Epidemiology and Molecular Characterization of Circulating Foot-and-Mouth Disease Viruses of Cattle and Vaccine Development in Bangladesh

PhD. Thesis-2017

SUBMITTED BY

HUZZAT ULLAH

REGISTRATION NO.: **111**

SESSION: **2011-2012**

DEPARTMENT OF MICROBIOLOGY, FACULTY OF BIOLOGICAL SCIENCES

UNIVERSITY OF DHAKA, DHAKA-1000, BANGLADESH

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

DEPARTMENT OF MICROBIOLOGY
FACULTY OF BIOLOGICAL SCIENCES
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Dedicated to…

My Beloved Parents, Who Cherished My Life with Their Blessings

Quotation…

"A little science estranges man from God, but much science leads them back to Him."

Louis Pasteur

Certification

It is hereby certified that student bearing Reg. No. 111, Session 2011-2012 has carried out the research work entitled "**Epidemiology and Molecular Characterization of Circulating Foot-and-Mouth Disease Viruses of Cattle and Vaccine Development 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.

..…………………………………

Md. Anwar Hossain, PhD
Professor
Department of Microbiology
University of Dhaka
Dhaka-1000, Bangladesh

………………………………..

Md. Alimul Islam, PhD
Professor
Department of Microbiology and Hygiene
Bangladesh Agricultural University
Mymensingh-2202, Bangladesh

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Abstract

Foot-and Mouth Disease (FMD) is the major impediment of livestock production and trade worldwide. Currently there are seven FMDV serotypes, namely O, A, C, Asia-1 and Southern African Territories (SAT) 1-3, which infect cloven-hoofed animals. Due to huge local demand for meat, milk and sacrificing animals, livestock industries are rapidly growing in Bangladesh. Furthermore, there is a great prospect of healthy meat export in the Middle East. To control FMD, mass vaccination of cattle every six months with a trivalent FMD vaccine (locally produced or imported from type O, A, and Asia-1) is practiced in Bangladesh. But the current vaccination strategy does not provide sufficient protection of the herd. Although FMD vaccines need to be adequately matched to the field virus to ensure sufficient protection against a challenge with a field virus, but limited/ no step is adopted in Bangladesh so far to match vaccines before implementation. Moreover, FAO and OIE have proposed a time-frame strategy for the progressive control of FMD (PCP-FMD) road map and accordingly, Bangladesh will achieve stage 2 in 2016- provided that epidemiological studies, risk identification, and fixed risk-based control plan are completed. Unfortunately, due to poor/no reporting system by the appropriate authorities of FMD to the OIE, inappropriate vaccination, and poor veterinary service care, Bangladesh is placed at stage 0 (non-reporting stage). It is hypothesized that proper epidemiological studies and characterization of circulatory FMDVs to select the appropriate vaccine candidate in Bangladesh is the prime step to fix risk-based strategies to implement FMD-control program and achieve the PCP-FMD goal. To address the hypothesis, demographic data were collected using a questionnaire at field level infected farms; 283 FMDV-infected tissues were sampled from the 39 individual FMD outbreaks within May 2012 to April 2016 and characterization was done by PCR based identification of VP1 gene, isolation of viruses in BHK-21 cell-culture, genome sequencing and analysis. The results demonstrated that the disease is endemic in Bangladesh. The husbandry systems, practiced in the investigated herds were intensive, semi-intensive with free animal movement or extensive. Demographic data revealed that the average morbidity rate of FMD was 53.8% with a higher incidence in indigenous cattle (55.7%) than that of crossbred cattle (49.2%). The highest number of outbreaks occurred in October (23.1%), followed by December (12.8%) and March or September (10.3%) which was decreased gradually up to mid-August, and in April (0%). Among seven serotypes distributed worldwide, FMDV type O, A and Asia-1 were circulating in Bangladesh and type O FMDV accounted for the most outbreaks (87%), followed by Asia-1 (8%) and A (5%) type virus. Phylogenetic analysis revealed a single lineage and topotype of FMDV serotypes O (Ind2001 lineage and O/ME-SA topotype), A (genotype VII of Asia topotype) and Asia-1 (genetic lineage C) circulatory in Bangladesh; and intrusion of FMDV occurred from India and vice versa. The complete genome of serotype O [KF985189] and A [KJ754939] were retrieved to be 8131 nucleotides (nt) and 8220 nt in length, respectively. Comparative genome analyses with reference sequence or vaccine strain revealed that within serotype O, 82 nt deletion in S-fragment and 43 nt insertion in 5'-UTR resulting introduction of an extra pseudoknot structure, whereas a 84 nt insertion within the 5′-UTR, a lengthened polyC tract was observed in serotype A. Within VP1, variation in B-C loop (40~60), G-H loop (133~160) in both serotypes and a 10 amino acid insertion (position 92~101) in 3A protein within serotype O were found. In summary-(i) three types of FMDVs are prevailing in Bangladesh (ii) intrusion of FMDV occurred from neighboring countries and (iii) inappropriate vaccination with mismatch virus strain caused vaccine failure. Finally, for effective control and prevention of FMD, proper epidemiology data, disease reporting, animals' movement quarantine, appropriate vaccination and strong political will of the government are required.

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ABBREVIATIONS

3D – Three Dimensional

BLAST- Basic Local Alignment Search Tool

BHK - Baby Hamster Kidney

CFT- Complement Fixation Test

CPE-Cytopathic Effect

DDBJ- DNA Data Bank of Japan

EIF - Eukaryotic Initiation Factor

ELISA - Enzyme Linked Immunosorbent Assay

FMD – Foot and Mouth Disease

FMDV – Foot and Mouth Disease Virus

Fig. - Figure

IRES - Internal Ribosome Entry Site

MEGA - Molecular Evolutionary Genetics Analysis

MSA - Multiple Sequence Alignment

NCBI- National Centre for Biotechnology Information

NTP - Nucleotide Phosphate

PDB- Protein Data Bank

PVS- Protein Variability Server

OIE - Office Des Epizooties

ORF - Open Reading Frame

PCR - Polymerase Chain Reaction

PDFMD- Project Directorate on Foot-and Mouth Disease

SAT - South African Territories

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

DLS-Department of Livestock Services.

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

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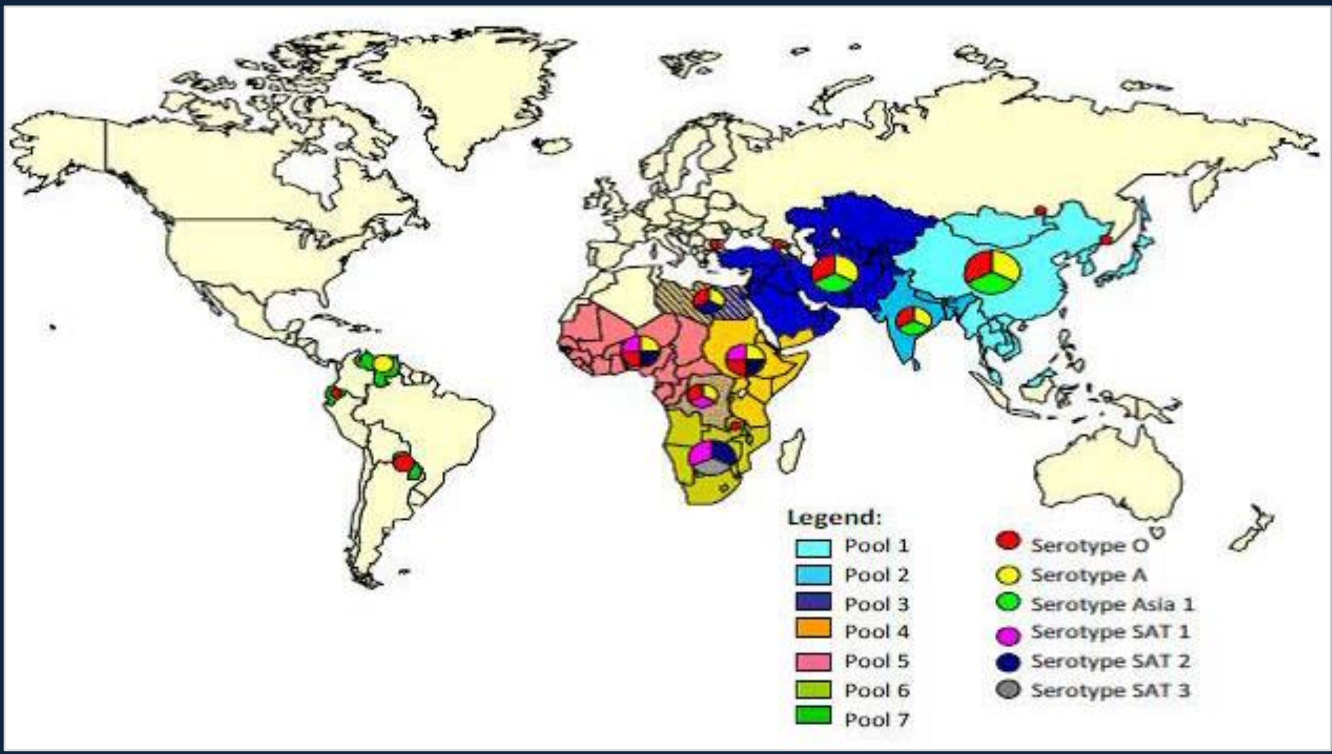
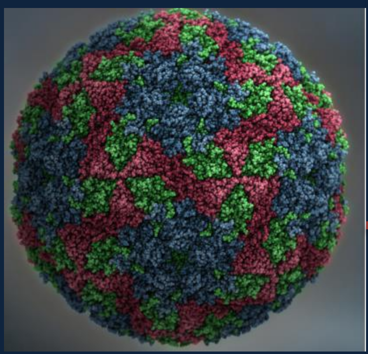




Chapter 1

Introduction

1.1 General Introduction

Foot-and-mouth disease (FMD) is a highly infectious, severe debilitating and clinically acute disease that affects more than 70 species of domestic and wild cloven-hoofed animals (Alexandersen *et al.*, 2003). The disease is caused by foot-and-mouth disease virus (FMDV), the most important animal pathogen worldwide which was discovered at the end of the 19th century. The most sensitive hosts of FMDV are cattle and swine developing severe clinical sings, because of their extreme sensitivity to respiratory infection. The clinical signs in sheep, goats, and wild ruminants are milder than in cattle (Geering and Lubroth, 2002). Horses, dogs and cats are not susceptible hosts but they can inevitably spread the virus via their fur if contaminated. This virus is not a human pathogen (OIE, 2009), although there have been rare reports of infection in human. It can spread over many kilometers depending on weather conditions (Donaldson and Alexandersen, 2002). The disease has direct and indirect economic consequences resulting in substantial economic losses in terms of reduced milk and meat production, death of animals, weight loss and loss of draught power in animals taking typically a long recovery period following the disease (Grubman and Baxt, 2004; James and Rushton, 2002).

Livestock sector is an important contributor in Bangladesh economy and its overall contribution to the agricultural Gross Domestic Products (GDP) is 10.11% and to national GDP is 3.2%; and 20% country’s labour force is associated in raising the livestock animals (Epidemiology Unit, DLS, 2014). In other words, a major portion of the rural population is directly or indirectly linked to the livestock sector which plays a pivotal role in people’s day to day economy. In Bangladesh there are about 53.6 million domesticated animals with 23.49 million cattle, 1.46 million buffalo, 25.44 million goats and 3.21 million sheep (Epidemiology Unit, DLS, 2014). These animals have great genetic potential for high quality meat production and milk yield but unplanned breeding, a poor market system, a shortage of animal feed and abundance of infectious diseases like FMD are the main obstacles in the development of the livestock sector to its optimum capacity. Bangladesh is endemic for FMD (WAHID, 2009) and is seen almost throughout the year in all parts of the country (Nandi *et al.*, 2015) and the gravity of the economic losses due to this disease was estimated 125 million USD/annum (Epidemiology section, DLS 2014). Because of its associated economic impact and the difficulties in its effective control, FMD ranks first in the A list of infectious diseases of animals (OIE 2009).

FMDV is a small, non-enveloped, positive-sense, single stranded RNA (8.4 kb in length) virus belonging to the genus *Aphthovirus* of the family *Picornaviridae* (Racaniello, 2001). According to the antigenic properties of the capsid proteins, there are seven immunologically distinct FMDV serotypes globally namely O, A, C, Asia1, SAT-1, SAT-2 and SAT-3 (Bachrach, 1968; Domingo *et al.*, 2002; Knowles and Samuel, 2003). Among the seven, FMDV serotypes O, A, Asia1 and C have been reported in the Asian continent in a wide span of time (Kitching 1999; Knowles and Samuel, 2003; Mittal *et al.*, 2005). The genome contains a single open reading frame (ORF) and translated into a polyprotein which is post-translationally cleaved to yield 12 mature proteins (Belsham, 1993; Sáiz *et al.*, 2002). These are; 4 structural (VP1, VP2, VP3, VP4 also known as 1D, 1B, 1C and 1A respectively) and 8 nonstructural proteins (L^{pro}, 2A, 2B, 2C, 3A, 3B, 3Cpro, and 3Dpol). The genome is encapsidated by sixty copies of each of the four structural proteins and with the exception of VP4 that is antigenically distinct and unable to form pentamers. All other three surface exposed components remain associated in a protein complex which acts as a monomer for the self-assembly of five monomers into the pentameric capsid subunit (Acharya *et al.*, 1989).

The molecular epidemiology of FMDV has been extensively studied using the VP1 coding region of the virus genome (Knowles and Samuel, 2003). VP1, the most variable capsid protein includes a major immunogenic site of the virus which has been used to genotype the seven serotypes of FMDV into geographically distinct groups called topotypes. Furthermore, comparison of VP1 coding sequences from isolates obtained during outbreaks provides evidence of relatedness between individual FMDV strains and hence the tracing of the spread and transmission of the virus from one region to another or across national borders (Knowles and Samuel, 2003). In Bangladesh, the molecular epidemiology of FMD is not well understood (Loth *et al.*, 2011) because the molecular characterization of FMDV is not routine. There is therefore limited data on the epidemiology of FMDV although the outbreaks are frequent and difficult to control. Moreover, animals frequently cross Bangladesh's boarders from other neighboring countries especially India and Myanmar (Nandi *et al.*, 2015). While these animals may be reservoirs of FMDV, their contribution to the introduction and maintenance of FMDV is unknown. Many recent studies have shown the predominance of FMDV serotype O

(80-85%) followed by serotype A (10-15%) and Asia1 (up to 5%) in this country (Nandi *et al.*, 2015). Other serotype C that was last recorded in early 1995 (Biswal *et al.*, 2012).

No single strategy for control of FMD in Bangladesh is practiced. The control strategy focused on ethno veterinary treatment and vaccination of animals. But insufficient sampling and lack of immediate typing and viral risk analysis affects the capability to rapidly implement control measures. Moreover, underreporting of FMD by the livestock department of the Government of Bangladesh implicated that the status of Bangladesh at PCP-FMD road map recommended by OIE/FAO always showed 'non reported' i.e. FMD control program in Bangladesh is at early stage or no-control program implemented stage; and also the current strains circulating throughout country are in many cases unknown as well or not known at all. In addition, imported vaccines are available in the local markets that are produced against the FMD virus of foreign strain. Sometimes this causes the emergence of new virus strain. On the other hand there is no genetic data for seed virus of locally (Livestock Research Institute, Mohakhali, Dhaka) produced FMD vaccines to match the circulatory serotypes in our territory. As results, locally prepared or imported FMD vaccines did not protect animals at acceptable protection covers against the disease as FMD outbreaks have been commonly observed in vaccinated and non-vaccinated herds more or less alike. A recent incidence of FMD due to serotype Asia-1 in 38 days post vaccinated animals was observed (Ullah *et al.*, 2015). In this case ELISA assay of local field serum sample revealed antibody titer level of >2·4 (log10) but failed to protect the cattle [cutoff value ≥1.64 (log10)] from infection occurred by the virus. The circulatory genotype Asia-1 showed VP1 protein sequence heterogeneity of eight amino acid substitutions within the G-H loop with the vaccine strain [IND 63/72 (AY304994)] used in vaccination programme. This investigation focused that the amino acid substitution in VP1 protein at G-H loop of the locally circulated FMDV serotype Asia-1 strain may be a reason for current vaccination failure. As new strains have been emerging constantly within each serotype owing to high mutation rate and the quasispecies nature of this RNA virus (Tosh *et al.*, 2002), molecular characterization of all the serotypes prevalent in Bangladesh are needed to prevent FMD through vaccination with appropriate virus strain(s) circulating in Bangladesh. So, this research work gave emphasis on the epidemiological study of circulating serotypes and lineage of FMD viruses, isolation and genome-wide analysis of FMD serotypes and appropriate vaccine strain selection for safe livestock practices.

1.2 Review of Literature

Agriculture is an economically important sector of Bangladesh and livestock sector is an integral part of agriculture. Livestock meets the demands of meat, milk, fat and hides and is also a source of ready cash for the villagers to fulfill their daily requirements. This sector is under continuous stress and threat of different viral, bacterial, parasitic and metabolic diseases. Among these, foot-and-mouth disease (FMD) and its etiological agent FMDV is the most notorious problems in the world including Bangladesh.

