGGATCTGAGAAGGGGAC
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
TTCGGACAGGGTGCTCGCTCAGTTTGACCTGTCTTTGGCAGCAAAGCACATGTCAAACACCTT
CCTGGCAGGTCTCGCCCAGTACTACACACAGTACAGCGGCACCATCAACCTGCATTTCATGTT
CACAGGCCCCACTGACGCGAAAGCGCGTTACATGATTGCATATGCCCCGCCTGGCATGAAGC
CGCCTAAAACACCTGAGGCGGCTGCTCACTGCATTCATGCAGAGTGGGACACAGGGCTGAAC
TCAAAGTTCACATTTTCAATCCCTTACCTTTCGGCGGCTGATTACGCGTACACCGCGTCTGAC
GCTGCCGAAACCACAAATGTGCAGGGATGGGTGTGCTTGTTCCAAATAACACACGGGAAAGC
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TGACGCCCGCACACAGACCACCTCCACAGGTGAGTCCGCTGATCCCGTGACCACCACCGTTG
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GACAGATTTGTGAAAGTAATACCGAAAGACCAAATCAATGTGTTGGACCTGATGCAAACCCC
TGCTCACACTTTGGTAGGCGCACTCCTCCGCACCGCCACTTACTACTTCGCAGACCTAGAAGT
GGCAGTGAAGCACGAGGGCAACCTCACCTGGGTCCCGAACGGGGCGCCCGAGGCGGCGCTG
GACAACACCACCAACCCAACGGCCTACCACAAGGCACCGCTCACCCGTCTTGCTCTGCCTTAC
ACAGCACCACACCGTGTTCTGGCTACTGTTTACAACGGGAACTGCAAGTATGGCAAGGGCGC
TGTGACCAACGTGAGGGGTGACTTGCAAGTGTTGGCTCAGAAGGCAGCAAGAACGCTGCCCA
CCTCCTTTAACTACGGTGCCATCAAGGCTACCCGGGTGACTGAACTGCTTTACCGCATGAAGA
GGGCCGAAACATACTGCCCTCGGCCTCTGCTGGCCATTCACCCGGAACAAGCCAGACACAAG
CAGAAGATTGTGGCACCTGTGAAACAGTTGTTGAATTTCGACCTGCTCAAGTTGGCGGGAGA
CGTTGAGTCCAACCCTGGGCCCTTCTTCTTCTCCGACGTCAGATCAAACTTTTCCAAACTTGTA
GAAACCATCAACCAGATGCAGGAAGACATGTCAACAAAACACGGACCCGACTTTAACCGGTT
GGTGTCCGCGTTTGAGGAATTGGCCACTGGAGTGAAAGCCATCAGGACCGGCCTCGACGAGG
CCAAGCCCTGGTACAAGCTCATCAAACTCCTAAGCCGCCTGTCGTGTATGGCCGCTGTAGCAG
CACGGTCAAAGGACCCAGTCCTTGTGGCCATCATGCTGGCTGACACCGGTCTCGAGATTCTGG
ACAGCACATTTGTCGTGAAGAAGATCTCCGACTCACTCTCCAGTCTCTTTCACGTGCCGGCCC
CTGTCTTCAGTTTCGGAGCCCCGATTCTGTTGGCCGGGTTGGTCAAAGTCGCCTCGAGTTTCTT
CCGGTCCACGCCCGAAGACCTTGAGAGAGCAGAGAAACAGCTCAAAGCACGTGACATCAAC
GACATTTTCGCCATTCTCAAGAACGGCGAGTGGCTGGTCAAGTTGATCCTTGCTATCCGCGAC
TGGATTAAAGCATGGATCGCCTCAGAAGAGAAGTTTGTCACCATGACAGACTTGGTGCCTGG
CATCCTCGAAAAGCAGCGGGACCTCAACGACCCGAGCAAGTACAAGGAAGCCAAGGAGTGG
CTCGACAACGCGCGCCAAGCGTGTTTGAAGAGTGGGAACGTCCACATTGCAAATCTCTGCAA
AGTGGTCGCCCCGGCGCCCAGCAAGTCGAGACCCGAACCTGTAGTCGTTTGCCTCCGCGGCA
AATCTGGCCAGGGCAAGAGTTTCCTTGCGAACGTGCTCGCACAAGCAATCTCCACTCACTTCA
CTGGCAGAATCGATTCAGTTTGGTACTGTCCACCTGACCCCGACCACTTCGACGGTTACAACC
AACAGACCGTTGTCGTGATGGACGATTTGGGCCAGAACCCTGACGGCAAGGACTTCAAGTAC
TTTGCACAGATGGTTTCCACCACAGGGTTCATCCCGCCCATGGCCTCGCTCGAGGACAAAGGT
AAGCCTTTTAACAGTAAGGTCATCATAGCTACCACCAACCTGTACTCGGGGTTCACCCCGAGA
ACAATGGTGTGCCCTGATGCACTGAACCGCAGGTTTCACTTTGACATTGATGTGAGCGCCAAG
GATGGGTACAAAATTAACAACAAATTGGACATAATCAAAGCTCTTGAAGACACCCACACTAA
CCCAGTGGCAATGTTTCAATATGACTGTGCCCTTCTCAACGGCATGGCCGTTGAAATGAAGAG
AATGCAACAAGACATGTTCAAGCCTCAACCACCCCTCCAGAACGTCTACCAACTTGTTCAGG
AGGTGATTGAACGGGTCGAGCTCCACGAGAAAGTGTCGAGCCACCCAATCTTCAAGCAGATC
TCAATTCCTTCCCAAAAATCTGTGTTGTACTTCCTCATTGAGAAAGGTCAGCACGAAGCAGCA
ATTGAATTCTTTGAGGGGATGGTCCACGACTCCATCAAGGAGGAGCTCCGACCCCTCATCCAA
CAGACATCATTTGTGAAACGCGCTTTCAAGCGCCTGAAGGAAAATTTTGAGATTGTTGCCCTG
TGTTTGACTCTCCTGGCAAACATAGTGATTATGATCCGCGAGACTCGCAAGAGACAGCAGAT
GGTGGATAACGCAGTGAATGAATACATTGAGAAAGCAAACATCACCACGGATGACAAGACT
CTTGACGAGGCGGAAAAGAACCCTCTGGAGACCAGCGGTGCCAGCACCGTCGGTTTCAGAGA
GAGAACTCTCCCAGGACACAAGGCGAGTGGTGACGTGAACTCCGAACCCGCCAAACCTGTGG
AGGAACAACCACAAGCTGAAGGACCCTACGCCGGACCACTTGAGCGCCAGAAACCTCTGAA
AGTGCGCGCCAAACTGCCACAACAAGAGGGGCCTTACGCTGGTCCGATGGAGAGACAGAAA
CCACTGAAAGTGAAAGCAAAAGCCCCGGTCGTTAAGGAAGGACCTTACGAGGGGCCAGTGA
AGAAGCCTGTCGCTTTGAAAGTGAAAGCAAAGAACTTGATTGTCACTGAGAGTGGCGCCCCA
CCGACCGACTTGCAAAAGATGGTTATGGGCAACACAAAGCCTGTTGAGCTCATCCTCGACGG
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ACTTTACCTGCGTTGGGTGAACGCCGTGTGCGGTGACGCATAATCCCTTAGAGACCACATTGG
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TCCTATTCCAAAAAAAAAAAAAAAAAAAAAAAAAA

Appendix VIII

BAN/MY/My-466/2021, complete genome (accession no. OP957418)

TTGAAAGGGGGCGCTAGGGTCTCACCCCCAGTATGCCACCGGCAGCTCCTGCGTTGCACTCCACAC
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AAAAAAAAAA

Appendix IX

Evolutionary Divergence between established sub-lineages of Ind-2001 lineage and groups of isolates calculated in MEGA11.