1.2.1 Foot-and-Mouth Disease (FMD)

1.2.1.1 Diversity of etiological agents of Foot-and-Mouth Diseases

The disease has different names in different regions of the world which include: Aphtous fever, Epizotic aphtae, Infectious aphtous stomatitis, Aftosa (Italian and Spanish), fievere aphtheuse (French), Maul and Klavenseuch (German) (Timoney *et al.,* 1988). The greatest advances in our knowledge of FMD and its control have been made during the last 100 years or so as demonstrated by the substantial international literature on all aspects of the disease. Regrettably, it will be possible to review here only a few of the most important findings. One of the first and most significant discoveries was made by Loeffler and Frosch (1897) who demonstrated that the etiological agent was a filterable particle, and, effectively, FMD was the first animal disease to be attributed to a virus. Although in 350 B.C Aristotle mentioned a cattle plague that could have been foot-and-mouth disease or rinderpest, another similar destructive bovine disease and Italian physician Hieronymus Fracastorius (1546) gave the first clear description of foot-and-mouth disease. During the early part of the 20th century, the diverse antigenic nature of the virus was recognized and led to the description of the seven serotypes over the next 50 years. Initially, Vallee and Carre (1922) first showed the existence of two immunological types of FMDV by cross-immunity tests in cattle. They were designated by their areas of origin, O (Oise, a department in northern France) and A (Allemagne, Germany). Soon after Waldmann and Trautwein (1926) reported the existence of three immunologically distinct types, A, B and C. Comparison of these virus types revealed that Waldmann and Trautwein's types A and B were the same as Vallée and Carré's types O and A, respectively; type C was distinct. Thus the three types became known, by international agreement, as Vallée O, Vallée A and Waldmann C and later simply as O, A and C. Many atypical virus strains were later described, mainly from Africa, until in 1948 a sample submitted to the WRL from Bechuanaland yielded a virus (BEC/1/48) which in cross-protection tests in cattle and guinea pigs was found to be distinct from O, A and C. Subsequently a virus isolate from Northern Rhodesia (RHO/1/48) was identified as yet another distinct type. Retrospective testing of viruses isolated between 1931 and 1937 revealed isolates from Southern Rhodesia in 1937 (RV/11/37) and 1931 (RV/1/31) which were similar to the 1948 isolates from Bechuanaland and Northern Rhodesia, respectively (Brooksby, 1982). A further virus isolate from Southern Rhodesia in 1934 (RV/7/34) was found to be a third new type. These new types were designated SAT (Southern African Territories) types 1, 2 and 3. The seventh serotype, designated Asia-1, was first recognized in the early 1950's as viruses isolated from India in 1951 and 1952 (Dhanda *et al.*, 1957) and Pakistan in 1954 (Brooksby and Rogers, 1957). Within each serotype there is considerable diversity and antisera against one strain of a serotype may not recognize other strains of the same serotype. Isolates were classified into antigenically related “subtype” within a serotype and each serotype contains a number of subtypes (Buxton and Fraser, 1977).

1.2.1.2 Consequences of the FMD

FMD is not a particularly serious disease in terms of mortality and that its relevance is largely in terms of production loss, international trade and economy. While the trade consequences are certainly extremely damaging for some countries, FMD causes significant distress and suffering to animals and impacts on the livelihood of all farmers, regardless of the size and sophistication of their livestock unit (**Figure 1.2.1.2**). Bangladesh has no recent assessment of losses to FMD. It is the number one barrier to export animal products. It causes abortion in pregnant animals, death to young calves without showing any clinical sign, economic losses for milking cows by sharp decline in milk production and damage to teats, hamper in traction power and high treatment cost. The morbidity rate can approach 100% in naïve cattle or swine herds. Adult livestock do not usually die from FMD (the case fatality rate is approximately 1-5%), in some groups of calves mortality rate may reach up to 80% and 100% in suckling piglets (Epidemiology Unit, DLS, 2014).

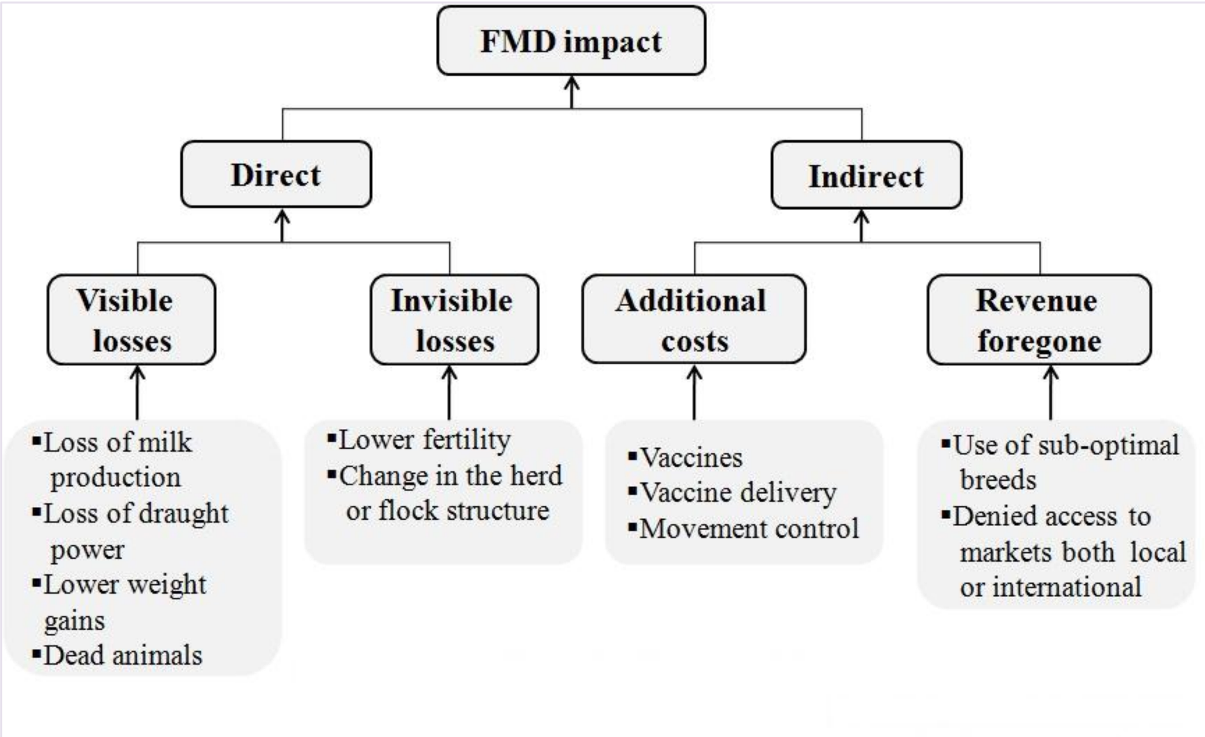


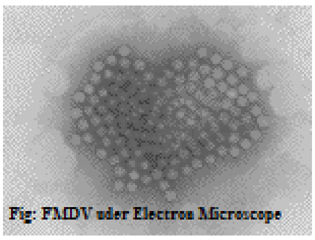
Figure 1.2.1.2 Economic impact of FMD outbreaks in Bangladesh

1.2.2 Foot-and-Mouth Disease Virus (FMDV)

1.2.2.1 Taxonomy

FMDV was documented in 1963 by the International Committee on Taxonomy of Viruses (ICTV) as member of *Aphthovirus* in the *Picornaviridae*. The name, *Picornaviridae* is derived from a Latin word ‘Pico’ which means very small and ‘rna’ for RNA, which refers to the size and genome type of the virus. The genus name ‘*Aphthovirus*’ refers to the vesicular lesions developed in cloven-footed animals (OIE, 2009).

1.2.2.2 Morphology and Genome Structure



The FMD viruses are among the smallest known RNA viruses and have non enveloped, ether-resistant, icosahedral nucleocapsid (protein shell) with symmetry of 22-30 nm in diameter (Melnick *et al.*, 1975; Cooper *et al.*, 1978). Under the transmission electron microscope (TEM) at a magnification of 350,000X, the FMD virion appears to be a round particle with a smooth surface. FMDV is distinguished from other Picornaviruses by its lack of a surface canyon, receptor binding site for entero and cardio-viruses. Another feature is the presence of a channel at fivefold axis which permits the entry of small molecules such as CsCl into the capsid. Buoyant density of FMDV is highest among the Picornaviruses (Grubman and Baxt, 2004).

The genome is linear and non-segmented. It consists of a single molecule of positive sense RNA, with a 5' genome linked protein (VPg) associated to the genome via a phosphodiester bond linked to a tyrosine residue (Grubman and Baxt, 2004). The 5' end of the genome has a 'poly C' region, while the 3' end is polyadenylated (Agol *et al.*, 1999).

1.2.2.3 Biophysical Characteristics of Foot-and-Mouth Disease Virus

In acidic conditions, the FMDV particles are disrupted into pentameric subunits composed of five copies each of the virus structural capsid proteins (VP1-3) with the liberation of the internal capsid protein (VP4) and the RNA. FMDV is also unstable at pH>11 and when treated by heat or by gamma radiation loose infectivity for susceptible cells (Newman *et al.*, 1973; Acharya *et al.*, 1990).

1.2.2.4 Genome organization

The FMDV is a 146S particle consisting of a single-stranded RNA genome of approximately 8500 nucleotides and 60 copies each of four structural proteins (VP1, VP2, VP3 and VP4). In addition, a precursor protein (VP0), and a genome-linked protein, (VPg) covalently attached to the 5' terminus of the RNA are also present (Grubman and Baxt, 2004). The genome of FMDV comprises of 5' un-translated region (UTR) of about 1150 nucleotides followed by a single open reading frame (ORF) of 6996 nucleotides (excluding stop codon) and a 3' un-translated region (UTR) of approximately 160 nucleotides. The ORF can be translated into a single polyprotein, that can be cleaved into four structural proteins (VP4, VP2, VP3 and VP1), and 8 non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C and 3D). In the **figure 1.2.2.4**, L is an additional N-terminal leader protein (Feng *et al.*, 2004).

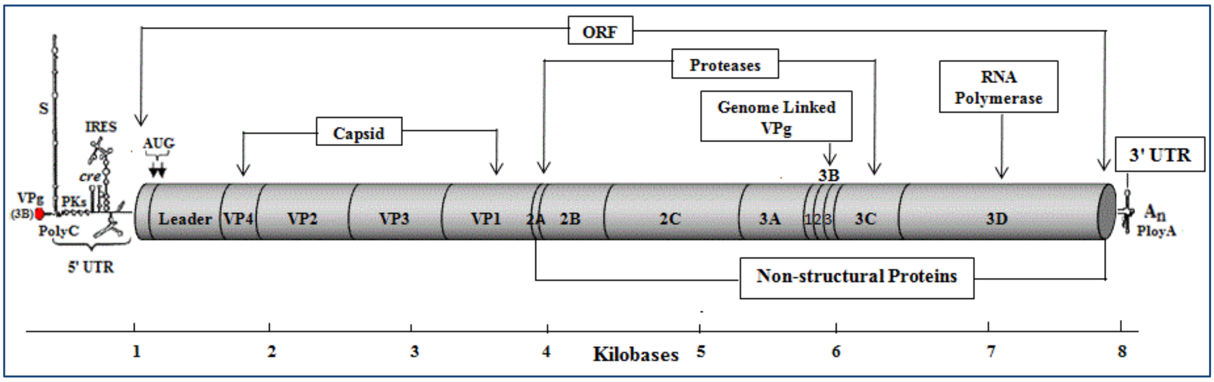


Figure 1.2.2.4 Schematic structure of FMDV genome

1.2.2.4.1 5' Un-translated Region (UTR)

The 5' UTR is about 1100-1300 nucleotides and plays important roles in FMDV replication. It attached to the 5' end covalently with avirus encoded protein, 3B, called genome linked virus protein (VPg) (Sangar *et al.*, 1977). The protein 3B occurs in three different forms, 3B1, 3B2 and 3B3 (Sangar *et al.*, 1977; Belsham, 1993). The aphthoviruses are unique in having these three similar, but not identical, VPg-encoding genes in tandem (Palmenberg, 1987). The 5' UTR consists of a short S-fragment (400 bases in length) is capable of forming a large hairpin structure, the cytidyl (polyC) tract (50-100 nucleotides) followed by a non-translated segment (genomic long fragment, LF, of about 720 bases) which is predicted to form three tandemly repeated pseudoknots (Clarke *et al.*, 1987), a stem loop *cis*-acting replication element (*cre*) also referred to as a 3B-uridylylation site (bus) and a type II Internal Ribosome Entry Site (IRES). However, the specific contribution of S-fragment, polyC tract, and L fragment pseudoknots to FMDV biology is unknown. The *cre* is essential for picornaviral replication and contains a conserved AAACA motif which in aphthovirus functions as a template for 3D-pol-mediated uridylylation of 3B (Murray and Barton, 2003).

1.2.2.4.2. ORF Encoded FMDV Virus Polyprotein

1.2.2.4.2.1 The Leader Protease (L^{pro})

The first component of FMDV polyprotein is the Leader protein (L^{pro}). FMDV is unique in having a protease as the Leader protein. Two isoforms, Lab^{pro} and Lb^{pro}, arise from the presence of two in-frame AUG codons (separated usually by 84 nucleotides) for the initiation of protein synthesis on the viral RNA (Sangar *et al.*, 1987). It has been shown that these two forms of the L protein share the known major functions of this papain-like cysteine protease (Medina *et al.*, 1993). They can both cleave the L/P1 junction, in *trans* (intermolecular) and probably in *cis* (intramolecular) as well. They can also both induce the cleavage of the eukaryotic initiation factor 4G (eIF4G) which is an essential component of the cap-binding complex resulting blockage of host cap-dependent mRNA translation but the modified complex is still able to support cap-independent translation initiation on the FMDV mRNA IRES element (Belsham, 2005).

1.2.2.4.2.2 FMDV Structural Proteins

The P1-2A gene product is the precursor of the capsid proteins 1A, 1B, 1C, and 1D. The capsid is composed of the four major structural proteins (VP4, VP2, VP3 and VP1) and contains 60 copies of each (Cooper *et al.*, 1978). Five copies of VP1 are clustered around the fivefold axis of symmetry; while VP2 and VP3 are positioned at the two and three-fold axis of symmetry (Acharya *et al.*, 1990) **(Figure 1.2.2.4.2.2)**. These proteins are elements of identical four-segmented protein subunits called protomers which are defined as the smallest identical subunit of an oligomeric protein (Monod *et al.*, 1965).

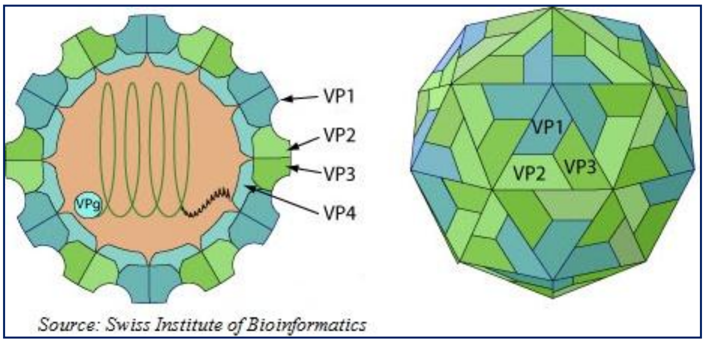


Figure 1.2.2.4.2.2 The virion of FMDV

To establish the icosahedral symmetry structure in the virus capsid the structural proteins are usually assembled as 12 pentamers. The basic building block of the icosahedral capsid is a pentamer made up of five copies of VP1 to VP4. The pentamers are stable through the interactions involving the N and C terminus of VP1 and VP3 along with VP4. These three proteins together with VP2 form the protomeric subunit and adjacent pentamers are held together by hydrogen bonds between parts of VP2 and VP3. It has been reported that the relative weakness of these interactions may facilitate the uncoating of the viruses during replication (Stanway, 1990). VP1, VP2 and VP3 are situated at the surface of the FMD virus. The smallest capsid protein, VP4, is internal and can be thought of as an N-terminal extension of VP2 which is cleaved from the VP2/VP4 precursor at the final stage of maturation of the virus particle. The VP4 protein interacts with the viral RNA (Strohmaier and Adam, 1974; Chow *et al.*, 1987; Acharya *et al.*, 1989). VP3 is the most conserved surface exposed structural protein among different FMD viruses (Acharya *et al.*, 1990). VP1 is the most important protein for epidemiological studies of FMD viruses both for its antigenic properties and as the virus-cell attachment site. Two regions of VP1, amino acids 140-160 and 200-213, have been shown to induce antibodies involved in neutralization of viral infectivity (Bittle *et al.*, 1982; Strohmaier *et al.*, 1982). The electron density map has also revealed that the immunogenic site (140-154) is exposed on the surface of the virus particle and located at a highly disordered region (GH-loop) of the capsid (Acharya *et al.*, 1989), while the C-terminus antigenic residues (200-213) are highly ordered. Several subsequent studies have shown that purified VP1 alone can elicit neutralizing and protective antibodies in mice, guinea pigs, cattle and pigs although the titre was low when compared when the whole virus particle was used (Bittle *et al.*, 1982; Strohmaier *et al.*, 1982; Acharya *et al.*, 1989). The 201-213 sequence at the C-terminus also elicited neutralizing antibody but the levels were lower than those obtained with the 141-160 (G-H loop) sequence.

Several studies have reported that the RGD sequence within the G-H-loop of the VP1 is involved in attachment of the virus to susceptible cell receptors (Leippert *et al.*, 1997). The cleavage of VP1 by trypsin abolished the ability of the FMDV to bind and infect susceptible cell cultures (Baxt *et al.*, 1989). Similar work conducted by Liebermann and co-workers (1991) on Type O1 Kaufbeuren reported that the highly conserved triplet, RGD within the G-H-loop is responsible for binding of FMD virus to pig kidney cell receptors. FMD virus infection of susceptible cells is successfully blocked following the binding of antibodies directed against the RGD region as well as peptides representing part of its sequence. In addition, many studies have reported that the RGD binds to a large family of integrin receptors and many extracellular substrate proteins (Ruoslahti and Pierschbacher, 1987). Although, the RGD has been reported to play the main role in cell-virus attachment, it is not the sole element in the binding process since removal of the C-terminus of VP1 in the absence of cleavage within the FMD virus loop also affects the attachment (Fox *et al.*, 1989).

1.2.2.4.2.3. Non-Structural Proteins (NSP)

The P2 and P3 precursors are processed into non-structural proteins **(Figure 2.2.4)** which are involved in viral RNA replication and protein processing (Brown, 1976; Sangar, 1979; Forss *et al.,* 1984; Acharya *et al.,* 1989; Belsham, 1993). The genome segments P2 and P3 encode the non-structural proteins 2A, 2B, 2C and 3A, 3B, 3C, 3D, respectively (Ryan *et al.,* 1989). The protein 2A is a protease and cleaves itself liberating the precursor of the capsid proteins whilst 3C carries out the majority of the processing of the polyprotein (Stanway, 1990). The protein 3D is the RNA-dependent RNA polymerase (RdRp) and is required for the replicative intermediate (RI) stage during replication (Caliguiri, 1974). The roles of the proteins are described in **Table 1.2.2.4.2.3.**

Table 1.2.2.4.2.3 Characteristics and functions of Non-structural proteins of FMDV

Name	Characteristics	Functions
NSP-2A	Protease	An 18-amino acid peptide which induces a modification of the cellular translation apparatus (Donnelly, *et al.,* 2001a **)**
NSP-2B	Integral membrane protein	Enhances membrane permeability, blocks protein secretory pathways, suppresses apoptotic responses by affecting intracellular Ca^{2+} homeostasis, and is implicated in virus-induced cytopathic effects (Doedens and Kirkegaard**,** 1995; Jecht *et al.*,1998; van Kuppeveld *et al.*, 1997)
NSP-2C	An ATPase	2C localizes to membrane-associated virus-replicating complexes (Tesar and Marquardt, 1989) and affecting the initiation of minus-strand RNA synthesis in the cytoplasm (Klein *et al.*, 1999).

Name	Characteristics	Functions
NSP-3A	A negative regulator of virus	FMDV 3A has been implicated in virus virulence and host range (Graff *et al.*, 1994; Lama *et al.*, 1998)
NSP-3B	Encode multiple 3B proteins (3B1-3)	Primes genomic RNA synthesis during virus replication (Falk *et al.*,1992; Paul *et al.*, 2003)
NSP-3C	A member of the trypsin like family of serine proteases	Responsible for most of the proteolytic processing of the viral poly protein. The 3C also modifies certain cellular proteins e.g. the histone H3 was shown to be cleaved by this protease (Falk *et al.*, 1990), and more recently it has been shown that 3C protease also cleaves the translation initiation factors eIF4A and eIF4GI within FMDV-infected cells (Belsham *et al.*, 2000).
NSP-3D	Viral RdRp ($3D^{pol}$)	Responsible for generating minus-and-plus sense genomic RNA

1.2.2.4.2.4. 3' Un-translated Region (UTR)

The 3' UTR, which follows the ORF termination codon, contains a short stretch of RNA which folds into a specific stem-loop structure (Pilipenko *et al.*,1992) followed by a polyA tract of variable length carried on the genome (Dorsch-Hasler *et al.*,1975). The 3' UTR also appears to be important for genome replication (Melchers *et al.*, 1997; Pilipenko *et al.*, 1996; Rohll *et al.*, 1995). This is supported by studies showing that the 3' UTR can bind a number of picornaviral proteins that are involved in RNA replication (Cui and Porter, 1995; Cui *et al.*, 1993; Harris *et al.*, 1994). Gutierrez and coworkers (1994) demonstrated that hybridization of antisense RNA to the 3' UTR of FMDV did not effect in vitro translation of viral RNA but did inhibit RNA replication in infected cells. In contrast, more recent studies have demonstrated that deletion of the FMDV 3' UTR reduced the efficiency of in vitro translation (Lopez de Quinto *et al.*, 2002) and blocked the ability to recover viable virus from transfected cells (Saiz *et al.*, 2001). Replacing the FMDV 3' UTR with that of the enterovirus, SVD virus, resulted in a nonviable genome (Saiz *et al.*, 2001), suggesting that the 3' UTR is specific for each picornavirus. The polyA tract probably functions in FMDV translation (Lopez de Quinto *et al.*, 2002) and may also play a role in picornavirus RNA replication (Barton *et al.*, 2001; Herold and Andino, 2001).

1.2.2.5 Virus Replication

Aphthoviruses replicate in a similar fashion to all picornaviruses. Replication is cytoplasmic and involves attachment of the exogenous virus to the cell membrane. Attachment to the membrane and subsequent entry into the cell is mediated by a membrane receptor. After genome replication within the cytoplasm, virion assembly occurs and new virus particles aggregate within the cell. Release of new virus particles is mediated by cell lysis (**Figure 1.2.2.5**).

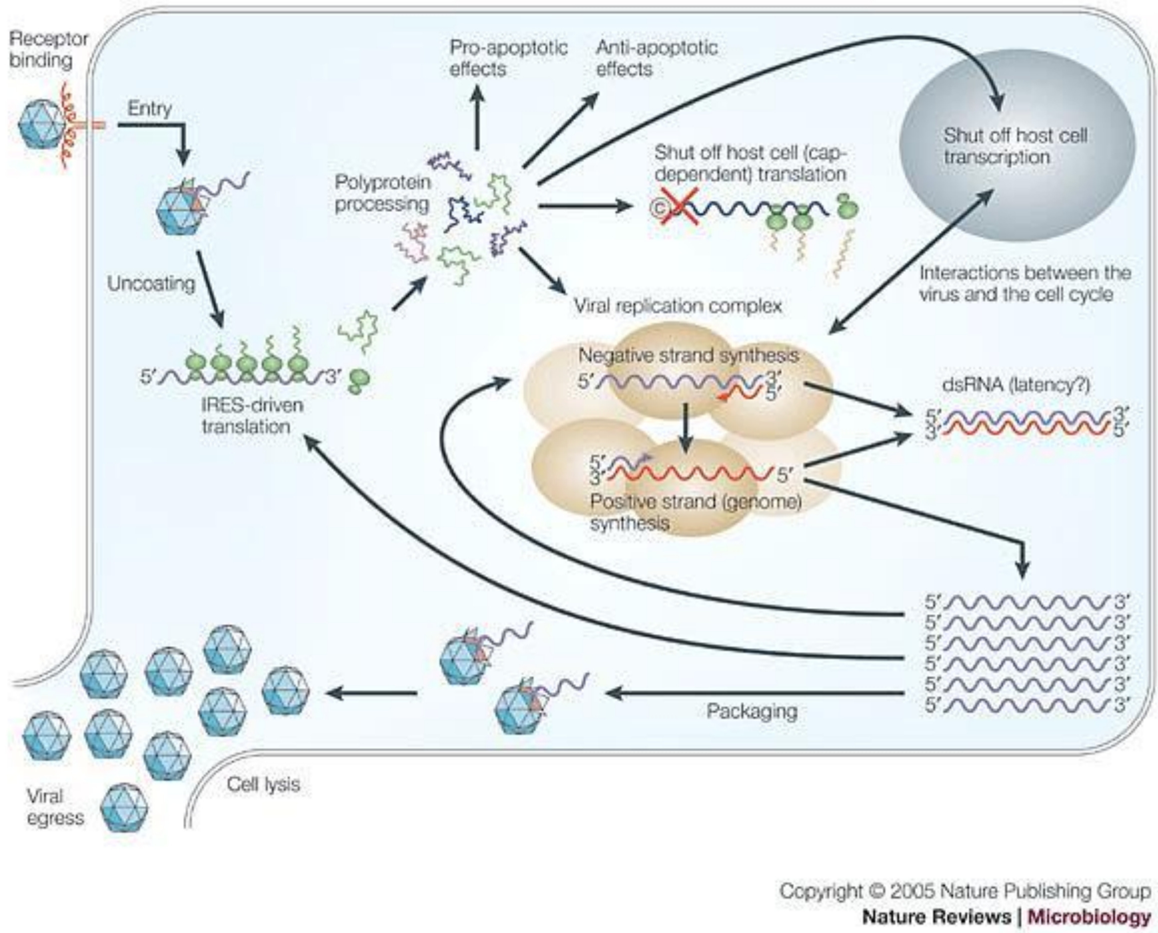


Figure 1.2.2.5 The Life cycle of a member of family *Picornaviridae* (Whitton *et al.*, 2005).

In the initial event of the replication process, FMD virus uses highly conserved triplet sequence (Arg-Gly-Asp) motif on the G-H loop to attach to specific receptors on the cell membrane (Fox *et al.*, 1989; Mateu *et al.*, 1996). These receptors mediate the release of the viral genome from the protein shell into the cytoplasm, which is the site of the genome replication. The incoming RNA uses the host cell protein-synthesizing machinery causing shut down of host cell replication. Complementary negative (-) RNA strand synthesis of the positive (+) RNA strand is initiated by a virally-encoded RNA polymerase. Further synthesis of (+) RNA strands leads to the formation of multi-stranded replicative intermediates (RI) structures with a 3' poly A which are transcribed from the poly U tract in the RI. The RI generates a pool of (+) RNA for translation and some for synthesis of additional (-) RNA. As the protein level increases, some (+) RNAs are packaged into virions (Rueckert, 1985). During packaging, a single molecule of new viral RNA is inserted in the so called procapsid, which is a preformed, empty capsid. The new virus particle can finally be released through cell lysis.

1.2.2.6 Susceptible Host Range

Aphthovirus can infect at least 200 species of mammal belonging to more than 20 families (Murphy *et al.,* 1995) particularly cloven-hoofed animals domestic and wild such as; cattle, swine, sheep, goats, camels, deer, moose, llama, chamois, alpaca, vicuna, giraffe and others. The most sensitive hosts of FMDV are cattle and swine, because of their extreme sensitivity to respiratory infection, these animals due to their sensitivities develop sever clinical sings. The clinical signs in sheep, goats, and wild ruminants are milder than in cattle (Geering and Lubroth, 2002). Horses, dogs and cats are not susceptible hosts but they can inevitably spread the virus via their fur if contaminated. It has been reported more frequently in the Indian elephant. Amongst wildlife, the disease can be severe or subclinical in impala making the impala a possible transmission route of FMD virus from buffalo to cattle (Bastos *et al.*, 2000).

1.2.2.7 Transmission and Pathogenesis

In general, the FMDV can be carried by animals, animal products, people, vehicles and contaminated equipments to susceptible animals (Kitching, 2005). FMDV can become airborne and spread by wind under specific weather condition and a humidity higher than 60% (Donaldson and Alexandersen, 2002; Alexandersen *et al.*, 2003; Mahy, 2005), especially in highly dense livestock in areas (Cannon and Garner, 1999). The FMDV can be spread by direct and indirect contact. For direct contact, the virus spreads from animal to animal at grazing areas, at water sources, and other places. For indirect contact, FMDV contaminates equipments, animal products, and by aerosol under suitable condition (Geering and Lubroth, 2002). The incubation period of FMD is highly variable, 2-14 days, and depends on the strain and dose of virus, the route of transmission, the animal species and the husbandry conditions (Alexandersen and Mowat, 2005). Charleston *et al.* (2011) found that period of infectiousness in cattle is only 1.7 days and animals are not infectious until 0.5 days after the appearance of clinical signs.

Some immunized animals, no matter whether of by vaccination or by natural infection, may carry FMDV without clinical signs. The carrier stage can last up to six months for cattle, nine months for sheep, four months for goats and one month for swine (Aftosa, 2007). It has been reported that people can carry the virus in the nasal passages for a short period of time. FMDV can be transmitted by human via contaminated clothes, shoes, and equipments (Mahy, 2005). The epithelial cells of the dorsal soft palate, the roof of the pharynx and part of the tonsil are thought to play a special role in the primary infection (Alexandersen *et al.* 2003). This includes the feet and mammary teats (Alexandersen *et al.* 2003). Less commonly affected epithelial lesion sites include external genitalia and rumen. During this secondary stage, lesions which are observed initially as a blanched area subsequently develop into vesicles that cause lesions at the mouth, feet and teats (Seibold 1963; Alexandersen and Mowat 2005; Arzt *et al.* 2009). There is often secondary bacterial infection which delays healing of the lesions. Myocardial infection, when it does occur, is typically during the viremic phase in young pigs, small ruminants, and wildlife (Arzt *et al.* 2011a). Clearance of viraemia and viral tissue load is achieved by the induction of an effective immune response, and is characterised by the generation of virus specific antibody and may be dependent on the interaction of virus-antibody complex with phagocytic cells of reticuloendothelial system (McCullough *et al.* 1986, 1988, 1992).

1.2.2.8 Genetic Variation of FMDV

The genetic variation in FMD viral genome exists due to absence of proof-reading in the 3D-encoded RNA dependent RNA polymerase and competitive selection.

1.2.2.8.1 Mutations

Antigenic variation can be caused by nucleotide mutations or recombination in the RNA viral genome. Mutants with a selective advantage in the prevailing environment are better represented than other viruses (Sahle *et al.,* 2004). Mutation rate in FMDV ranges from 10^{-3} to 10^{-5} per nucleotide site per genome replication. This high error rate leads to differences in replicated genomes from the original parental genome of the virus (Grubman and Baxt, 2004). Studies revealed that the rates of mutations of the European serotype FMDV RNA genome can reach 10^{-2} substitutions per nucleotide site per year (s/n/y) (Gebauer *et al.,* 1988). Similar studies conducted on SAT1 and SAT2 FMD viruses have estimated nucleotide changes of 1.64 % and 1.54 %, respectively per year for the VP1 gene (Vosloo *et al.,* 1996). This rapid mutation rate is a million times greater than the rates in their natural hosts (10^{-8} to 10^{-9} nucleotide substitution per year). These mutations may give rise to variant viruses that can be a source of new outbreaks (Vosloo *et al.,* 1992 and 1996). Mutations that lead to conformational changes produce a population of neutralizing escape variants (Stave *et al.,* 1988).

1.2.2.8.2. Recombination

Recombination was first reported in picornaviruses following the replication of a mixture of mutants in the same cell monolayer (Hirst, 1962). Later, Pringle (1965) presented evidence of genetic recombination between immunologically distinct strains of FMD virus SAT2 (Kenya-3 and Rho-1) multiplying in the same tissue. The mechanism of recombination in FMD virus has been described as the modification of the surface proteins due to segment crossing over during co-infection of the animal cells by more than one FMD virus serotype (Krebs and Marquardt, 1992). Crossing over or reciprocal recombination involves the even exchange of homologous sequences. Non-reciprocal recombination, on the other hand, involves the uneven replacement of a sequence which results in the loss of one of the variant sequences involved in the recombination event. This procedure has been reported to be responsible for severe types of mutational change that may affect the susceptibility of the natural hosts (King *et al.,* 1980).

1.2.2.8.3 Quasispecies Concept

The quasispecies concept was proposed by Eigen in 1971. RNA viruses have genomes that replicate in the absence of repair mechanisms; they evolve very rapidly with a mutation frequency per nucleotide site of 10^{-3} to 10^{-5} substitutions per year (Van Regenmortel *et al.,* 1997). The high rate of error during RNA replication in the picornaviruses gives rise to a range of multiple co-circulating viral genomes within a host. A dominant virus genome sequence called a master or consensus sequence in regard to the specific environment emerges and competes with the others in the virus population. This is defined as the quasi-species principle of RNA viruses (van Regenmortel *et al.,* 1997). At a certain time, a shift or change in the equilibrium will alter the consensus sequence and cause one of the variants to dominate. FMDV populations exist as mixtures of related but non-identical genomes that can have a competitive potential, and generate a dominant variant in a viral population (Domingo *et al.*, 1992).

Continual modification of the genome of FMD viruses isolated from persistently infected cattle and buffalo have been reported (Vosloo *et al.*, 1996). Antigenic changes occur following replication of a virulent FMD virus in partially immune populations of cattle and a large number of genetic and phenotypic variants have also been generated after limited replication in cell cultures (Sobrino *et al.*, 1983).