	1	2	3	4	5	6	7	8	9
1. 2020-21 isolates		0.010	0.007	0.013	0.016	0.015	0.017	0.014	0.009
2. 2019 isolates	0.058		0.006	0.014	0.017	0.015	0.018	0.013	0.003
3. Ind-2001BD1	0.035	0.032		0.012	0.015	0.013	0.016	0.012	0.005
4. Ind-2001BD2	0.084	0.090	0.079		0.013	0.011	0.014	0.011	0.013
5. Ind-2001a	0.114	0.118	0.103	0.079		0.008	0.011	0.012	0.015
6. Ind-2001b	0.105	0.103	0.089	0.068	0.044		0.012	0.013	0.013
7. Ind-2001c	0.119	0.124	0.115	0.095	0.069	0.078		0.014	0.016
8. Ind-2001d	0.099	0.091	0.085	0.073	0.088	0.092	0.106		0.011
9. Ind-2001e	0.046	0.018	0.026	0.084	0.105	0.093	0.114	0.082	

Supplementary Table S3 Evolutionary Divergence among groups of established lineages (PanAsia, PanAsia-2, SA-2018) of serotype O, MYMBD21 and uncharacterized Bangladeshi isolates calculated in MEGA11:

	1	2	3	4	5
1. PanAsia		0.009	0.013	0.015	0.014
2. PanAsia-2	0.102		0.013	0.014	0.012
3. Uncharacterized BDisolates	0.110	0.119		0.014	0.013
4. SA-2018	0.129	0.124	0.101		0.010
5. MYMBD21	0.108	0.105	0.089	0.062	

Appendix X

Mutational Analysis

➢ **Variations in the VP1 nucleotide sequence of MYMBD21 with SA-2018:**

> ### MYMBD21 vs SA-2018

Range 1: 1 to 639 Graphics   ▼ Next Match ▲

NW Score	Identities	Gaps	Strand
1123	608/639(95%)	0/639(0%)	Plus/Plus

```
Query    1   ACAACCTCCACAGGTGAGTCGGCTGACCCCGTGACCGCCACCGTTGAGAACTACGGGGGC   60
Sbjct    1   .......................T.......T...........A........A...   60

Query   61   GAAACACAGGTCCAGAGACGTCAGCACACGGACGTTTCTTTCATATTGGACAGATTTGTG   120
Sbjct   61   ..G................C..............C.........................A   120

Query  121   AAAGTGACACCAAAAGACCAAATCAATGTATTGGACCTGATGCAAACCCCCGCTCACACT   180
Sbjct  121   ....................T.....C..................T.........   180

Query  181   TTGGTGGGTGCCCTTCTTCGCACCGCTACCTACTACTTCGCAGATTTAGAGGTGGCAGTG   240
Sbjct  181   ................C.......C.......................   240

Query  241   AAACACGAGGGGGACCTCACCTGGGTCCCGAACGGAGCACCCGAAAAAGCCTTGGACAAC   300
Sbjct  241   .....T......A................G..G...........   300

Query  301   ACCACTAATCCAACGGCTTACCACAAGGCACCACTCACCCGACTTGCACTGCCGTACACG   360
Sbjct  301   .....C.................G...................   360

Query  361   GCACCACACCGTGTCTTGGCTACTGTCTACAACGGGAACTGCAAGTACGGCGAGAGCCGC   420
Sbjct  361   .....C.........A..........T...................A.   420

Query  421   GCAACTAGTGTGAGAGGTGACCTGCAAGTGTTGGCCCAGAAGGCGGCAAGGACGCTGCCT   480
Sbjct  421   ........C..........................A.............   480

Query  481   ACCTCCTTTAACTATGGTGCCATCAAAGCTACTCGGGTGACTGAACTGCTTTACCGCATG   540
Sbjct  481   ........C.....C........................T......   540

Query  541   AAGAGGGCTGAAACATACTGCCCTCGGCCTCTTCTGGCCATCCACCCGAGTGAAGCTAGA   600
Sbjct  541   ..........................T..........   600

Query  601   CACAAACAGAAGATTGTGGCACCTGTGAAACAACTTCTG   639
Sbjct  601   .......A.................G.........   639
```

S3 Nucleotide changes of MYMBD21 against SA-2018 consensus VP1 sequences (5% divergence) calculated using Needleman-Wunsch algorithm in BLAST global alignment tool [Subject=MYMBD21; Query: SA-2018]
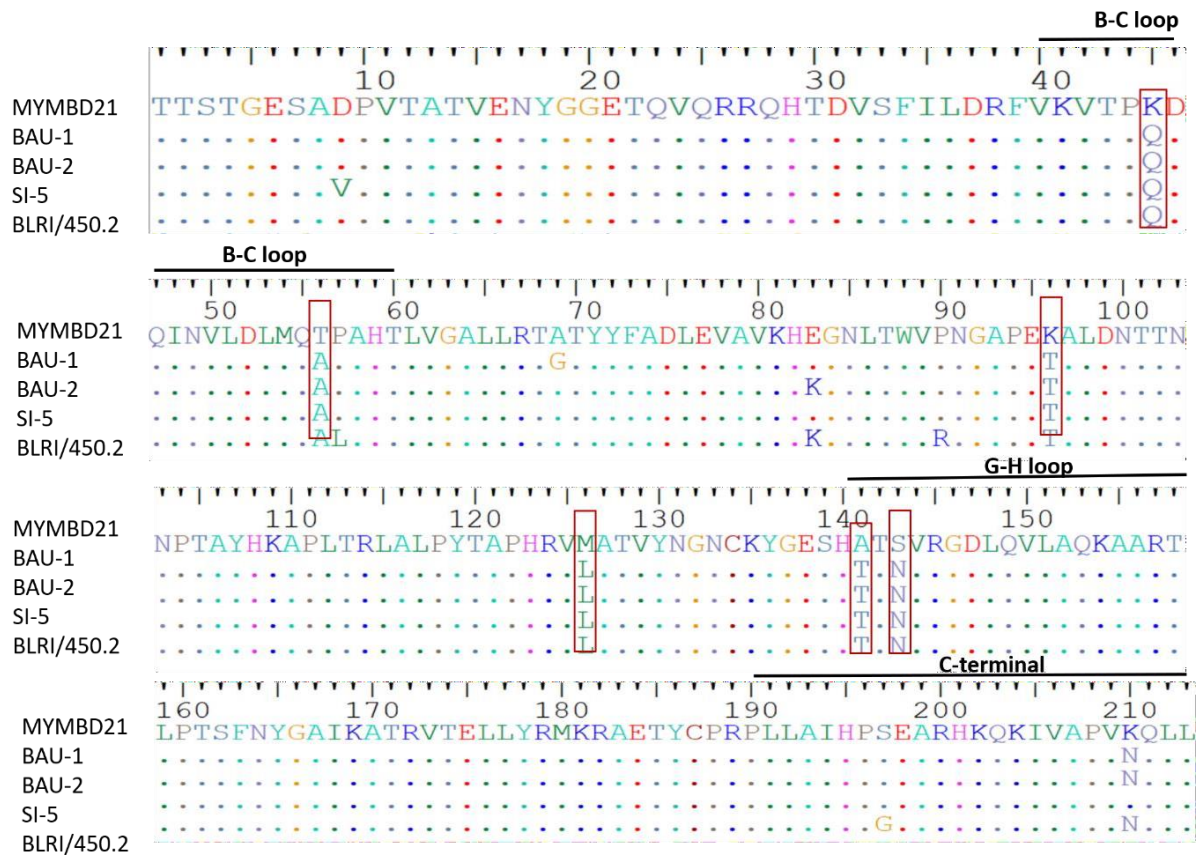
Variations in the VP1 nucleotide sequence of MYMBD21 with uncategorized Bangladeshi isolates:

MYMBD21 vs BD_BAU_ML1_2013 (BAU-1)

Range 1: 1 to 639 Graphics   ▼ Next Match ▲

NW Score	Identities	Gaps	Strand
1033	590/639(92%)	0/639(0%)	Plus/Plus

```
Query   1    ACCACCTCCACAGGTGAGTCAGCTGACCCCGTGACTGCCACTGTTGAGAACTACGGTGGC   60
Sbjct   1    ..A...............G....T...........C.....A........A...        60

Query   61   GAGACGCAGGTCCAGAGACGCCAGCACACGGATGTCTCTTTCATATTGGACAGATTTGTA  120
Sbjct   61   .....A...................C..................................  120

Query   121  AAGGTAACACCACAAGACCAAATCAATGTACTGGACCTGATGCAAGCCCCTGCTCACACT  180
Sbjct   121  ..A..G......A.......T..................A...................  180

Query   181  TTGGTGGGTGCCCTTCTTCGCACCGGCACCTACTATTTCGCAGATTTAGAGGTGGCGGTG  240
Sbjct   181  ...............C.......C.......C...............A...          240

Query   241  AAACACGAAGGGAACCTTACCTGGGTCCCGAATGGAGCACCCGAAACGGCCTTGGACAAC  300
Sbjct   241  .....T..G......C...........C..G..G.......AA...............  300

Query   301  ACCACCAATCCAACGGCCTACCACAAGGCACCACTCACCCGGCTTGCGCTGCCTTACACG  360
Sbjct   301  ...............T.......G.......A....A.....G.....          360

Query   361  GCACCCCACCGTGTTTTAGCTACTGTTTACAACGGGAACTGCAAGTATGGCGAGAGCCAC  420
Sbjct   361  ...........CA.G.......................C.............        420

Query   421  ACAACCAATGTGAGAGGTGACCTGCAAGTGTTGGCCCAGAAAGCGGCAAGAACGCTGCCT  480
Sbjct   421  G....T.GC....................G..A.....G.........           480

Query   481  ACCTCCTTCAACTACGGTGCCATCAAAGCCACTCGGGTGACTGAACTGCTTTACCGCATG  540
Sbjct   481  ....................T....................T...             540

Query   541  AAGAGGGCTGAAACATACTGCCCTCGGCCTCTTCTAGCCATCCACCCGAGCGAAGCTAGA  600
Sbjct   541  ...................T.G...............T.....                600

Query   601  CACAAACAAAAGATTGTGGCACCTGTGAACCAGCTTTTG  639
Sbjct   601  ...........................G..A...C..   639
```

MYMBD21 vs BD_BAU_ML2_2013 (BAU-2)

Range 1: 1 to 639 Graphics   ▼ Next Match ▲

NW Score	Identities	Gaps	Strand
1038	591/639(92%)	0/639(0%)	Plus/Plus

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Query   1    ACCACCTCCACAGGTGAGTCAGCTGACCCCGTGACTGCCACTGTTGAGAACTACGGTGGC   60
Sbjct   1    ..A...............G....T...........C.....A........A...        60

Query   61   GAGACGCAGGTCCAGAGACGCCAGCACACGGATGTCTCTTTCATATTGGACAGATTTGTA  120
Sbjct   61   .....A...................C..................................  120

Query   121  AAGGTAACACCACAAGACCAAATCAATGTACTGGACCTGATGCAAGCCCCTGCTCACACT  180
Sbjct   121  ..A..G......A.......T..................A...................  180

Query   181  TTGGTGGGTGCCCTTCTTCGCACCGGCACCTACTATTTCGCAGATTTAGAGGTGGCGGTG  240
Sbjct   181  ...............C...........C...............A...          240

Query   241  AAACACAAAGGGAACCTTACCTGGGTCCCGAATGGAGCACCCGAAACGGCCTTGGACAAC  300
Sbjct   241  .....TG.G.......C.......C..G..G.......AA...............  300

Query   301  ACCACCAATCCAACGGCCTACCACAAGGCACCACTCACCCGGCTTGCACTGCCTTACACG  360
Sbjct   301  ...............T.......G.......A.............G.....        360

Query   361  GCACCCCACCGTGTTTTAGCTACTGTTTACAACGGGAACTGCAAGTATGGCGAGAGCCAC  420
Sbjct   361  ...........CA.G.......................C.............        420

Query   421  ACAACCAATGTGAGAGGTGACCTGCAAGTGTTGGCCCAGAAAGCGGCAAGAACGCTGCCT  480
Sbjct   421  G....T.GC....................G..A.....G.........           480

Query   481  ACCTCCTTCAACTACGGTGCCATCAAAGCCACTCGGGTGACTGAACTGCTTTACCGCATG  540
Sbjct   481  ....................T....................T...             540

Query   541  AAGAGGGCTGAAACATACTGCCCTCGGCCTCTTCTAGCCATCCACCCGAGCGAAGCTAGA  600
Sbjct   541  ...................T.G...............T.....                600

Query   601  CACAAACAAAAGATTGTGGCACCTGTGAACCAGCTTTTG  639
Sbjct   601  ...........................G..A...C..   639
```

MYMBD21 vs BD_SI_5_2013 (SI-5)

Range 1: 1 to 639 Graphics   ▼ Next Match

NW Score	Identities	Gaps	Strand
1033	590/639(92%)	0/639(0%)	Plus/Plus

```
Query   1    ACCACCTCCACAGGTGAGTCAGCTGTCCCCGTGACTGCCACTGTTGAGAACTACGGTGGC   60
Sbjct   1    ..A...............G...AT...........C.....A........A...        60

Query   61   GAGACGCAGGTCCAGAGACGCCAGCACACGGATGTCTCTTTCATATTGGACAGATTTGTA  120
Sbjct   61   .....A...................C..................................  120

Query   121  AAGGTAACACCACAAGACCAAATCAATGTATTGGACCTGATGCAAGCCCCTGCTCACACT  180
Sbjct   121  ..A..G......A.......T......C..................A...........  180

Query   181  TTGGTGGGTGCCCTTCTTCGCACCGGCACCTACTATTTCGCAGATTTAGAGGTGGCGGTG  240
Sbjct   181  ...............C...........................A...          240

Query   241  AAACACGAAGGGAACCTTACCTGGGTCCCGAATGGAGCACCCGAAACGGCCTTGGACAAC  300
Sbjct   241  .....T..G......C...........C..G..G.......AA...............  300

Query   301  ACCACTAATCCAACGGCCTACCACAAGGCACCACTCACCCGGCTTGCACTGCCTTACACG  360
Sbjct   301  .....C.......T.......G.......A.............G.....        360

Query   361  GCACCCCACCGTGTTTTTGGCTACTGTTTACAACGGGAACTGCAAGTATGGCGAGAGCCAC  420
Sbjct   361  ...........CA.....................................        420

Query   421  ACAACCAACGTGAGAGGTGACCTGCAAGTGTTGGCCCAGAAAGCGGCAAGAACGCTGCCT  480
Sbjct   421  G....T.G....................G..A.....G.........           480