1.2.2.9 Laboratory diagnosis of FMD

Complement fixation test was the earliest laboratory test used to diagnose FMD. This test was later replaced by ELISA due to its sensitivity and specificity. This method is able to confirm the clinical diagnosis and identify the FMDV serotypes (Ferris *et al.*, 1988). Molecular methods, such as conventional RT-PCR assays have been developed and also can provide serotype-specific results. However, the number of samples that can be analyzed simultaneously with this technique is limited and this approach may not be able to cope with samples that might be received during an epidemic. Therefore, real time RT-PCR was developed and has been shown to have high sensitivity and specificity for the detection of FMDV genomes of all seven serotypes (Reid *et al.*, 2002). This assay has been used on a large number of tissues samples, serum samples, swab samples and tissue culture supernatants. Another antigen detection method that has been developed is the "lateral flow device" (LFD) which has been evaluated and shown to be pan-reactive to all FMDV serotype except for serotype SAT 2. Since this technique was easy and rapid, it has the potential to be used for pen-side diagnosis for FMD suspected outbreak (Ferris *et al.*, 2009).

1.2.3 Epidemiology of FMD

The epidemiology of FMD is complex, and it is affected by different viral, host, and environmental factors, among them, variations in virus virulence (severity of lesions, amount, and duration of virus release), particle stability in different microenvironments, and chances of long-term persistence. FMDV multiplication and spread also depend on the host species, nutritional and immunological status, population density, animal movements, and contacts between different domestic and wild host species and animals capable of mechanical dissemination of the virus (Nishiura and Omori, 2010). The environment can provide geographical barriers to virus dissemination or, alternatively, can promote virus transmission when appropriate atmospheric conditions prevail. In this multifactorial scenario (Sobrino and Domingo, 2001), the high potential for FMDV variation and adaptation has modeled complex evolutionary patterns that are being revealed by molecular epidemiology analyses, mostly based on nucleotide sequencing of capsid protein genes.

1.2.3.1 FMD Virus Pools

Despite the propensity and opportunities for spread of FMDVs into new regions, comparison of the VP1 coding nucleotide sequences reveals a tendency for similar viruses to recur in the same geographical area. This tendency apparently reflects some degree of ecological isolation, likely reflecting patterns of animal movement and trade or specific wildlife reservoirs (e.g. African buffalo) within a region. Based on genetic and antigenic analyses, FMDVs throughout the world have been sub-divided into seven regional pools (Di Nardo *et al.*, 2011; Sumption *et al.*, 2012).

Certain countries share viruses belonging to two different pools, for example, Egypt and Libya **(Table: 1.2.3.1).** Virus circulation and evolution within these regional virus pools result in changing needs for appropriate vaccine selection.

Table: 1.2.3.1 List of countries representing each virus pool for the period 2011-2015

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 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

Geographical distribution of seven pools of foot-and-mouth disease viruses (**Figure 1.2.3.1)** showed that FMDV Serotype O is the most widely distributed serotype of the virus (in 6 of the 7 indicated virus pools) whereas, in contrast, SAT3 is only present in pool 6 (within southern Africa). The Asia-1, SAT1 and SAT2 serotypes also have quite limited geographical distribution. However, individual countries can have multiple serotypes in circulation at the same time and hence it is necessary to be able to determine which serotype is responsible for an outbreak if vaccination is to be used. Countries which are normally free of the disease (marked in yellow) can still suffer incursions of the virus which can have high economic costs.

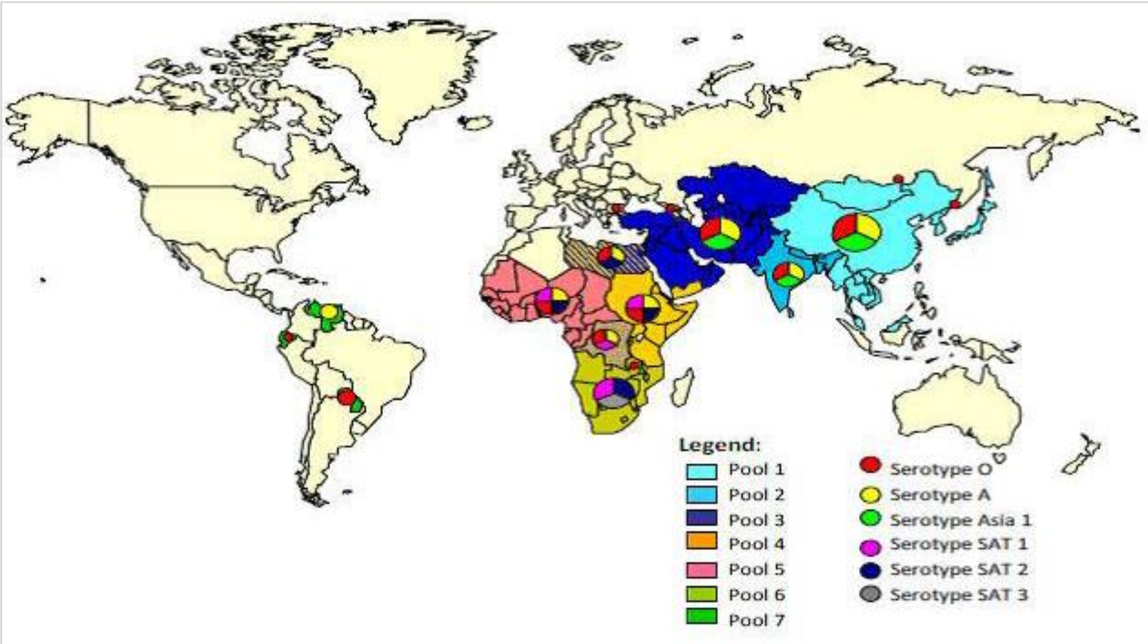


Figure 1.2.3.1 Foot and mouth disease (FMD) virus pools: World distribution by serotype in 2011-2015

1.2.3.2 Global Distribution of FMDV

The serotypes of FMDV are not distributed uniformly around the world. Recently, FMD has been endemic in several parts of the world, particularly in Asia, South Africa, the Middle East, and South America (**Figure 1.2.3.2)**. The serotype O, A and C viruses have had the widest distribution and have been responsible for outbreaks in Europe, America, Asia and Africa. However, the serotype O is the most distributed strain in many countries (Kitching, 1999) and the last reported outbreak due to serotype C was in Ethiopia during 2005 and serotype C viruses may no longer exist outside of laboratories. The SAT1-3 viruses are normally restricted to sub-Saharan Africa although there have been some limited outbreaks due to SAT1 viruses in the Middle East between 1962-1965 and 1969-1970 and then in Greece in 1962 (Knowles and Samuel, 2003). Similarly, there have been reports of minor incursions of the serotype SAT2 in Yemen in 1990 and in Kuwait and Saudi Arabia in 2000 (Grubman and Baxt, 2004). More recently, spreading of FMDV byserotype SAT2 occurs from sub-Saharan Africa through northern African countries (Egypt and Libya) and into Palestine (Valdazo-Gonzalez *et al.*, 2012). This serotype was also detected in Bahrain. The serotype Asia-1 is generally confined to Asia, except two incursions into Greece, one in 1984 and a second in 2000 had been reported.

The pandemic serotype O virus (designated as the PanAsia strain) belongs to the ME-SA topotype which has spread rapidly and vigorously. This lineage replaced the other lineages of FMDV previously circulating in the Middle East. This lineage has been responsible for disease outbreaks everywhere in the world where FMD is endemic or sporadic except for South America and been responsible for incursions into previously disease-free countries. The PanAsia lineage was first detected in India in 1982 (Hemadri *et al.*, 2002) but was confined to India until 1990. Its predominance in field outbreaks in India was, however, noticed from 1996 onwards (Hemadri *et al.*, 2002). It spread northwards to Nepal in 1990 and again 1997-1999 and to Bhutan in 1998 and also towards the west into Bahrain, Kuwait, Saudi Arabia, Syria, Yemen, Iran and Lebanon in 1998 and to UAE, Israel and Turkey in 1999 (Knowles *et al.*, 2005). The lineage spread further to China in 1999 and into South East Asian countries causing outbreaks in Thailand in 1999, Malaysia and Laos (PDR) in 2000 and Vietnam in 2002. The virus also caused disease outbreaks in South Korea (Shin *et al.*, 2003) and in Japan 2000 (Sakamoto *et al.*, 2002).

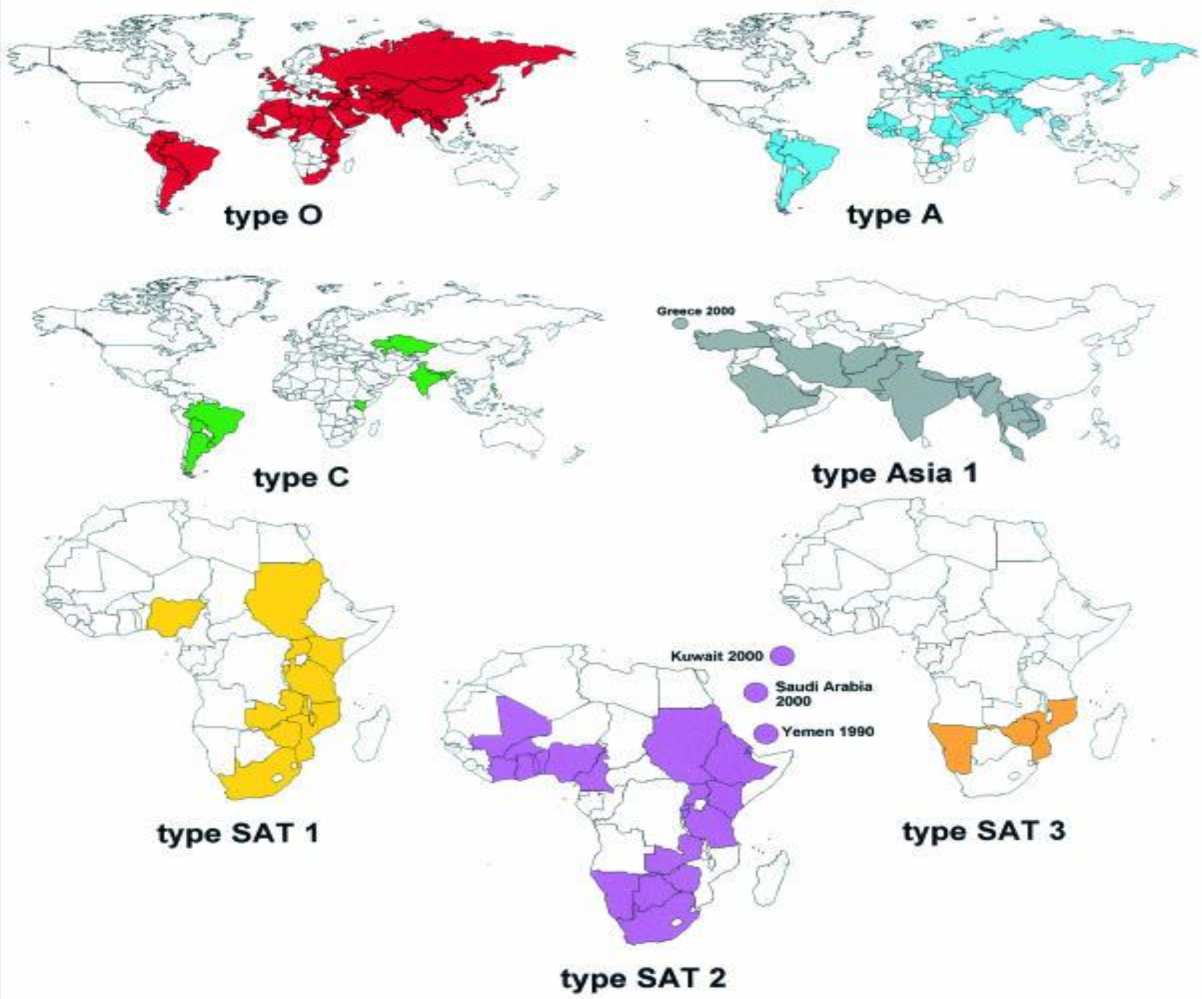


Figure 1.2.3.2 Countries in which FMD was reported to the OIE between 1990 and 2002. (www.iah.bbsrc.ac.uk/virus/picornaviridae/aphthovirus). **The data and maps were compiled by Nick Knowles.**

South Korea faced outbreaks again in 2002 and in 2010. The 2002 outbreaks were caused by serotype O virus, belonging to the PanAsia lineage, whereas, both serotype O (topotype SEA, lineage MYA-98) and A (topotype ASIA, genotype SEA, lineage MYA-97) viruses were responsible for the 2010-2011 outbreaks (Yoon *et al.*, 2012). South Korea appears to have had three independent introductions of the virus in 2010. Firstly there was an incursion of FMDV serotype A in January 2010. The disease was controlled using a stamping out policy. There had been no reported outbreaks caused by serotype A in eastern Asia since 1973. This incursion was followed by second introduction of FMDV in April 2010, in this case serotype O. South Korea was declared free without vaccination by OIE on September 2010 after implementing a stamping out policy. The third incursion took place in November 2010 and then spread throughout the country.

Similarly, Japan was hit by FMD ten years after the previous outbreak (Nishiura and Omori, 2010), FMDV type O, belonging to the MYA-98 lineage within the SEA topotype, was detected on April 2010 at a beef feeding farm in southern Japan. The disease spread to the surrounding areas and the VP1 sequence data indicate that mainland Southeast Asia is the source of FMDV serotypes O and A in Eastern Asia (Knowles *et al.*, 2012).

1.2.3.3 FMDV in Bangladesh

In Bangladesh, FMD remains endemic and was first officially documented in 1958 (Pirbright Laboratory 2010) (**Figure: 1.2.3.3)** during extensive outbreaks in many parts of the country. However, FMDV Asia-1 serotype was isolated in 1987 and 1996 (Marquardt *et al.,* 2000) and again between 2012 and 2013 (Ullah *et al.,* 2015) but not in later studies. FMDV serotype C was last found in Bangladesh in 1992 (FAO World Reference Laboratory for Foot-and-Mouth Disease, 2010). There were type A and type O viruses isolated between 1987 and 1997 in Bangladesh (Freiberg *et al.,* 1999), again between 1998 and 2000 (Islam *et al.,* 2001) and between 2011 and 2013 (Nandi *et al.,* 2015; Sultana *et al.,* 2014 and Ullah *et al.,* 2014). According to the FAO Reference Centre for South-Asia, in the field of FMD diagnosis, epidemiology and research, serotypes O, A and Asia-1 of the FMD virus are currently prevalent in Bangladesh and the disease is reported throughout the year.

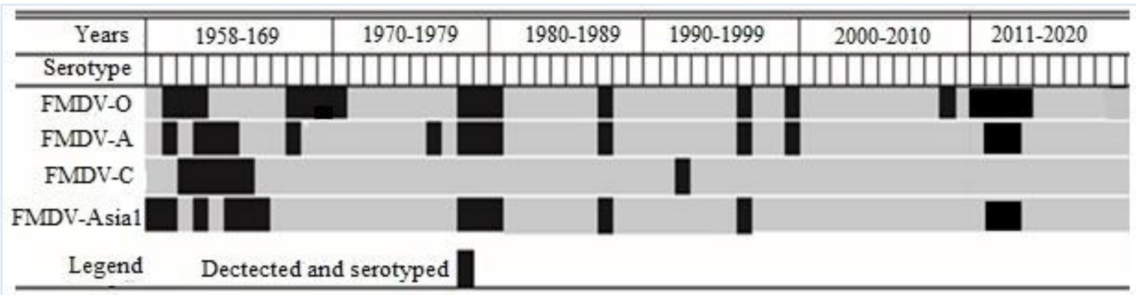


Figure: 1.2.3.3 Occurrence of FMD outbreaks in Bangladesh

1.2.4 Cell Culture and Virus Isolation

The suspensions obtained from the specimens are inoculated onto susceptible cells (e.g. BHK-21 cells) incubated at 37^{o}C and examined for cytopathic effect (CPE) 24 to 48 hours post infection. Revenson and Segura (1963) reported that FMDV grew well on BHK-21 cell line enabling large-scale production of antigen with good complement fixing properties. The BHK-21 cell culture provides better growth for FMDV than the suspension culture (Clarke and Spier, 1980; Girard, 1975; Clarke and Spier, 1977). It has also been reported that with subsequent passage in BHK-21 clone 13 cell line, the titre of FMDV increased significantly (Sellers, 1955). Nair (1987) reported that the susceptibility and infectivity titers of IBRS-2 and MVPK cell lines were less as compared to BHK-21 cells, and thus had no advantage over BHK-21 cell line for vaccine production. Mishra *et al.*, (1995) also adapted FMDV field isolates to BHK-21 clone 13 cells in 3-7 serial passage.

The CPE usually develops within 48 hours, but it can be seen as early as 12 hours post infection as cell detachment and destruction with high virus concentration. If no CPE is observed the cells are frozen and thawed followed by 2 blind passages on fresh cell culture and examined for CPE for another 48 hours (Kahrs, 1981; Westbury *et al.*, 1988). However, the cell culture system is laborious, time consuming, and relatively low sensitive. It also requires careful handling of specimens and a biosafety laboratory.

1.2.5 Molecular Characterization of FMDV

When epithelium tissue is not available from ruminant animals e.g. in advanced or convalescent cases and infection is suspected in the absence of clinical signs, samples of oesophageal-pharyngeal (OP) fluid is collected by means of a probang and used for virus isolation.