Query   481  ACCTCCTTCAACTACGGTGCCATCAAAGCCACTCGGGTGACTGAACTGCTTTACCGCATG  540
Sbjct   481  ....................T....................               540

Query   541  AAGAGGGCTGAAACATACTGTCCTCGGCCTCTTCTAGCCATCCACCCGAGCGAAGCTAGA  600
Sbjct   541  ...................C...........T.G...........T.........      600

Query   601  CACAAACAAAAGATTGTGGCACCTGTGAACCAGCTTTTG  639
Sbjct   601  ...........................G..A...C..   639
```

MYMBD21 vs O/BAN/BLRI/450.2/2018 (BLRI/450.2)

NW Score	Identities	Gaps	Strand
1023	588/639(92%)	0/639(0%)	Plus/Plus

```
Query   1    ACCACCTCCACAGGTGAGTCAGCTGACCCCGTGACTGCCACTGTTGAGAACTACGGTGGC   60
Sbjct   1    ..A...............G....T...........C.....A........A...        60

Query   61   GAGACGCAGGTCCAGAGACGCCAGCACACGGATGTCTCTTTCATATTGGACAGATTTGTA  120
Sbjct   61   .....A...................C..................................  120

Query   121  AAGGTAACACCACAAGACCAAATCAATGTACTGGACCTGATGCAAGCCCCTTGCTCACACT  180
Sbjct   121  ..A..G......A.......T..................A...C...........  180

Query   181  TTGGTGGGTGCCCTTCTTCGCACCGCCACCTACTATTTCGCAGATTTAGAGGTGGCGGTG  240
Sbjct   181  ...............C...........................A...          240

Query   241  AAACACAAAGGGAACCTTACCTGGGTCCGGAATGGAGCACCCGAAACGGCCTTGGACAAC  300
Sbjct   241  .....TG.G.......C..........C..C..G..G.......AA...........  300

Query   301  ACCACCAATCCAACGGCCTACCACAAGGCACCACTCACCCGGCTTGCACTGCCTTACACG  360
Sbjct   301  ...............T.......G.......A.............G.....        360

Query   361  GCACCCCACCGTGTTTTAGCTACTGTTTACAACGGGAACTGCAAGTATGGCGAGAGCCAC  420
Sbjct   361  ...........CA.G.......................C.............        420

Query   421  ACAACCAATGTGAGAGGTGACCTGCAAGTGTTGGCCCAGAAAGCGGCAAGAACGCTGCCT  480
Sbjct   421  G....T.GC....................G..A.....G.........           480

Query   481  ACCTCCTTCAACTACGGTGCCATCAAAGCCACTCGGGTGACTGAACTGCTTTACCGCATG  540
Sbjct   481  ....................T....................T...             540

Query   541  AAGAGGGCTGAAACATACTGCCCTCGGCCTCTTCTAGCCATCCACCCGGGCGAAGCTAGA  600
Sbjct   541  ...................T.G...........A.T.........              600

Query   601  CACAAACAAAAGATTGTGGCACCTGTGAACCAGCTTTTG  639
Sbjct   601  ...........................G..A...C..   639
```

S4 Nucleotide changes of VP1 of MYMBD21 against uncharacterized Bangladeshi isolates (BD_BAU_ML1_2013; BD_BAU_ML2_2013; BD_SI_5_2013; O/BAN/BLRI/450.2/2018) showing 92%identity or 8% divergence calculated using Needleman-Wunsch algorithm [7] in BLAST global alignment tool. [Subject=MYMBD21; Query: BAU-1; BAU-2, SI-5; BLRI/450.2]

Amino acid variations

Supplementary Figure S5 Amino acid variations against MYMBD21 against uncharacterized Bangladeshi isolates that were reported during 2013 and 2018. VP1 amino acid sequences were aligned and viewed using BioEdit B-C loop:40-60 amino acid; G-H loop:130-160 amino acid; Cterminal:190-213 amino acid [BAU-1: BD_BAU_ML1_2013; BAU-2: BD_BAU_ML1_2013; SI-5:BD_SI_5_2013; BLRI/450.2:O/BAN/BLRI/450.2/2018]

❖ **Comparison with vaccine strains**

Variations in the VP1 nucleotide sequence of MYMBD21 with both current field vaccine and proposed local vaccine strains:

```
Range 1: 1 to 639  Graphics                                      ▼ Next Match

NW Score        Identities            Gaps             Strand
908             565/639(88%)          0/639(0%)        Plus/Plus

Query  1    ACCACCTCCACAGGTGAGTCAGCTGACCCCGTGACCGCCACTGTTGAAAACTACGGCGGT  60
Sbjct  1    ..A...............G....T........T....C..............A..C     60

Query  61   GAGACACAGGTCCAGAGGCGCCAACACACGGACGTCTCATTCATTTTGGACAGATTTGTA  120
Sbjct  61   ................A.....G..............T.....A..............    120

Query  121  AAAGTGACGCCAAAAGACCAAATTAATGTACTGGACCTGATGCAAACCCCCGCTCACACT  180
Sbjct  121  ........A............................................T.......  180

Query  181  CTGGTGGGAGCGCTCCTTCGTACTGCCACTTACTATTTCGCTGACTTAGAAGTGGCAGTG  240
Sbjct  181  T.......T..C..T..C..C..C.....C.....C.....A..T.....G.........  240

Query  241  AAATACGAGGGAAACCTCACTTGGGTCCCGAATGGGGCGCCTGAAAACGCGTTGGATAAC  300
Sbjct  241  ...C.T.....G.......C............C.....C.....A..C.....C...     300

Query  301  ACCACCAACCCAACGGCATACCACAAGGCACCACTCACCCGGCTTGCATTGCCGTACACG  360
Sbjct  301  ........T.......T..........G.....A.....C...........         360

Query  361  GCACCACAACGTGTGTTGGCAACCGTTTACAACGGGAACTGCAAGTACGGTGATGGTTCG  420
Sbjct  361  .....C..C.....CA....T..T.....................C..GA.CCAC      420

Query  421  GTGACCAACATAAGAGGTGACCTACAAGTGTTGGCCCAGAAGGCGGCGAGAACGCTGCCT  480
Sbjct  421  .CA..T.G.G.G.........G....................A..A..G.........    480

Query  481  ACCTCCTTCAACTACGGTGCCATCAAAGCTACTCGGGTGACTGAACTGCTTTACCGCATG  540
Sbjct  481  ...........................................T......           540

Query  541  AAGAGGGCTGAGACGTACTGCCCCCGGCCTCTTTTGGCCATTCACCCGAACGAGGCCAGA  600
Sbjct  541  ...........A..A........T...........C.......GT..A..T...       600

Query  601  CACAAACAGAAGATTGTGGCACCTGTGAAGCAGCTCCTG    639
Sbjct  601  ........A....................A..T....    639
```

S6 Nucleotide changes of MYMBD21 against current field vaccine strain (O/India/R2/75) showing 88% VP1 nucleotide identity that was calculated using Needleman-Wunsch algorithm in BLAST global alignment tool. [Subject=MYMBD21; Query: vaccine strain]

Range 1: 1 to 639 Graphics

NW Score	Identities	Gaps	Strand
878	559/639(87%)	0/639(0%)	Plus/Plus

```
Query  1    ACCACCTCCACAGGTGAGTCCGCTGATCCCGTGACCACCACCGTTGAGAACTACGGTGGA  60
Sbjct  1    ..A.................G.................TG..........A........A..C  60