1.2.5.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

A variety of RT-PCR methods have been reported in recent years for the early detection of FMDV RNA in epithelium, cell culture isolates and other tissues using universal primers for all seven serotypes (Meyer *et al.*,1991). Typing of FMDV by RT-PCR was first demonstrated by Rodriguez *et al.*, 1992 for the differentiation of the serotypes O, A and C. Serotype specific primers have since been designed for the detection of all seven FMDV serotypes by RT-PCR (Vangrysperre and De Clercq, 1996; Callens, and De Clercq, 1997).

1.2.5.2 Nucleotide Sequencing

Unlike many living organisms where the hereditary information is encoded within a DNA genome, FMD virus has a RNA genome that can be sequenced directly, but RNA is unstable and it usually first transcribed into cDNA prior of performing the nucleotide sequence. Reverse transcription (RT) when combined with PCR provides a rapid and powerful technique for studying diverse RNA genomes. In epidemiological studies of FMD virus, nucleotide sequencing of the VP1 gene has been used extensively to determine the relationships between the field isolates. The technique is also routinely used to investigate genetic variation, molecular evolution in carrier animals, and to identify the source of an infection in outbreak conditions (Domingo *et al.*, 1985; Beck and Strohmaier, 1987; Dopazo *et al.*, 1988; Vosloo *et al.*, 1992; Saiz *et al.*, 1993; Bastos, 1998). The first genetic relationships of FMD virus type A, O, and C were constructed using this approach (Beck and Strohmaier, 1987).

The nucleotide sequence of the major immunogenic protein, VP1 was also used to subtype the European FMD viruses type A and O recovered from different outbreaks (Beck and Strohmaier, 1987). They reported that the use of nucleotide sequences is not only a rapid and accurate technique for subtyping FMD virus but also differentiates variants of a given subtype. They also demonstrated that a single nucleotide change could be detected in the nucleotide sequencing of the isolate from Germany in 1984 (O Zusmarshausen) and strain O1 Kaufbeuren. Subsequent studies using this approach have provided crucial epidemiological insights which include among others, the use of nucleotide sequences for the identification of virus variants arising from laboratory cell passage (Sáíz *et al.*, 1993), the identification of trans-boundary virus transmission (Sáíz *et al.*, 1993; Samuel *et al.*, 1999), and evidence of prolonged persistence of a particular virus type in the field (Freiberg, *et al.*, 1999; Samuel *et al.*, 1999). Sequence data has also been instrumental in identifying outbreaks resulting from inadequately inactivated vaccines (Beck and Strohmaier, 1987; Krebs and Marquardt, 1992) and for refuting vaccine involvement in outbreaks (Suryanarayana *et al.*, 1998).

1.2.6 Selection of Candidate for the Development of Vaccine

Attempts to develop FMDV vaccines started in the early years of the 20th century when Belin (1927) described the experiments with attenuation of the virus. Later researchers also worked on attenuated FMD vaccines, including intensive studies in the 1960s, but major problems were encountered such as unpredictable virulence in the field. Effectively, this undermined any belief that a safe and stable attenuated product could be realized within a reasonable time frame. Given current knowledge and with the molecular biological tools at our disposal, it is conceivable that such a vaccine virus could now be developed.

The earliest report on the development of an experimental vaccine for FMDV was published by Vallee *et al.*, (1925), who showed that FMDV in the vesicular fluid from naturally infected calves could be inactivated with formaldehyde without losing its immunizing ability. This procedure was further developed by Waldmann *et al.*, (1937), who collected vesicular fluid from tongue of cattle which had been deliberately infected with FMDV, and subsequently inactivated it with formaldehyde in the presence of aluminium hydroxide gel. The aluminium hydroxide functioned as an adjuvant as well as facilitating inactivation of virus and providing a simple method of concentration. This resulted in the first, large scale production of an inactivated vaccine against FMDV. Large scale production of FMD vaccines was hindered due to the lack of in vitro systems for propagation of the virus. Clearly, the need to deliberately infect cattle was undesirable and production of Waldmann type vaccines was greatly assisted by the work of Frenkel (1947) who used epithelium obtained from the tongues of recently slaughtered healthy cattle. Suspension of the epithelial cells were prepared and maintained in vitro subsequently infected in a manner similar to that used today with baby hamster kidney (BHK) cells. Some national FMD laboratories in Europe soon introduced this technology and eventually this method was commercialized in France (Girard and Mackowiak, 1953) and Argentina (Rosenbusch, 1960). Availability of sufficient vaccine using this method resulted in the introduction of general vaccination programmes in many

European countries and achieved significant reductions in the number of FMD outbreaks (Fogedby, 1962). Other methods for growth of the virus for vaccine production were also investigated, for example, growing the virus in cattle skin in vivo (Belin, 1953). This method gained considerable application in France (Fogedby, 1962). A lapinized FMD vaccine was produced by growing the virus in rabbits which was then adsorbed to aluminum hydroxide and inactivated using formalin in 1954 in the former USSR. Mass vaccination using this vaccine resulted in eradication of the disease in Siberia (Fogedby, 1962). Industrial production of FMD virus in primary calf kidney cell culture monolayers was begun in 1960 (Ubertini *et al.*, 1960). Formaldehyde was initially used as inactivant for vaccine production, however, it was later replaced by binary ethyleneimine (Bahnemann, 1975 and 1990) when it was found that some residual infectious viruses were still present in the vaccines produced using the former inactivant.

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

The Progressive Control Pathway for Foot and Mouth Disease (PCP-FMD) is a set of FMD control activity stages that, if implemented, should enable countries to progressively increase the level of FMD control to the point where an application for OIE-endorsement of a national control programme vaccination (in an advanced phase of Stage 3) or official freedom from FMD with or without vaccination (end of Stages 4 and 5, respectively) may be successful and the status sustainable. FAO collaboration with OIE developed PCP-FMD as a working tool in the design of FMD country (and some regional) control programs to assist and facilitate countries where FMD is still endemic to progressively reduce the impact of FMD and the load of FMD virus. The PCP is not intended to be prescriptive; rather it is outcome-oriented and the key outcomes might be different in different countries and regions. Under this background the tool designed several stages assigning a definite goal (**Figure 1.2.7**). FAO also planned a tentative time-frame (**Table 1.2.7**) for each of the FMD endemic country for fulfilling the defined goal. Bangladesh has officially written plan in place to study the epidemiology and socioeconomic impact of FMD which is required to enter into stage-1 of PCP-FMD road map and it is revealed that with little more effort the country fulfilled the entire requirements (both essential and recommended) set for stage-1 of PCP and is ready to move to stage-2 at the moment. Unfortunately, poor reporting system by the related authorities or government about the disease to the OIE has made us difficult to earn credit in this concern. So, the plan does not include activities to estimate FMD incidence which is one of the required activities.

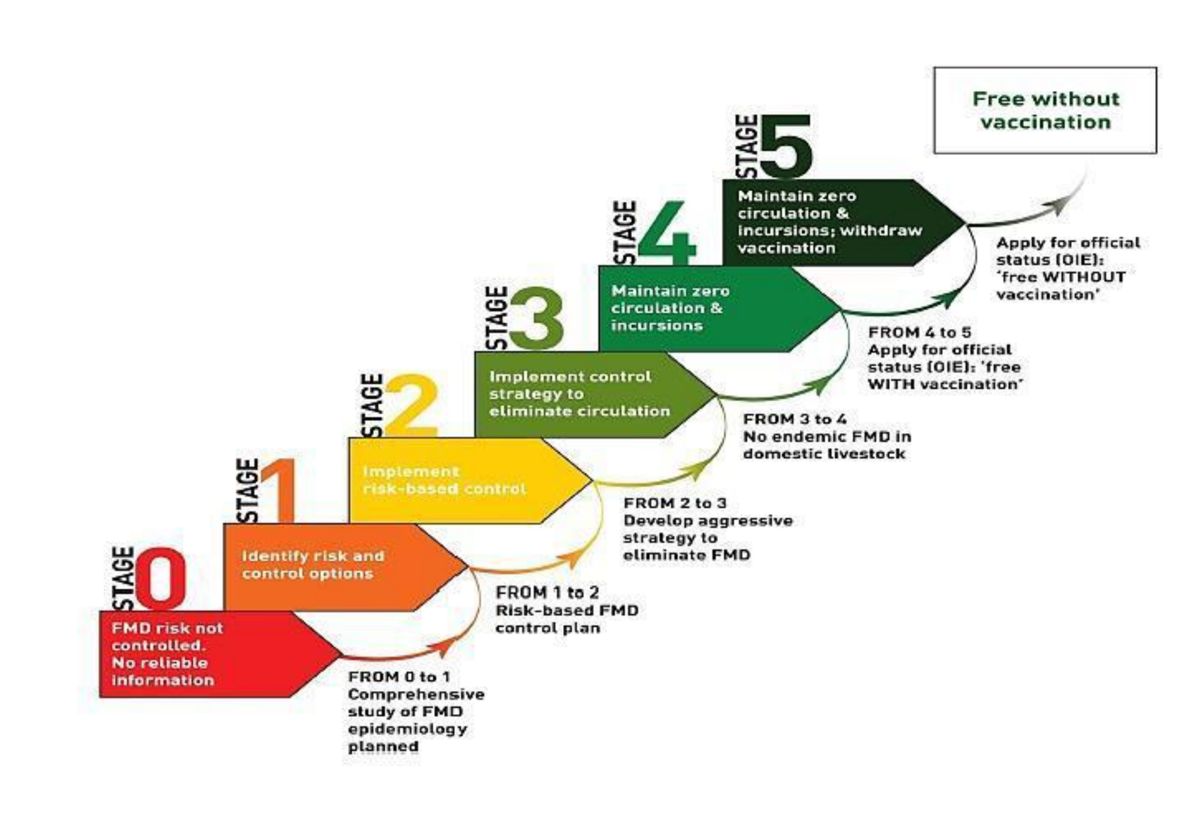


Figure 1.2.7 Stage progression in the Progressive Control Pathway for FMD

The revised agreed time frame given in **Table 1.2.6** showed that all countries but India consider themselves in PCP-FMD stage-1 and practically Bangladesh and Sri Lanka should have moved to stage-2 by 2014 though it is not officially affiliated by the OIE. India claimed to be at stage-3 since 2011 and in stage-4 since 2015, i.e. free from FMD with vaccination. India has prepared a FMD Control Programme for onward submission to OIE for its endorsement. Afghanistan and Pakistan were not invited to this event as they follow the West Eurasian road map being in gene pool-3 of FMD virus.

Table 1.2.7 Revised Time frame for PCP-FMD road map (2011-2020) for SAARC countries developed in 2013

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

1.2.8 Significance of the Study

FMD has direct and indirect impacts, which have been highlighted from different perspectives. Direct impact includes effect on animal health (high levels of morbidity and mortality, decreased rates of reproduction, weight gain and milk production) control or eradication programme costs and restrictions to trade in livestock and livestock products. Indirect impact has been divided into agricultural and other products (decreased draught efficiency in agricultural production), natural resources (limited to moderate changes in biodiversity) and human-welfare effects (loss of income and assets). All together it costs 2-30 billion USD per annum worldwide.

Bangladesh is not well equipped to control transboundary animal diseases like FMD because of the lack of infrastructure, effective animal health personnel those who are capable of correctly interpreting and analyzing the datasets produced and financial resources. For this reason, the current situation of FMD status in Bangladesh is almost unknown and majority of outbreaks remains unrecorded, and has little success to report for the FMD control. Moreover, very few laboratories in Bangladesh have the means to diagnose FMD adequately that lead to another limitation in the early diagnosis of FMD in an endemic situation.

For successful FMD control programme, sincere and honest approach, stable plan, skilled manpower, long-term Government commitment and adequate resources are necessary. A sustainable programme will also need farmer education, commitment and financial contributions.

1.2.9 Problem Statement

According to the study, by April 2016 many FMD outbreaks were reported spreading to more than 26 districts in different parts of Bangladesh. Since molecular characterization of FMDV is not routine in Bangladesh, the serotype and the genetic relationships among the viruses responsible for the recent FMD outbreaks is not known. It is not known whether the outbreaks in the different districts are due to a common source or they are new and independent introductions. To address this problem in the light of PCP-FMD road map, this investigation focused on understanding the molecular epidemiology of the disease, genome analysis of the local circulatory viruses, and the selection of suitable vaccine candidate.

1.2.10 Aim and Objectives

Based on the research focus, the aim and objectives of this study are-

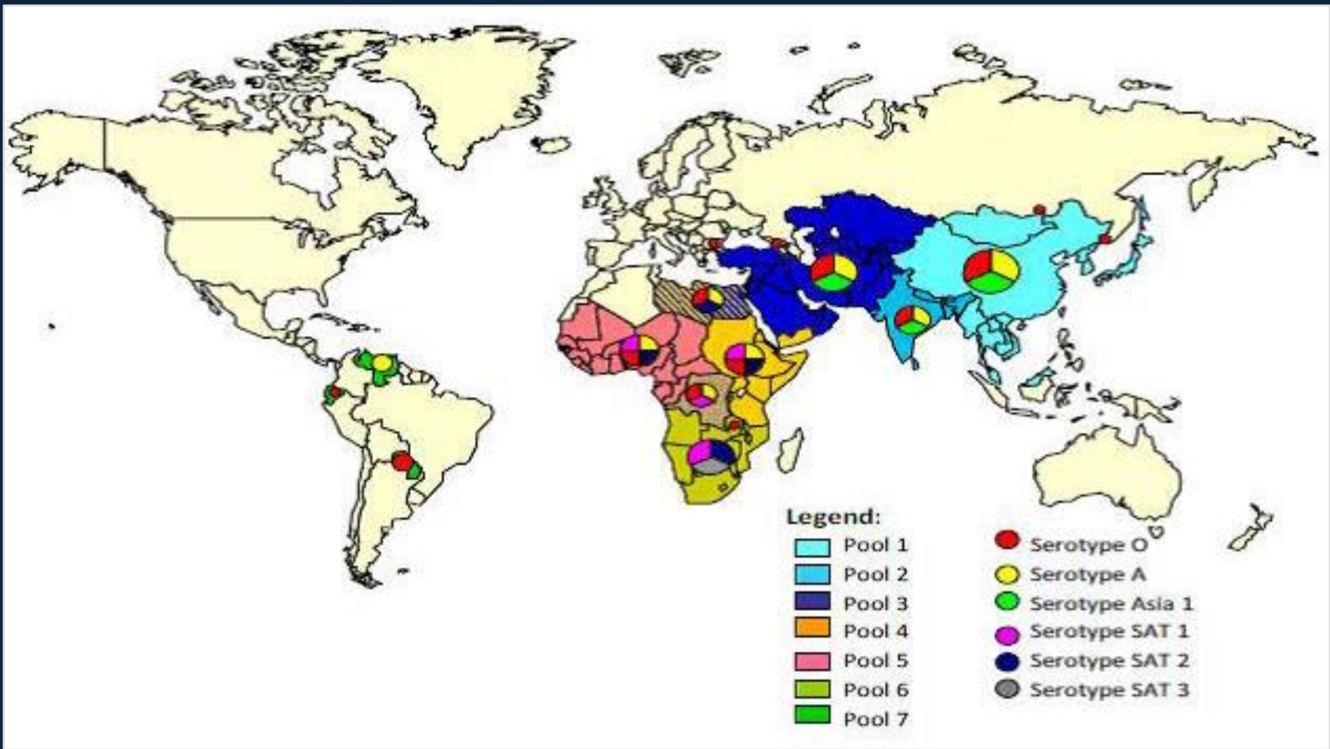
i. To learn the epidemiology of FMD in Bangladesh followed by molecular characterization of circulatory FMDVs;

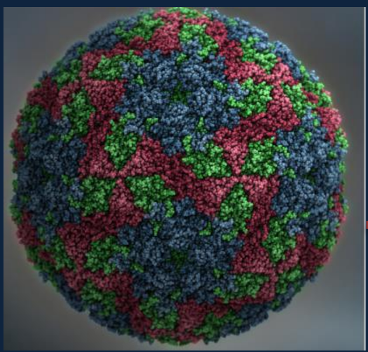
ii. To isolate the FMDVs circulating in cattle population of Bangladesh.

iii. To characterize the complete genome and genome wide analysis of the isolated FMDV serotypes; and

iv. To select the effective vaccine candidate from the outcomes of this study.

2





Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Research plan

The following figure contains all the information required to carry out the experiments described in the thesis chapter by chapter. All protocols required are described where appropriate and the lists of chemicals and equipment required are given in appendices.

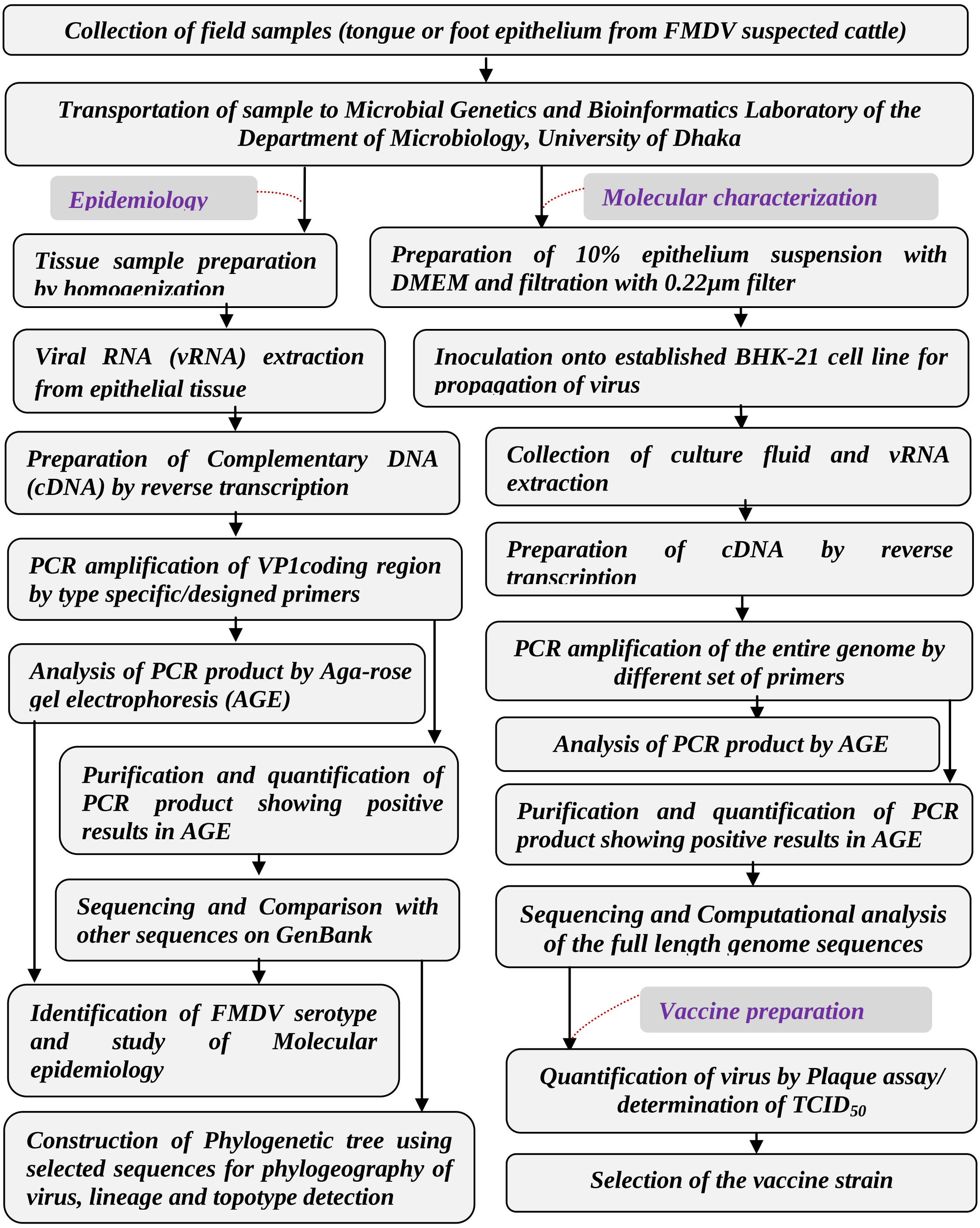


Figure 2.1 Research plan for the study of epidemiology and analysis of complete genome sequence of FMDV.

2.2 Study Sites and Sample Collection

FMD viruses from independent outbreaks in 26 different districts of Bangladesh **(Figure 2.2)** were obtained as tongue or foot epithelium tissue samples.

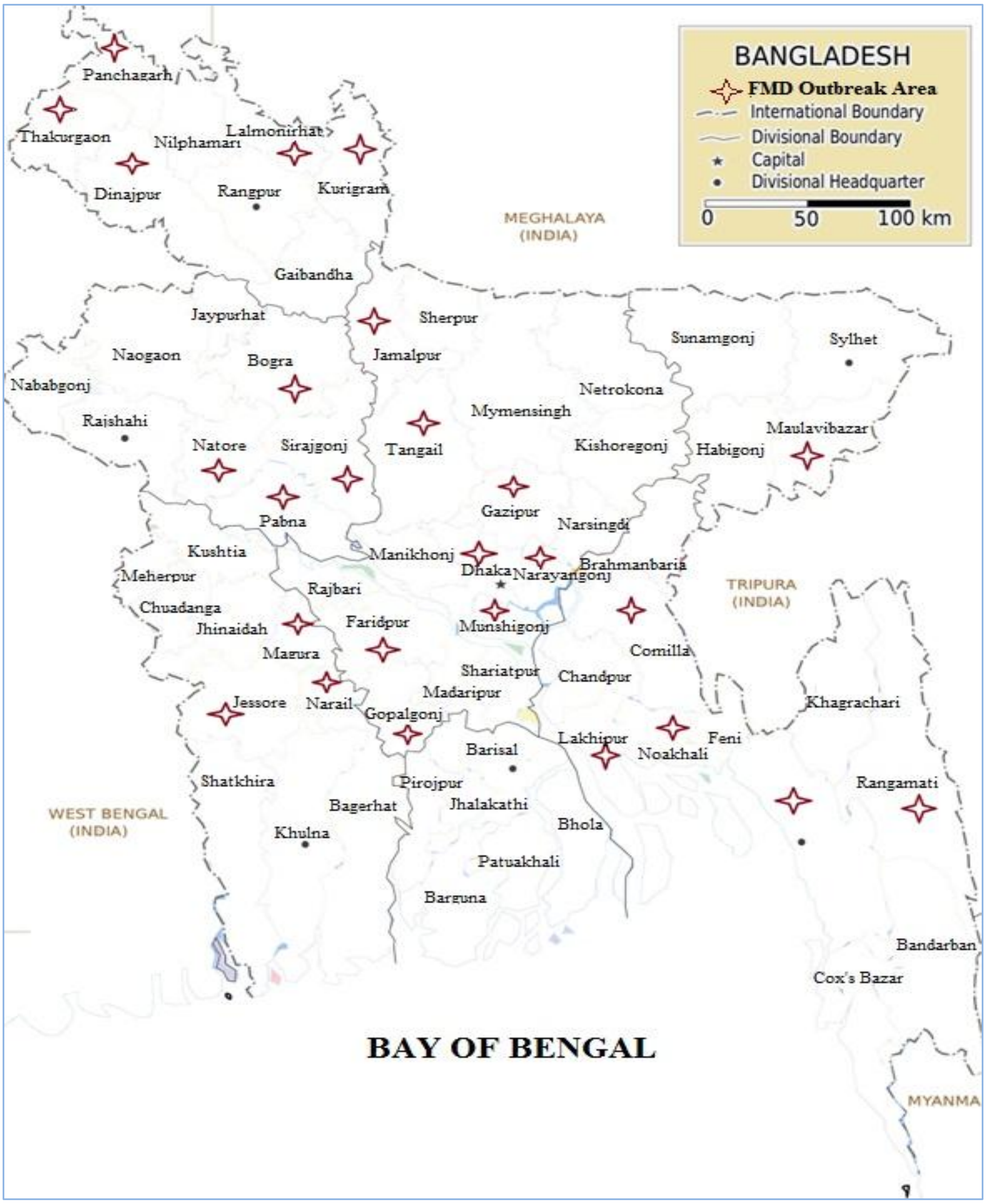


Figure 2.2 The locations of different FMD outbreak areas of Bangladesh (marked with stars) from which oral tissue samples were collected.

Between May 2012 and April 2016, a total of 283 samples were collected in cryogenic vials from cattle with clinical signs of FMD and immediately after collection, these samples containing vials were labeled using waterproof ink and placed in a cool box to ensure the cool chain transportation to the laboratory and finally stored at -80^{0}C until RNA extraction or further processing was performed. Collection dates with other related information are presented in **Appendix-1I.** The samples were named according to three letter country code (i.e. BAN for Bangladesh), followed by a two letter district code GA (for Gazipur), followed by upazilla and laboratory record number (for Sa-197) and finally by year of outbreak (e.g. BAN/GA/Sa-197/2013, representing FMDV from Bangladesh, Gazipur, Sadar-laboratory record number 197 and collected in 2013).

2.3 Epidemiology Study of Foot and Mouth Disease virus

A prerequisite to develop strategies for FMD detection and control is a thorough understanding of the nature and extent at which changes in the FMDV genome are related to epidemiological factors. Following steps were attempted for molecular epidemiology study.

2.3.1 Study of Demography Epidemiology

The questionnaire format was designed for the baseline survey of cattle of FMDV affected areas during sample collection **(Figure 2.3.1)**. The target of this questionnaire is to determine the risk factors associated with FMD of cattle in Bangladesh prefecture from May 2012 to April 2016. The results of this study will provide initial information for epidemiological study and based on this to set up control and eradication programs, and prevent spreading of FMD outbreaks in the future.

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 7.2 Pre-winter: Yes/ No

7.3 Winter: Yes/ No 7.4 Others (Specify):

8. Treatment (Specify):

9. Vaccination (Specify):

Investigated and Completed by

____

Signature and Name:
Date:
Designation:

Figure 2.3.1 Questionnaire that filled up during sample collection

2.3.2 Study of Molecular Epidemiology

2.3.2.1 Tissue sample preparation

Homogenization followed by RNA extraction from all tongue or foot epithelial tissue samples preserved at -80^0 C in the laboratory was carried out using automated Maxwell® 16 RNA extraction instrument (Promega, USA) following the manufacturer's instructions. Briefly, Elution tubes containing 462 µl lysis buffers, supplied with the kit, per 70 mg thawed tissue samples (66 µl/10 mg tissue) were thoroughly homogenized with the instrument for 10 minutes or until completely lysed. The supernatant (homogenized lysate, approx. 450-500 µl) was incubated on ice for 10 minutes and used for RNA extraction. The Kit (Promega, USA) comprises the disruptive and protective properties of guanidine thiocyanate (GTC), a chaotropic agent most commonly used for the extraction of RNA and DNA, that lyses samples, denature nucleoprotein complexes and inactivate ribonucleases.

2.3.2.2 RNA extraction from epithelial tissue

Approximately 500 µl homogenized tissue lysate was transferred to a 2 ml micro centrifuge tube (ExtraGene, USA). A volume of 835 µl of blue RNA dilution buffer (supplied with the kit, **Appendix I**) was added to lysates. The mixture was kept on ice as much as possible (minimum 10 minutes). Then the clearing agent, supplied with kit, was first vigorously vortexed until re-suspended resin completely followed by 125 µl solutions was added into the mixture and vortexed for 30 seconds to selectively removed genomic DNA. The mixture was heated in a pre-set 70^0 C heat block for 3 minutes. After heating, vortex was done again for 30 seconds and incubated at room temperature for 5 minutes for proper cooling. Clearing column assembly was done by placing one clearing column into a collection tube. About 700 µl of sample was first loaded to clearing column without disrupting the sediment of clearing agent and centrifuged at 12000 x g for 2 minutes. Flow through from the collection tube was loaded to well #1 of the Maxwell® 16 SEV RNA Cartridge. Same step was repeated for remaining volume of each sample. The cartridge was then placed inside the automated Maxwell® 16 RNA extraction instrument. A volume of 300 µl of nuclease free water was loaded in the elution tube and placed in the specified chamber of the instrument prior to the extraction procedure. The instrument was programmed to extract total nucleic acid from each sample. Extracted nucleic acid from each sample was eluted in elution tube containing nuclease free water (300 µl). Positive and negative extraction control samples were included in each group of RNA extractions. The extracted RNA was stored in -80^0 C until reverse transcription PCR for cDNA synthesis.

2.3.2.3 Preparation of Complementary DNA (cDNA)

The extracted RNA was reverse transcribed into complementary DNA (cDNA) by using ImPro-II™ Reverse Transcription System (Promega, USA; **Appendix I**) as per manufacture's instruction. Both hexameric random primers and oligo(dT) 15 primers were used to reverse transcribe the RNA. To prepare cDNA, experimental RNA was combined with the random and oligo(dT) 15 primer, and an aliquot of the positive control RNA was combined with oligo(dT) 15 primer. A negative control (no RNA template) was set to check the unwanted contamination. The primer/template mix was thermally denatured at 70°C for 5 minutes and chilled on ice. This step was performed on heat block (Veriti 96 well Thermal cycler, Applied Biosystem, USA). A reverse transcription reaction mix was prepared on ice to contain nuclease-free water, 5X reaction buffer, ImProm-II™ reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor. In experimental systems, the addition of 1unit/µl of Recombinant RNasin® Ribonuclease Inhibitor was recommended but optional. As a final step, the template-primer combination was added to the reaction mix on ice and run on thermal cycler for conversion of RNA to cDNA. Following an initial annealing at 25°C for 5 minutes, the reaction is incubated at 42°C for up to one hour. Because no cleanup or dilution was done following the cDNA synthesis, so the product was directly added to amplification reactions.

The detailed method of cDNA preparation is deciphered below:

2.3.2.3.1 Target RNA and Primer Combination and Denaturation

Commercially autoclaved, nuclease-free, thin-walled sterile dilution tubes (ExtraGene, USA) and reaction tubes (Eppendorf, USA) were placed on ice. The extracted RNA and the 1.2 kb Kanamycin positive control RNA were thawed on ice and the experimental RNA (upto1µg) was combined with the cDNA primer in Nuclease-Free Water for a final volume of 10µl per RT reaction. The volume was multiplied to accommodate multiple reactions when more than one reaction was planned using a single RNA: primer combination **(Table 2.3.2.3.1a)**.

Table: 2.3.2.3.1a RNA/Primer Mixture for cDNA Preparation

Reagents	Positive Control	Negative Control	Experimental Reaction
1.2kb Kanamycin Positive Control RNA (1µg)	5.0 µl	-	-
Random (Hexameric Primer)	-	2.0 µl	2.0 µl
Oligo(dT)$_{15}$Primer (0.5µg/reaction)	2.0 µl	2.0 µl	2.0 µl
Nuclease-FreeWater	3.0 µl	6.0 µl	1.0 µl
Experimental RNA	-	-	5.0 µl
Final Volume	**10.0 µl**	**10.0 µl**	**10.0 µl**

Each tube of RNA was closed tightly and placed into a preheated 70°C heat block for 5 minutes and immediately chilled in ice-water for at least five minutes. Then each tube was spin for 10 seconds in a mini centrifuge (ExtraGene, USA) to collect the condensate and maintained the original volume. The tubes were kept closed and on ice until the reverse transcription reaction mix were added.

2.3.2.3.2 Reverse Transcription

For reverse transcription, the reaction mix was prepared by combining the following components of the ImProm-II™ Reverse Transcription System in a sterile 1.5 ml micro centrifuge tube (Eppendorf, USA) on ice. 30 µl of reaction mix was prepared for each cDNA synthesis reaction to be performed. There action mix was vortexed gently to mix, pulse spin and placed on ice prior for allocating into the reaction tubes. The volumes needed for each component is described in **Table 2.3.2.3.2a**.

Table: 2.3.2.3.2a Reaction Mixture for cDNA Preparation

Reagents	Positive Control	Negative Control	Experimental Reaction
RNase Free H_2O	13.2 µl	13.2 µl	9.6 µl
5X Reaction Buffer	8.0 µl	8.0 µl	8 µl
$MgCl_2$ (6mM)	4.8 µl	4.8 µl	6.4 µl
dNTP Mix (final conc. 1.0 mM each dNTP)	2.0 µl	2.0 µl	2 µl
Recombinant RNasin® Ribonuclease Inhibitor	-	-	2.0 µl
ImPro Reverse Transcriptase	2.0 µl	2.0 µl	2.0 µl
Final Volume	**30.0 µl**	**30.0 µl**	**30.0 µl**

Reverse transcription reaction mix (30 µl aliquots) was then added to each reaction tube on ice. Careful handling in that step was adopted to prevent cross-contamination. A 10 µl of RNA and primer mix was added to each reaction for a final reaction volume of 40 µl per tube. For annealing of primers, the tubes were subsequently placed in a controlled-temperature heat block equilibrated at 25°C, and incubated for 5 minutes. The tubes were then incubated in a controlled-temperature heat block at 42°C for up to one hour for extension of cDNA product. The extension temperature may be optimized between 37°C and 55°C as per manufacture's instruction. The reaction tubes in a controlled-temperature heat block were incubatedat70°C for 15 minutes following extension step to inactivate the reverse transcriptase enzyme and stopped the reaction (**Table: 2.3.2.3.2b**).

Table 2.3.2.3.2b Optimum reaction condition for Reverse Transcription reaction

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

2.3.3 PCR Amplification

2.3.3.1 Designing of primer

To design primer for FMDV several reference sequences (Ref/seq) were downloaded from NCBI GenBank. The downloaded sequences were then aligned by ClustalW software. Following alignment conserved sequences were identified for primer design. The highest conserved sequence was selected for primer design. The conserved sequence was uploaded in online Primer3 plus website. The primer size was chosen randomly. The designed primer was then tested for best fitted melting temperature, thermodynamic properties, hairpin loop structure, dimmers, template complexity etc. by using IDT Oligo Analayzer 3.1 online server.

2.3.3.2 Optimization of PCR

In the study RT-PCR optimization was begun with selection of the DNA target and careful design of the PCR primers, and it ended in the laboratory by adjusting the annealing and elongation temperatures, the concentration of the PCR primers, dNPTs, and $MgCl_2$, and by selecting the most appropriate DNA polymerase. Template concentration was also used for optimization process. **Table 2.3.3.2** described the composition of reaction mixtures used in the PCR in this study.