Query  61   GAGACACAGGTCCAGAGACGTCAACACACCGACGTTTCTTTCATTTTGGACAGATTTGTG  120
Sbjct  61   ...................C..G.....G.....C.......A.............A  120

Query  121  AAAGTAATACCGAAAGACCAAATCAATGTGTTGGACCTGATGCAAACCCCTGCTCACACT  180
Sbjct  121  .....G.C...A.........T.....AC...........................  180

Query  181  TTGGTAGGCGCACTCCTCCGCACCGCCACTTACTACTTCGCAGACCTAGAAGTGGCAGTG  240
Sbjct  181  .....G..T..C..T.........C.............TT....G..........  240

Query  241  AAGCACGAGGGCAACCTCACCTGGGTCCCGAACGGGGCGCCCGAGGCGGCGCTGGACAAC  300
Sbjct  241  ..A..T.....G.....................AAAA..CT........  300

Query  301  ACCACCAACCCAACGGCCTACCACAAGGCACCGCTCACCCGTCTTGCTCTGCCTTACACA  360
Sbjct  301  ........T........T.................A.....A.....G.....G  360

Query  361  GCACCACACCGTGTTCTGGCTACTGTTTACAACGGGAACTGCAAGTATGGCAAGGGCGCT  420
Sbjct  361  .....C........CA.................C...G..A..CAC  420

Query  421  GTGACCAACGTGAGGGGTGACTTGCAAGTGTTGGCTCAGAAGGCAGCAAGAACGCTGCCC  480
Sbjct  421  .CA..T.G.....A......C............C............G.....T  480

Query  481  ACCTCCTTTAACTACGGTGCCATCAAGGCTACCCGGGTGACTGAACTGCTTTACCGCATG  540
Sbjct  481  .......C................A....T...................T......  540

Query  541  AAGAGGGCCGAAACATACTGCCCTCGGCCTCTGCTGGCCATTCACCCGGAACAAGCCAGA  600
Sbjct  541  .......T.................TT.......C......AGTG....T...  600

Query  601  CACAAGCAGAAGATTGTGGCACCTGTGAAACAGTTGTTG  639
Sbjct  601  .....A..A...................G..AC.TC..  639
```

S7: Nucleotide changes of MYMBD21 against proposed local vaccine strain (BAN/TA/Dh-301/2016) showing 87% VP1 nucleotide identity which was calculated using Needleman-Wunsch algorithm [7] in BLAST global alignment tool. [Subject=MYMBD21; Query: vaccine strain]

❖ **Amino acid variations with vaccine strains**

Range 1: 1 to 213 Graphics ▼ Next Match

NW Score	Identities	Positives	Gaps
1058	202/213(95%)	208/213(97%)	0/213(0%)

```
Query    1  TTSTGESADPVTATVENYGGETQVQRRQHTDVSFILDRFVKVTPKDQINVLDLMQTPAHT   60
Sbjct    1  ............................................................   60

Query   61  LVGALLRTATYYFADLEVAVKYEGNLTWVPNGAPENALDNTTNPTAYHKAPLTRLALPYT  120
Sbjct   61  ...................H.............K..........................  120

Query  121  APQRVLATVYNGNCKYGDGSVTNIRGDLQVLAQKAARTLPTSFNYGAIKATRVTELLYRM  180
Sbjct  121  ..H..M.......ESHA.SV........................................  180

Query  181  KRAETYCPRPLLAIHPNEARHKQKIVAPVKQLL  213
Sbjct  181  .................S..............  213
```

S8 Comparison of VP1 amino acid sequence between MYMBD21 (Subject) and current field vaccine strain (Query) showing 95% identity which was calculated using Needleman-Wunsch algorithm [7] in BLAST global alignment tool.

Range 1: 1 to 213 Graphics ▼ Next Match

NW Score	Identities	Positives	Gaps
1056	202/213(95%)	206/213(96%)	0/213(0%)

```
Query    1  TTSTGESADPVTTTVENYGGETQVQRRQHTDVSFILDRFVKVIPKDQINVLDLMQTPAHT   60
Sbjct    1  ...........A...........................T...................   60

Query   61  LVGALLRTATYYFADLEVAVKHEGNLTWVPNGAPEAALDNTTNPTAYHKAPLTRLALPYT  120
Sbjct   61  .................................K.........................  120

Query  121  APHRVLATVYNGNCKYGKGAVTNVRGDLQVLAQKAARTLPTSFNYGAIKATRVTELLYRM  180
Sbjct  121  .....M..........ESHA.S.....................................  180