Table 2.3.3.2 Preparation of PCR mix for the identification of FMDV serotypes

Component	Positive Control	NegativeControl	Experimental 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(10µM)	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)

2.3.3.3 PCR Amplification of VP1 Coding Region for Initial Identification of Serotype

A conventional PCR was performed to amplify cDNA corresponding to the entire VP1 coding sequence with the 16F:16R and/or VP1UF: NK61primerpairs (designed for the intended universal diagnosis of all seven serotypes of FMDV) for investigating the presence of FMD virus RNA in tissue (**Table 2.3.3.3)** using agarose gel electrophoresis (the protocol is discussed in next another **sub-section 2.3.4**). After initial confirmation, serotype specific primer pairs, O-1C564: NK61, A-1C562:NK61 and As1-1C505: NK61 were used to distinguish each of the three serotypes i.e. serotype O, A and Asia-1 (**Table 2.3.3.3)** prevailing in Bangladesh.

The cDNA was directly amplified by adding the products of the heat inactivated reverse transcription reaction to the PCR mixture and proceeding with thermal cycling. GoTaq® G2 Hot Start Colorless Master Mix 2X (Promega, USA) was used for PCR amplification that includes GoTaq® G2 Hot Start polymerase, dNTPs (400 mM each), $MgCl_2$ and reaction buffers (P^H 8.5) at optimal concentrations for efficient amplification of DNA templates by PCR.

PCR mix was prepared with the addition of 2X GoTaq® G2 Hot Start Colorless Master Mix, the upstream and downstream primers (**10 µM** each) and Nuclease free water **(Table 2.3.3.2)**. As GoTaq® HotStart Polymerase contains the GoTaq® DNA polymerase bound to a proprietary antibody that blocks polymerase activity at temperature below 70^{o}C and PCR mix was prepared by maintaining ice-cold condition. An initial denaturation step at 94^{o}C was required to inactivate the antibody and initiate hot-start PCR. Combined PCR components were mixed briefly by vortexing and short centrifugation at 12000g for 5 to 10 seconds was done to consolidate sample. After that, Reaction mix was aliquot into sterile, thin walled tubes and finally the DNA template was added to each tube. Both Positive and Negative controls were included to ensure there is no contamination in the reaction.

Table 2.3.3.3 Primers used for initial identification and serotyping of FMDV

FMDV Serotypes	Primers	Sequence(5'-3')	Annealing Temperature (^{0}C)	Location	Reference
All	16F	GAGAACTACG GWGGWGAGAC	55	VP1 (436bp)	Nandi *et al.*, 2015
All	16R	GCACCGWAGT TGAAGGAGGT	55		
All	VP1UF	GCRCAGTACTA CRCSCAGTAC	55	VP1	Ullah *et al.*, 2014
O	O-1C564	AATTACACATG GCAAGGCCGAC GG	60	1C (861bp)	Samuel and Knowles, 2001
A	A-1C562	TACCAAATTAC ACACGGGA	55	1C (863bp)	
Asia-1	As1-1C505	TACACTGCTTC TGACGTGGC	55	1C (908bp)	
All	NK61	GACATGTCCTC CTGCATCTG	55	2B	

All the PCR involves an initial denaturation step at 94^{o}C for 5 minutes in the thermal cycler (Applied Biosystem, USA) followed by 35 numbers of repeated PCR cycles (denaturation, annealing and elongation) under optimal reaction conditions along with final extension at 72^{o}C for 10 minutes. During the initial denaturation step, double stranded DNA (dsDNA) is separated into single DNA strands and the DNA polymerase becomes active as the chemical moiety blocking the enzyme activity is removed. In the annealing phase, the PCR primers bind to their targets and the PCR primers are subsequently extended by the DNA polymerase during the elongation phase. After this, PCR product containing tubes were stored at -20^{o}C until further analysis.

2.3.4 Analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) product

The amplified PCR products were analyzed by mixing 5 µl of the reaction mix with 1 µl of 6x orange loading dye solution and resolving the sample by run on 1.0 % agarose gel stained with ethidium bromide alongside a 1kb-size DNA marker (Promega, USA). To prepare 60 ml of 1.0% agarose solution 0.6g agarose was measured into a conical flask and 60ml 1xTAE buffer was added. The mixture was heated in microwave on rotating hot plate until agarose was dissolved and solution was clear. Then the solution was allowed to cool to about 45°C before pouring and 3 µl ethidium bromide was added at this point to a concentration of 10 ng/µl. On the flat surface, gel tray was prepared by sealing ends and placed well former (comb) in gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth were about 1-2 mm above the surface of the tray. The gel was poured onto gel tray to a depth of about 5 mm. The gel was then allowed to solidify about 15 minutes at room temperature. To run, the comb was removed gently from the gel, properly placed the tray in electrophoresis chamber, and cover (just until wells are submerged) with 1x TAE buffer (the same buffer used to prepare the agarose). Samples were prepared on parafilm (1 µl of 6x orange loading dye and 5 µl of PCR product). Molecular weight marker was prepared with 5 µl of molecular weight marker [0.5 µl molecular weight marker VI (Boehringer) and 4.5 µl H_2O] and 1 µl of 6x orange loading dye **(Table 2.3.4).** After well mixing of DNA and loading dye, 6 µl samples were loaded per well. Electrophoresis was started at 100 volts until dye markers had migrated an appropriate distance, depending on the size of DNA to visualized under short wave UV light (AlphaImager HPGel-documentation system, Cell Bioscience, USA).

Table 2.3.4 Preparation of 6x Orange Loading Dye

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		**1000.0 µl**

2.3.5 PCR clean-up and quantification

The DNA purification step is required to remove unincorporated primers after RT-PCR; however, it is not a totally efficient process.

2.3.5.1 Purification of RT-PCR product

Samples showing positive results in either of VP1UF/NK61 primer pairs or serotype specific primer pairs **(Table 2.3.3.3)** in conventional PCR to amplify cDNA corresponding to the entire VP1 coding sequence were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, USA) following centrifugation based protocol. The PCR product was also checked on 1.5% low melting agarose gel for the presence of spurious bands or primer dimers. If so the respective bands were cut and removed from the gel, placed in a 1.5 ml micro centrifuge tube, and 10 µl Membrane Binding Solution (MBS) per 10 mg of gel slice was added. Following vortex, the gel slice was incubated at 65^{o}C in a heat block until completely dissolved. For processing of PCR product an equal volume of MBS was added to PCR amplification. SV mini column into a collection tube was inserted and transferred the dissolved gel slice or prepared PCR product to the mini column assembly. Following 2 minutes of incubation at room temperature the mixture was centrifuged at16, 000×g (14,000rpm) for 1minute to discard the flow through and the mini column was reinserted to the collection tube. Then the SV mini column was subjected to wash for two times using centrifugation method with Membrane Wash Solution (Supplied in the kit, ethanol added). Following washing, the SV minicolumn was transferred carefully to a clean 1.5ml micro centrifuge tube and finally DNA was eluted in Nuclease Free Water as per the instructions supplied in kit. The SV minicolumn was discarded and the purified PCR product was stored at-20^{o}C until further processing.

2.3.5.2 Quantification of RT-PCR product

The amount of product was measured using a Nano Drop^{TM} spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). PCR product was measured as ng/µl. The reading of the ratio was between at 260 nm and 280nm (OD260/OD280). This OD 260/280 ratio provides an estimate of the purity of the nucleic acid (DNA) which is a value of 1.8.

2.3.6 Sequencing

The purified PCR products of representative samples were sent to gene sequence commercially in the First Base Laboratories Sdn, Malaysia with both forward and reverse primers (**Table 2.3.3.3**) used in PCR.

2.3.7 Sequence Comparison and Serotype Identification

The sequences (tracer files) were viewed using sequence viewer software like Chromas. Comparison of the sequences with other sequences from Genbank of National Biotechnology Information Centre (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool BLAST was performed for the identification viruses as well as their serotypes.

2.3.8 Study of Evolutionary History

2.3.8.1 Sequence Analysis and Construction of Phylogenetic Tree

Raw sequence data were assembled, proof-read and edited using SeqMan version 7.0 (DNASTAR, Inc., Madison, WI, USA). The consensus VP1 coding sequences (complete 1D region) of native FMDV serotypes were aligned using the ClustalW program with the related gene sequences from GenBank. The same program was used to calculate the nucleotide and amino acid (aa) identity matrices. A phylogenetic neighbour-joining tree was constructed (bootstrap replicates 1000) using MEGA 5.2 after determining the best-fitting nucleotide substitution model by Bayesian Information Criterion (BIC). The genetic heterogeneity of the viruses has been defined as genetic groups if the genetic relationship has <15% nucleotide divergence (Vosloo *et al.*, 1992; Samuel and Knowels 2001; Tosh *et al.*, 2002) and lineage of the genetic relationship has <7.5% nucleotide divergence (Mohapatra *et al.*, 2002).

2.3.8.2 VP1 Gene Based Phylogeny and Topotype Determination

The sequences VP1 region of different topotypes of respective viruses was downloaded on-line from the nucleotide database. All the selected sequences were assembled along with the VP1 gene sequence of native isolates like the procedure described in **sub-section 3.3.8.1.** ClustalW codon, built-in tool of MEGA 5.2 was used here**.** The model was selected and the bootstrap value was assigned. Reconstruction of the phylogeny was done using MEGA 5.2 software.

2.3.8.3 VP1 Gene Based Phylogeny and Lineage Determination

Smilarly the VP1 sequences different lineages under different topotypes were downloaded from public database. Alignment (pairwise and multiple alignment by ClustalW codon) of the sequences and isolated virus (VP1) followed by phylogeny reconstruction was performed according to protocol described in earlier section.

2.4 Molecular Characterization of Foot and Mouth Disease Virus

To understand the genetic characteristics of the virus causing FMD outbreak, and analyze the possible source for these antigenic variants, a complete genome characterization of isolates obtained from different geographically distinct regions affected by the outbreak was conducted.

2.4.1 Isolation of Foot and Mouth Disease Virus

BHK-21 cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics were used for these experiments.

2.4.1.1 Preparation of Media and Solutions

DMEM (Dulbecco's Modified Eagles' medium, Biochrome, Germany) liquid media with stable glutamine was used for preparation of cell line and virus isolation. Composition of Maintenance media, Growth media (Commercial formulation of the DMEM according to Dulbecco and Freeman 1959) with Fetal Bovine Serum (FBS), Trypsin-Versin solution, Phosphate Buffer Solution (PBS) and Antibiotic solution (Penicillin, Streptomycin, Gentamycin, Amphoterisin-B) are given in **Appendix-I**.

2.4.1.2 Sample Treatment and Preparation of Inoculum

500 mg sterilized sand with 3 ml of fresh 2% DMEM was ground in a mortar and pestle. Ground suspension was centrifuged in a bench centrifuge machine at 2000 g for 2 minutes followed by filtration of supernatant via 0.22 µm Millipore filter (Millipore Sterivex-GS 0.22 µm disposable filter units) and filtrate was used as negative control sample. On the other hand, about 500mg tongue epithelium was removed out from the cryogenic vial, previously stored at -80^{0}C, and was ground along with equal weight of the sterilized sand and 3ml of fresh 2% DMEM as like negative control in the same mortar and pestle for the preparation of positive sample. Both the negative control and positive samples were used to infect BHK-21 cell line.

2.4.1.3 Establishment of BHK-21 Cell Line

Baby hamster kidney cell line (BHK-21) was obtained from the repository maintained at Tissue Culture Laboratory of Center for Advanced Research and Science, University of Dhaka, Dhaka-1000, Bangladesh in cryo-preserved form (-196°C). These cells were revived and used for the FMD virus adaptation, propagation and vaccine production.

2.4.1.4 Revival of BHK-21 Cells

A cryogenic vial of 1.5 ml BHK-21 cell line, containing DMSO (Dimethyl Sulfoxide) plus growth media which was added during storage retrieved from liquid nitrogen storage tank (-196 ^{0}C), was placed in water bath at 37 °C till the complete thawing of the cell. Total contents of cryogenic vial transferred to the 15 ml sterile falcon tube and were mixed with sufficient amount of chilled growth medium (8.5 ml) and subjected to centrifugation at 2000 rpm for 2 minutes. The supernatant was discarded to remove the DMSO followed by 5 ml growth medium (fresh DMEM plus 10% FBS) was added to re-suspend the pellet and pipette up and down gently several times to break the pellet before passage of cells. Re-suspended media was placed at one corner of the 25 cm^2 cell culture flask by a serological pipette. After that media was softly spread over the entire inner bottom surface of the flask. The flask was incubated at 37º C for 24-48 hours for formation of monolayer in an incubator with humidified atmosphere of 5% CO_2 (Nuaire, USA). The flask having confluent monolayer of adherent BHK-21 cell line when observed under inverted microscope (Optika, Japan) was processed for harvesting and transferring to new culture vessels.

2.4.1.5 Sub-Passage of Cell

The growth medium overlying the cell monolayer was poured off in a sterile beaker under sterile conditions. The monolayer was rinsed, washed twice with 5 ml PBS (Phosphate Buffer Solution) to remove residual anti-protease activity of serum and covered with 800 µl of sterile 0.5% v/v trypsin solution. The mixture was allowed to react on the monolayer for about 3-5 minutes in an incubator at 37°C. The monolayer was periodically observed under an inverted microscope for rounding and detachment of cells. Mild tapping with finger in the side of flask was done to detach cells completely. Then 3.2 ml fresh 10% DMEM (growth medium) media was added into the flask for inactivation of protease. The cells were pipetted up and down several times to help break up the clumps of cells to form homogeneous cell suspension. Using the same pipette, the cell suspension was drawn up and quickly distributed in new flasks in required volume (0.5 to 1.0 µl). Fresh growth medium was added to make the final volume 5 ml. The flask was incubated at 37º C in an incubator with humidified atmosphere of 5% CO_2. After 24-48 hours of incubation, the flask had developed a confluent monolayer (log phase; 80-90% confluent) with typical cell sheet with light frosted glass appearance, having clearly visible fibroblastic whirls. The cells in that phase were selected to infect with the virus sample.

2.4.1.6 Culture of Virus

The medium of each cell culture flask having complete BHK-21 cell monolayer (log phase; 80-90% confluent) was removed and washed with PBS. One aliquot (200 µl) filtrate of virus sample was transferred to each of the tissue culture flask and 1.8 ml of maintenance media (fresh DMEM plus 2% FBS) was added. In negative control, blank media was used instead of sample filtrate. The flask (marked with sample code, number of passage and date) was incubated at 37º C in an incubator with humidified atmosphere of 5% CO2 for 60 minutes for proper infection with virus. Finally, 2% DMEM was added to the flasks to reach the volume up to 5ml. Each flask was incubated at 37°C for 24-48 hours. Periodically, cell culture flasks were monitored to observe cytopathic effect (CPE).

2.4.1.7 Harvesting of Virus

The samples showing CPE in contrast to negative control were selected and the fluid of each flask was harvested separately. A small scaled scrapper was used to dissociate adherent cell from the inner flat bottom surface of the flask. The total content of the flask was collected into a sterile 15 ml falcon tube. Centrifugation (1000 rpm for 2 minutes) was done to collect cell culture supernatant. Major portion of the supernatant was poured into another sterile 15ml falcon tube leaving cell pellet with 0.5 ml supernatant. Remaining cell pellet with supernatant was subjected to freeze-thaw for 2-3 times to disrupt the cells and release of intracellular viruses. After this step, previously poured cell culture supernatant was mixed with disrupted cell containing falcon tube. Finally, the viral suspension was clarified from the cell debris by centrifugation at 1000 rpm for 2 minutes to collect clear cell culture supernatant. Same procedure was followed for negative control.

2.4.1.8 Preservation of Virus

The cell culture supernatant of each falcon tube was further subdivided into aliquot in sterile 2 ml cryogenic vials (each containing 1.5 ml). The aliquots containing the virus suspension were stored at -80°C freezer (Nuaire, USA) till further use.

2.4.2 Characterization of Isolated Virus

2.4.2.1 Extraction of Viral RNA and cDNA Synthesis

450 µl of cell culture supernatant was mixed with 50 µl lysis buffer in a 2 ml micro centrifuge tube. The mixture was vortexed vigorously to lyse the cells and kept on ice as much as possible to facilitate complete lysis or until used for RNA extraction. RNA was extracted from cell culture supernatant like the procedure described in **sub-section 2.3.2.2**. Following RNA extraction, the preparation of complementary DNA (cDNA) was accomplished like the procedure described earlier in **sub-section 2.3.2.3**.

2.4.2.2 PCR Amplification of VP1 Region

cDNA was subjected to amplify with PCR using VP1UF and NK61 **(Table 2.3.3.3)** primer pairs according to procedure described in **sub-section 2.3.3.3.** PCR product was analyzed by mixing 5µl of the reaction mix with 1µl of 6x orange loading dye solution and resolving the sample by agarose gel electrophoresis alongside a DNA size marker according to protocol mentioned in **sub-section2.3.4**to ensure the presence of FMDV in cell culture supernatant.

2.4.2.3 PCR Amplification of Entire Genome

In the positive case of the presence of FMDV in cell supernatant the prepared RT product (cDNA) was subjected to amplify of the entire genome for the study of comparative genomics using following methods.

2.4.2.3.1 Designing PCR Primers

Oligo nucleotide primer design is critical in ensuring specific amplification of DNA. There are numerous specialist primer design programs and available on the internet. However, two basic approaches are considered for primer design in this study.