Query  181  KRAETYCPRPLLAIHPEQARHKQKIVAPVKQLL  213
Sbjct  181  ...............SE...............  213
```

S9 Comparison of VP1 amino acid sequence between MYMBD21 (Subject) and proposed local vaccine strain (Query) showing 95% identity which was calculated using Needleman-Wunsch algorithm [7] in BLAST global alignment tool.

❖ **Validating the quality of protein structure**

Ramachandran plot was developed using *Molprobityv4.4* [9] in SWISS-MODEL and Z-score
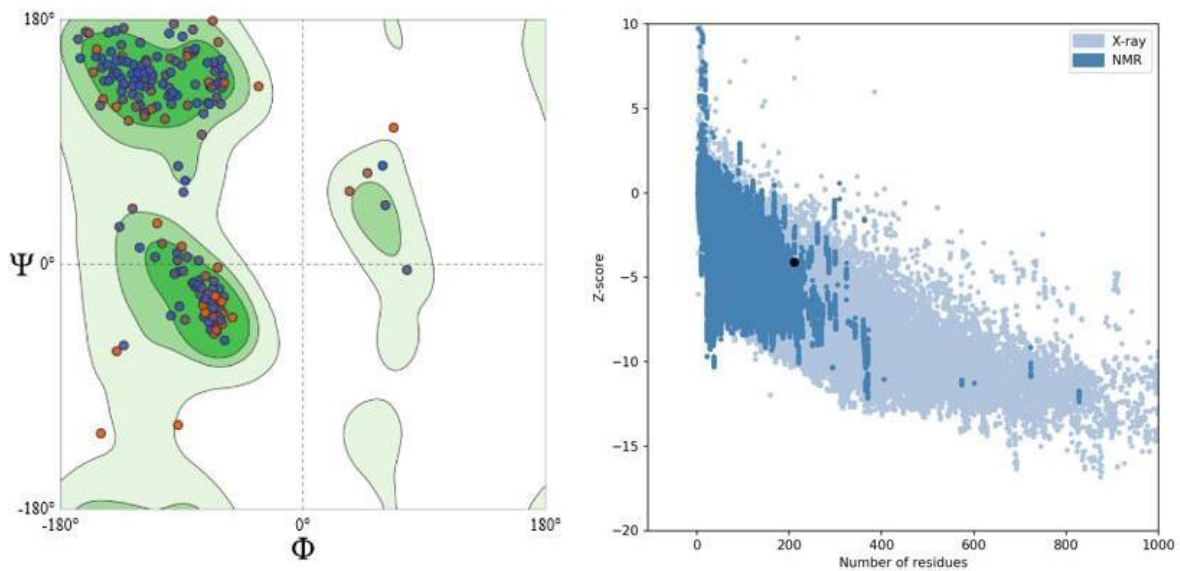
was calculated using ProSA-web [10]

MYMBD21:
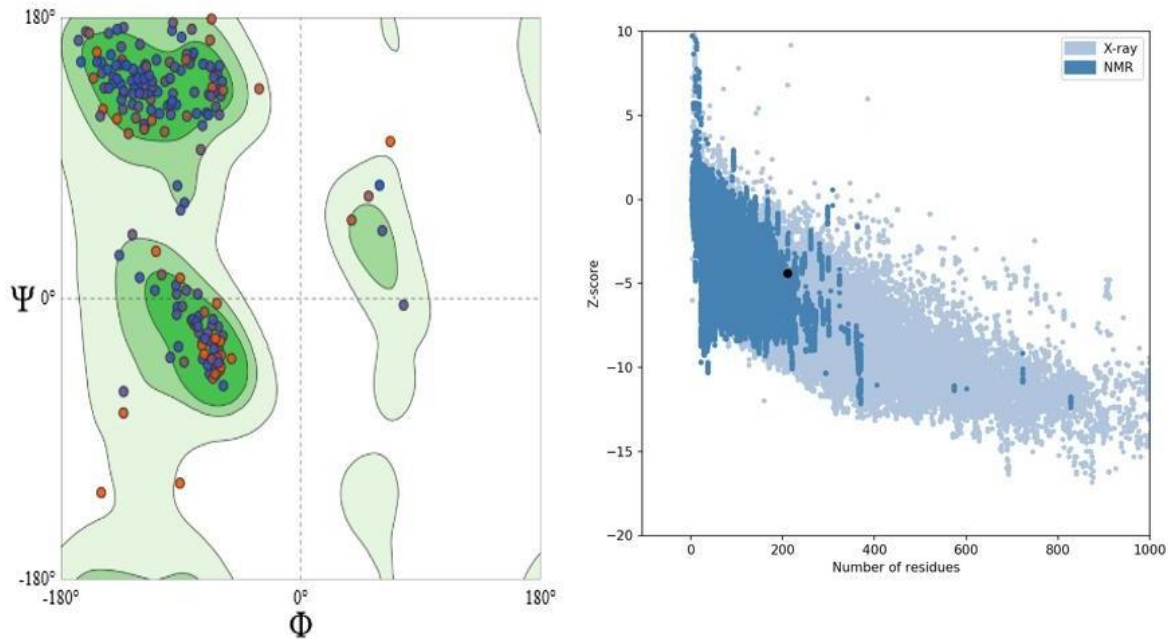
S10 Estimating the 3D model quality of consensus VP1 of MYMBD21 by Ramachandran plot and Z-score. In the plot, 94.23% of the amino acid residues were in the favored region with 1.44% outliers and Z-score was -4.41. [The deep green and pale green colors represent the favored and allowed regions for an amino acid of the protein, respectively. On the X and Y axes, Φ (phi) and Ψ (psi) represented the torsion angles around alpha carbon to amine and carboxyl groups of different amino acids]
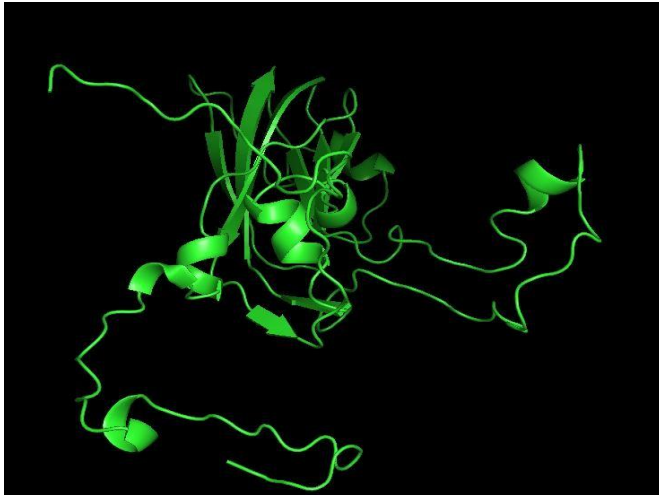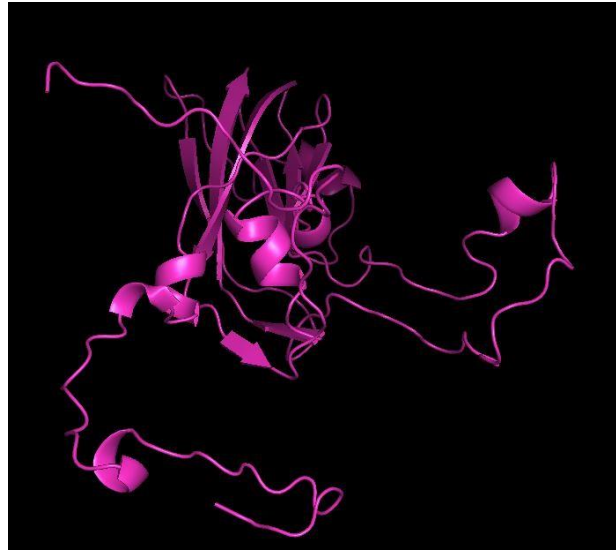
S11: 3D model of consensus VP1 of MYMBD21 isolate generated using PyMOL[11] .
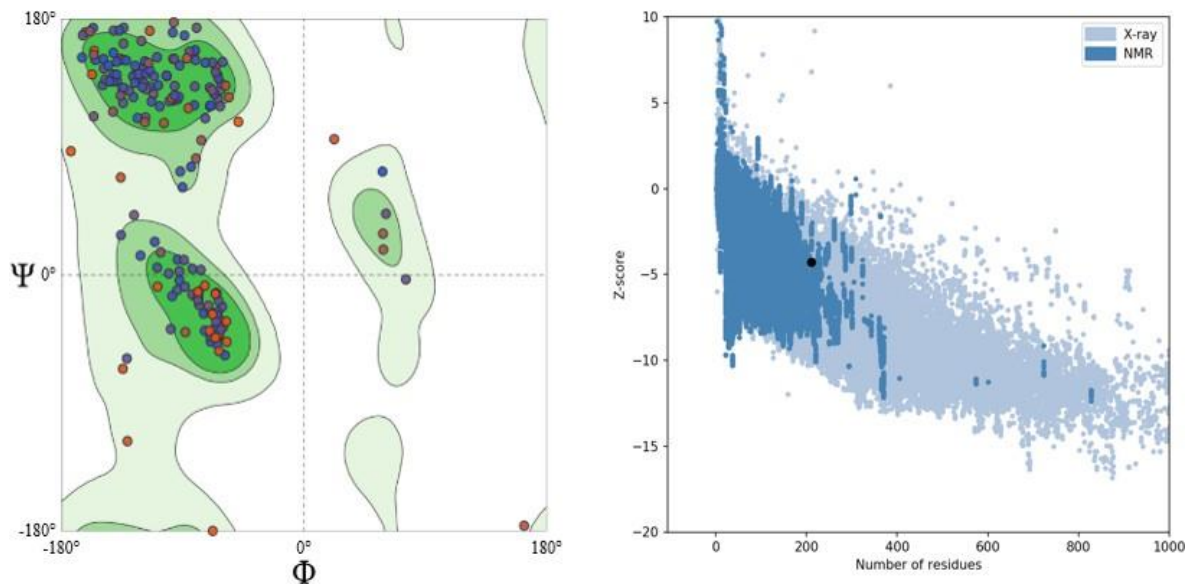
SA-2018:

S12: Estimating the 3D model quality of consensus VP1 of SA-2018 lineage by Ramachandran plot and Z-score. In the plot, 92.79% of the amino acid residues were in the favored region with 0.96% outliers and Z-score was -4.05.

S13: 3D model of consensus VP1 of SA-2018 isolate generated using PyMOL[11].

Uncategorized Bangladeshi isolates:

S14: Estimating the 3D model quality of consensus VP1 of uncategorized isolates by Ramachandran plot and Z-score. In the plot, 92.23% of the amino acid residues were in the favored region with 1.44% outliers and Z-score was -4.29.

S15 3D model of consensus VP1 of uncategorized Bangladeshi isolate generated using PyMOL [11].

Current field vaccine strain (O/India/R2/75):
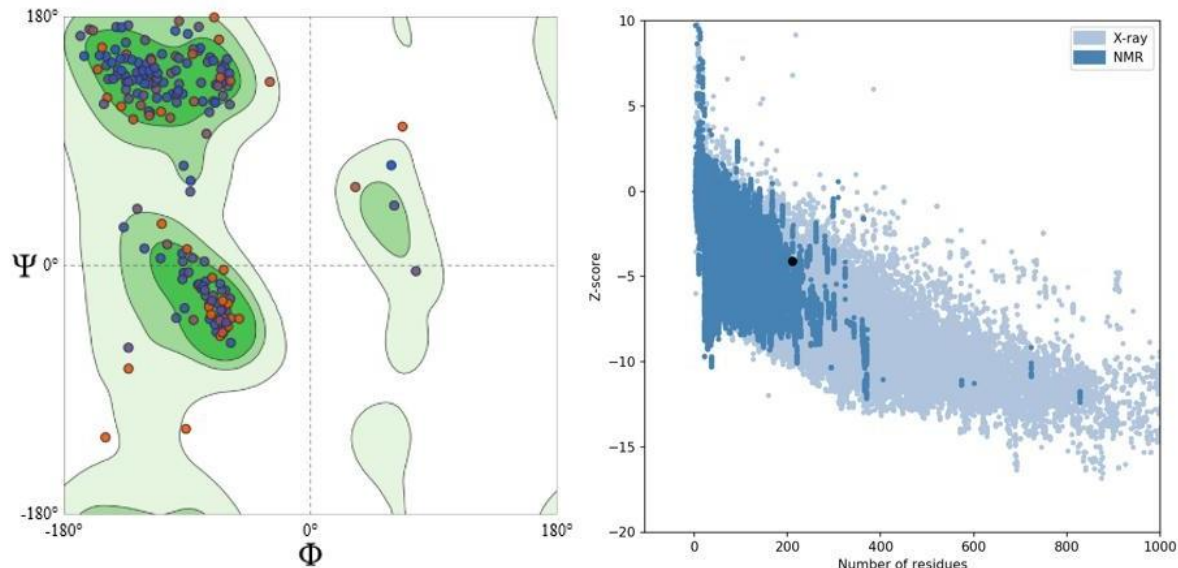
S16: Estimating the 3D model quality of VP1 of the current field vaccine strain (O/India/R2/75) by Ramachandran plot and Z-score. In the plot, 93.75% of the amino acid residues were in the favored region with 1.44% outliers and Z-score was -4.1.
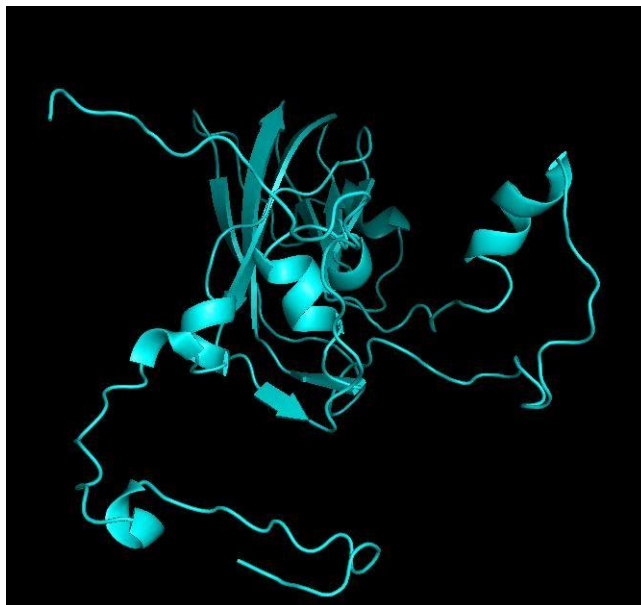
S17 :3D model of VP1 of the current field vaccine strain (O/India/R2/75) generated using PyMOL [11].

Local vaccine strain (BAN/TA/Dh-301/2016):
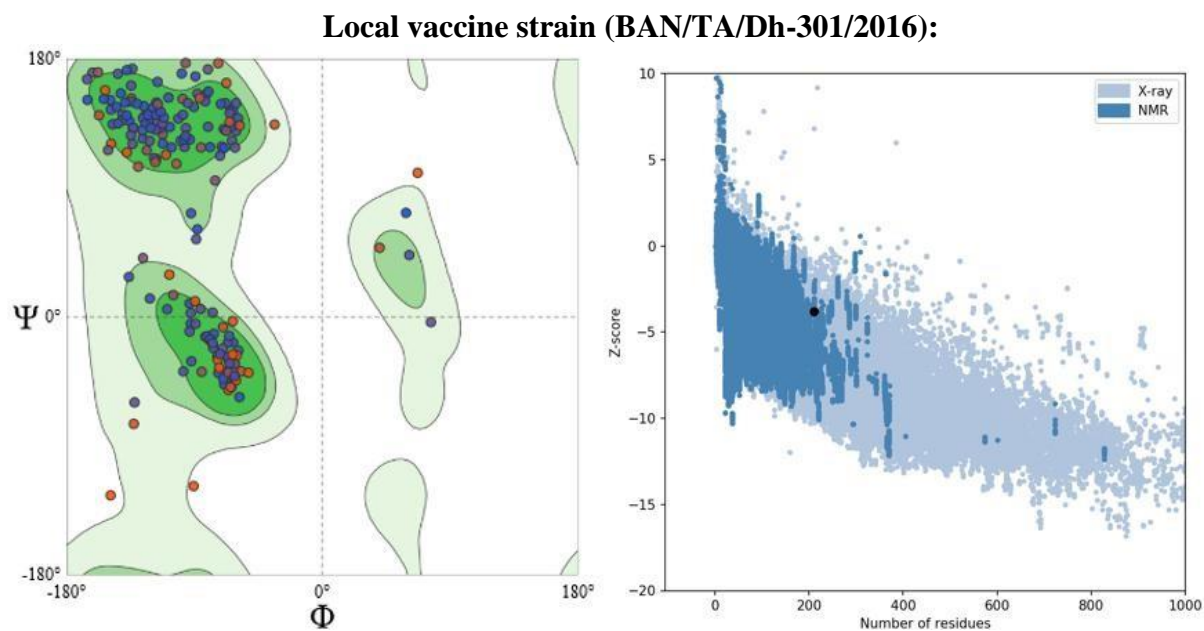
S18: Estimating the 3D model quality of VP1 of proposed local vaccine strain (BAN/TA/Dh-301/2016) by Ramachandran plot and Z-score. In the plot, 93.75% of the amino acid residues were in the favored region with 0.48% outliers and Z-score was -3.81.
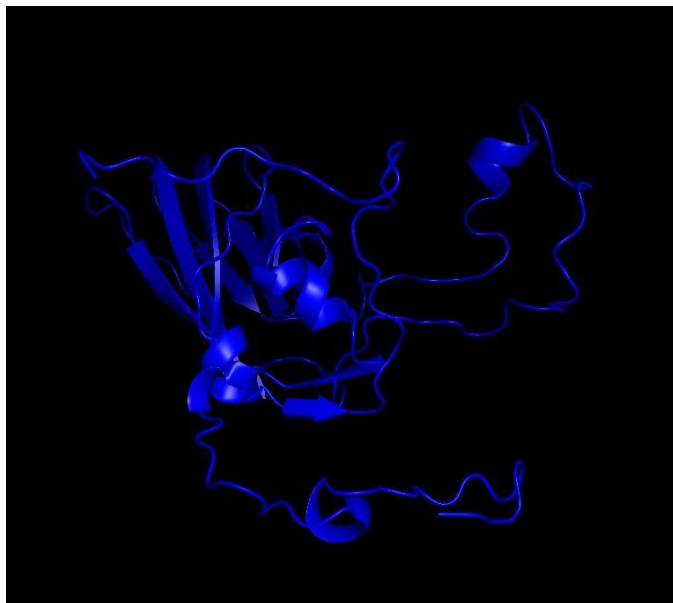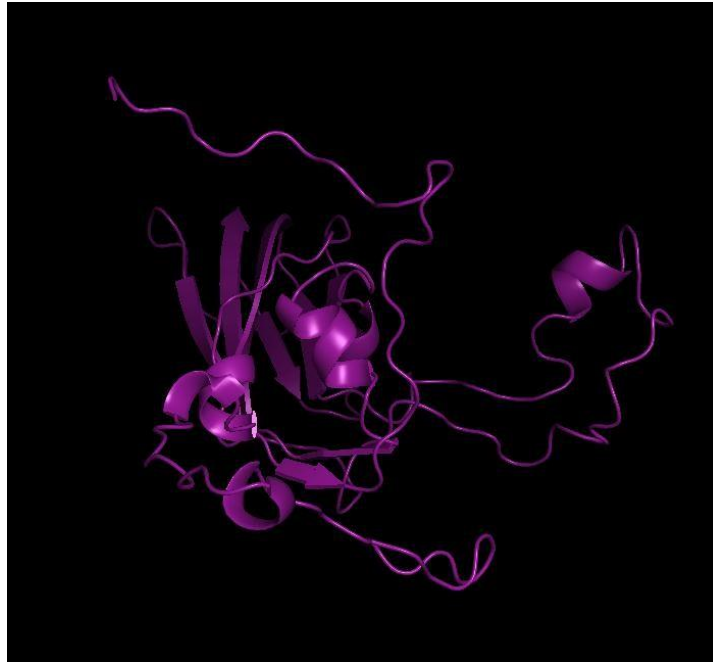
S19: 3D model of VP1 of proposed local vaccine strain (BAN/TA/Dh-301/2016) generated using PyMOL [11].
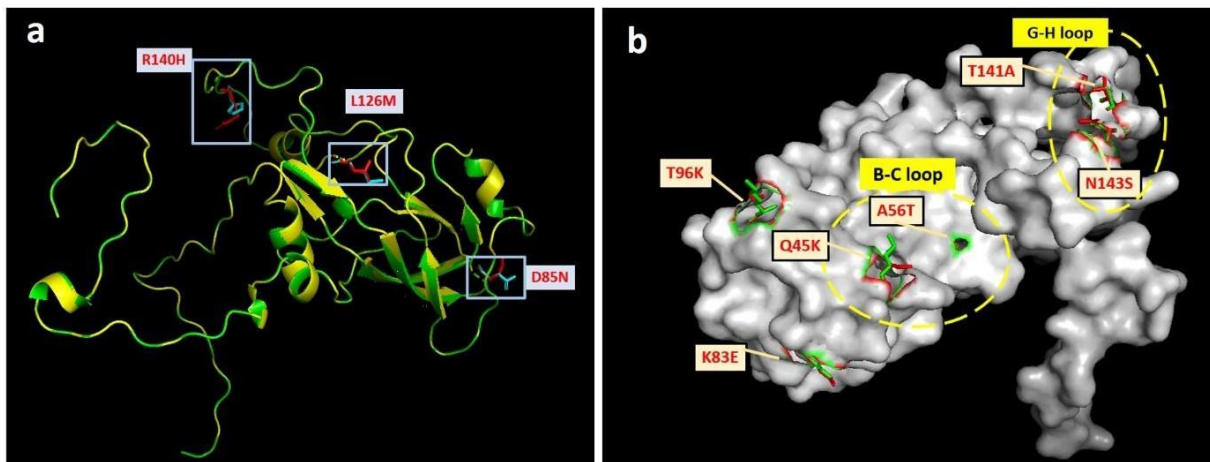
S20: Superimposed three-dimensional (3D) structure of VP1. (a) The 3D structure of superimposed MYMBD21 (colored in yellow) and SA-2018 (colored in green) consensus VP1. Unique amino acid substitution sites are represented with stick style (cyan color indicates MYMBD21 amino acids and red color indicates SA-2018 amino acids). (b) The 3D structure of consensus VP1 of superimposed MYMBD21 (colored in yellow) and uncharacterized Bangladeshi isolates (colored in green). Unique amino acid substitution sites are represented with stick style (green color indicates MYMBD21 amino acids and red color indicates uncharacterized Bangladeshi isolates' amino acids). Structures were visualized using PyMOL software [11].

Appendix-XI

Data Collection Sheet

1. Background information   **Date:**

1.1 Owner's Name: 1.2 Address:

Village: P/S: District:

2. Animal identification

2.1 Age group: Calf (up to 1 year):   Young Cattle (>1 year to before breeding):   Adult:

2.2 Sex: Male/ Female

2.3 Breed: Native   Exotic/Cross

3. Herd composition:

3.1 Herds of only cattle: Yes/ No   3.2 Herds of cattle and small ruminants: Yes/ No

4. Farming System/ Grazing habit of the Livestock

4.1 Intensive: Yes/ No   4.2 Semi intensive: Yes/ No   4.3 Extensive: Yes/ No

5. Movement of Animals

5.1 Limited Movement in the District: Yes /No 5.2 Cross Boundaries of District: Yes /No

5.3 Cross National Boundaries: Yes/ No

6. Investigation of Animals:

6.1 Clinical Signs in Animals Sampled

a._____ b._____

c._____ d._____

e._____ f._____

6.2 Total Number of Animals Examined:

Morbidity: Mortality: Case Fatality:

6.3 Collected Specimen (Specify):   6.4 Collected Sample ID No:

7. Climatic Condition during Sample Collection:

7.1 Summer: Yes/ No   7.2 Rainy Season: Yes/ No

7.3 Pre-winter: Yes/ No   7.4 Winter: Yes/ No   7.5 Others (Specify):

8. Treatment (Specify):

9. Vaccination (Specify):

Investigated and completed by

_____

Signature and Name:

Designation:

Date:

Appendix-XII

2/27/2019   FMDV KIT.jpg

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

Tel: 58613243, 9673387 (Office)
PABX: 9661900-73/4355, 7545
Fax: (+880-2)-8615583
E-mail: mimdadul07@yahoo.com
deanbio@du.ac.bd

Ref. ...66..../Biol.Scs./2018-2019   Date: 14.11.2018
৩০ কার্তিক, ১৪২৫

Ethical Review Committee

Dr. Munawar Sultana
Associate Professor
Department of Microbiology
University of Dhaka
Dhaka-1000, Bangladesh

Sub: Ethical Clearance.

Dear Munawar Sultana,

With reference to your application on the above subject, this is to inform you that your research proposal entitled **"Development of a simple low-cost diagnostic kit for foot and mouth disease virus"** has been reviewed and approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka.

I wish for the success of your research project.

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