2.4.2.3.1.1 Manual Primer Design

Manual assessment of the DNA sequence flanking the target region and selecting primers based on basic considerations such as GC content, length of primer, length of

PCR product and the absence of known polymorphisms was used for the successful design of primers.

2.4.2.3.1.2 Primer Design using Specific Primer Design Software

Primer-3 Plus software (Rozen and Skaletsky, 1999) was used for designing primers. Serotyping of the isolated virus was done by BLAST search. The closest hit of the isolated virus was taken as a gold standard to design primers. 14 primers were designed to amplify the complete structural part of the genome. The FASTA file of the standard (5'UTR to VP1 region) isolates was uploaded into Primer-3 Plus software window.

Regardless the method used to design the primers; several variables were taken into account to ensure high PCR success rates. Among the most critical were primer length/specificity (generally 18-25 nt), melting temperature, *Tm* (similar *Tm* value of both forward and reverse primers and ideally within 1^0C), annealing temperature (at least 50^0C and considered to be 5^0C lower than the *Tm*), complementary primer sequences (not contained more 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) [repeat stretches of poly pyrimidines and polypurines was more or less avoided] and the 3ˈend sequence (inclusion of GC residues at the 3ˈ end to ensure correct binding or for controlling mispriming).

Degenerate bases were incorporated in some of the primers to make the primers more universal for selected FMDV serotypes. To introduce degeneracy initial primers were aligned with the sequences those were closer to isolated virus using MEGA 5.2 software. The alignment file was saved and viewed by BioEdit software (Hall 2008) and degenerate bases were placed where variation at the nucleotide level was observed. The selected primers were tested for cross matching by BLAST search. The quality of the selected primers was checked using IDT Oligo Analyzer 3.1 version, an on-line tool. The free energy changes during different secondary structure formation like self-dimer, heterodimer was analyzed. The best possible primers were picked to place an order to oligo synthesizer (IDT- Integrated DNA Technology, USA).

2.4.2.3.2 PCR Amplification

PCR amplification was performed with different internal primer pairs spanning the entire genome of the isolated FMDV serotype O **(Appendix III Table 1)** and FMDV serotype A **(Appendix III Table 2)** to generate 16 and 22 overlapping fragments respectively.

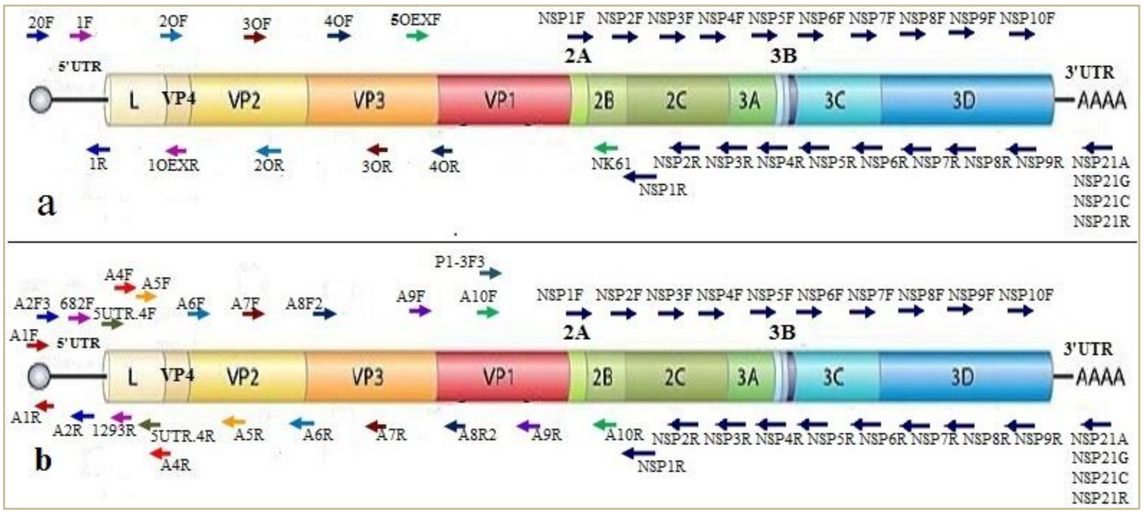


Figure 2.4.2.3.2 Primer pairs used to amplify the entire genome of (a) FMDV serotype O and (b) serotype A

The positioning of the overlapping primer pairs of both serotypes O and A is presented in the schematic representation in the **Figure 2.4.2.3.2.** PCR reaction mix was prepared with the addition of 2X GoTaq® Hot Start Colorless Master Mix (Promega, USA), 400nM of each of the primer pairs and Nuclease free water. Positive control was included alongside the test samples for optimizing reactions to eliminate poor quality DNA as a variable and no-DNA-template negative control was also included to ensure there was no contamination during operating PCR. The PCR conditions for cDNA amplification are an initial denaturation at 94^{0}C for 5 minutes followed by 35 cycles of denaturation at 94^{0}C for 1 minute, annealing at 55^{0}C for 1 minute, extension at 72^{0}C for 1 minute 30 seconds and a final extension at 72^{0}C for 7 minutes. For the amplification of 3' Un-Translated Region (UTR) 4 Rapid Amplification of cDNA Ends (RACE) primers was optimized against NSP 10F primer to get the desired amplicon (832 bp). Best one optimized primer for desired amplicon was included in this study.

PCR product was analyzed by mixing 5µl of the reaction mix with 1µl of 6x orange loading dye solution and resolving the sample by agarose gel electrophoresis alongside a DNA size marker according to protocol mentioned in **sub-section 2.3.4.**

2.4.2.3.3 PCR Product Purification

Purification of PCR product by Wizard® SV Gel and PCR Clean-Up System (Promega, USA) following centrifugation based protocol and quantification of PCR product concentration were decrypted like the **sub-section 2.3.5.** In case of the PCR product spanning 3'UTR, gel purification of the desired amplicon was required because of the presence of spurious bands on 1.5% low melting agarose gel. Respective bands were cut and removed by scalpel handle and blade placing the gel on UV-Illuminator (Biometra, USA) for visualization of bands. The gel slice containing desired band was taken in a 1.5 ml micro centrifuge tube and 10µl Membrane Binding Solution (MBS) per 10mg of gel slice was added to the tube. Following vortex the gel slice was incubated at 65^{o}C in a heat block until completely dissolved. Further steps of gel purification were preceded according to **sub-section 2.3.5.**

2.4.2.3.4 Sequencing of RT-PCR Products

The basic sequencing was followed according to the protocol described in the **sub-section 2.3.6.**

2.4.2.3.5 Assembling of Raw Sequences and Genome Annotation

Overlapping sequences spanning the entire genome was assembled in to complete genome sequence using SeqMan version 7.0.0 (Lasergene, DNAstar, USA). New project in SeqMan was performed to assemble all sequences into consensus sequences. All the parameters of the assembly project were set as default. Degenerate traces shown in the consensus were fixed by subsequent BLAST search. The assembly project was imported as both SeqMan and FASTA file format. Annotation of the complete genome was done according to National Centre for Biotechnology Information (NCBI) RefSeq (Reference sequence for Foot-and-Mouth Disease Virus serotype O). Pairwise and Multiple alignment of RefSeq and complete genome of native FMDV serotype O [BAN/NA/Ha-

156/2013] as well as same RefSeq and complete genome of native FMDV serotype A [BAN/GA/Sa-197/2013] in built-in ClustalW of MEGA 5.2 software was performed to annotate the complete genome. Open Reading Frame (ORF) of FMDV was annotated by pairwise and multiple alignment by the built-in ClustalW Codon of MEGA 5.2 software.

2.4.2.3.6 Sequence Comparison

Comparison of the complete genome was performed by searching nucleotide database via program BLASTN version 2.2.29. All the parameters of BLASTN program were kept default.

2.4.3 Recombination Analysis

In order to analyze for a possible recombination, the complete genome of local FMDV serotype O (BAN/NA/Ha-156/2013) and A (BAN/GA/Sa-197/2013) and other related complete genome sequences including reference strain and vaccine strains based on the multiple alignment result were checked for possible recombination breakpoints. Two methods were employed; boot-scan analysis using default parameters in **SimPlot software version 3.5.1** and GARD (Genetic Algorithm Recombination Detection), a genetic algorithm based statistical approach to search recombination breakpoints from multiple sequence alignment of homologous sequences hosted in Datamonkey.org server. Briefly, the complete genome nucleotide sequences generated in this study and other selected sequences were aligned using ClustalW implemented in MEGA 5.2. Pair-wise genetic similarities were plotted between the query sequence and a set of reference sequences. The recombination break points along the complete genome sequence were identified by examining the points at which the similarities between the query and reference sequences markedly changed. Each analysis was conducted twice to ensure repeatability of results.

2.5 Study of Structural Genomics

2.5.1 Prediction of the Secondary Structure of UTR

Secondary structure of S-fragment, Pseudoknots and Internal Ribosomal Entry Site (IRES) of 5' UTR of the local isolates and NCBI reference sequence (RefSeq) were computationally predicted using **Mfold Web Server**. Similarly, secondary structure of the 3' UTR was predicted using **RNAfold web server** (Vienna RNA Package, University of Vienna).

2.5.2 Prediction of the 3-D Structure

The amino acid sequence of 3-D structure of VP1 region and L^{pro} region of virion was uploaded separately to SWISS Model and Templates were selected. After that, the model was built up. The PDB file was visualized via PyMOL version 1.8.0.7.

2.6 Sequence submission to NCBI GenBank

VP1 sequences of the FMD positive tissue samples **(Appendix II)** and the complete genome sequences (**Appendix IV**) of local FMDV isolates were submitted to the NCBI GenBank database via on-line submission tool BankIt. The FMDV complete genome sequences described in this study assigned the accession numbers: KF985189 (BAN/NA/Ha-156/2013) and KJ754939 (BAN/GA/Sa-197/2013).

2.7 Characterization of circulatory FMDV seeds for vaccine preparation

2.7.1 Media and Equipments

Cell culture growth medium (DMEM high glucose with 4 to 6 mM glutamine and 10% fetal bovine serum) with addition of Penicillin, Streptomycin, Gentamicin, Fungizone (if required), Plaquing Medium (DMEM high glucose with 4 to 6mM glutamine and 2% fetal bovine serum), Host cells, Phosphate buffered saline (PBS), Plaque assay agarose (ultrapure), Staining dye for plaque (MTT or neutral red), Tissue culture grade sterile water, Micro pipettes (10 to 100 microliter), 96-well tissue culture plates (could be pre-coated with a collagen solution), Micro centrifuge tubes, Water bath, Microwave oven, Inverted microscope, Microbiological safety cabinet, CO_2 incubator.

2.7.2 Cell Counting

An aliquot of 0.5 ml cell culture supernatant was collected and mixed with 0.1 ml Trypan Blue 0.4% solution in a dilution tube. Hemocytometer was loaded so that fluid entirely covered the polished surface of each chamber. The cells in the center and four corner primary squares of each grid (ten primary squares) were counted. When the Hemocytometer was properly loaded, the volume of cell suspension that occupied one primary square was 0.1 mm cube (1 mm sq. x 0.1 mm) or 10^{-4} ml. The cells within 10 primary square (5 primary squares per chamber), were counted to give number of cells within 1 mm^3 (10 x 0.1mm^3) or 1 x 10^{-3} ml.

Total cell concentration in original suspension in cells ml^{-1} was then: Total count x 1000 x dilution factor.

2.7.3 Seeding of Culture Plate with Host Cells

1 x 10^6 cells per ml in growth media of each well of 96-well plates were inoculated. Culture plates were rocked gently back and forth and from side to side so that cells were distributed evenly. Once cells have been seeded, the cells were allowed to grow overnight. Next day, cells under a light microscope were visualized to confirm that cells were evenly distributed and reached >80% confluency.

2.7.4 Biological Titration of the Virus

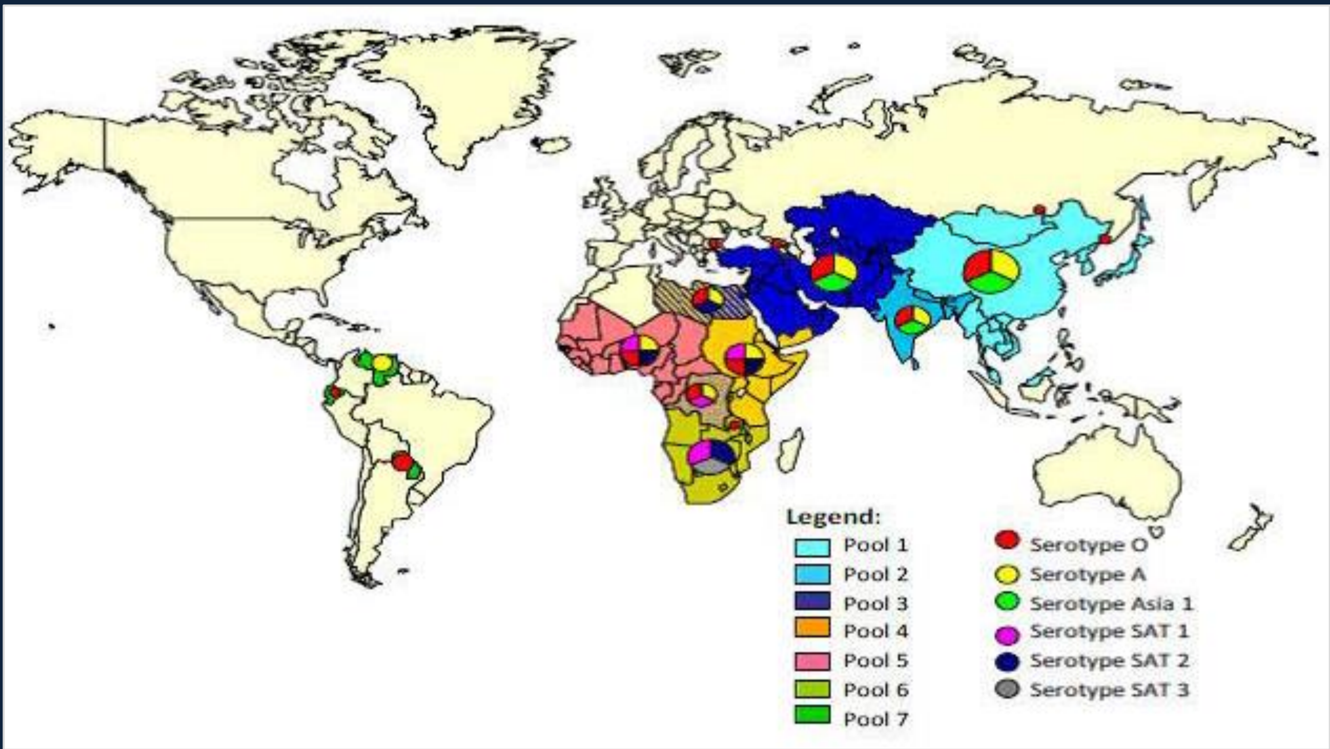
The BHK- 21 cells in one cell culture plate (Nunc) were used to calculate the biological titer (Tissue Culture Infective $Dose_{50}$: $TCID_{50}$) of the virus. The aliquot of serotype "O" stored at 4°C was taken out and 10-fold dilutions of the virus was prepared in 2.5 ml micro-centrifuge (eppendorf) tubes. Sterile 1.8 ml of maintenance medium was added to each of the tubes. In the first tube 0.2 ml of the virus was added and mixed properly. From the first tube 0.2 ml of the mixture was transferred to the next tube. In the same manner, a tenfold dilution was made in the till 10th tube. Growth medium was discarded from the previously cultured confluent monolayer of the cells present in 96 well cell culture plates. The cells were washed once with sterile PBS (pH 7.2). From the diluted virus suspension, an amount of 200 µl of the suspension was added to the respective well of the plate (8 wells per dilution) starting from the dilution of 10^{-1} till 10^{-10} **(Table 2.7.4)**. The 11th and 12th columns received the maintenance medium and were kept as cell control. The cell culture plate was incubated at 37°C for 72 hours and observed at 40 under inverted microscope after every 24 hours. Cells in each well were examined for the presence of any CPE. Number of wells in each row showing CPE was recorded.

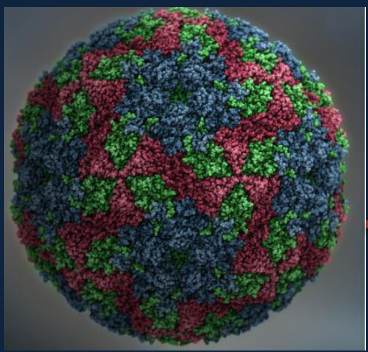
Table 2.7.4 Layout for $TCID_{50}$ Calculation

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Con
B	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Con
C	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Con
D	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Con
E	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Con
F	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Con
G	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Con
H	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Con

Molecular characterization was carried out by PCR amplification spanning the entire VP1 gene for type O and type A respectively according to protocol mentioned in **sub-section 2.4.2.3.2.** Then amplified sequences were purified and subjected to automated cycle sequencing reaction. After assembly, different bioinformatics study was carried out for the selection of effective vaccine candidate through the analysis of the genes related to protection of FMD.

3





Chapter 3

Results

3. Results

FMD virus replicates in the epithelial lining and thus the most suitable tissue for the isolation and identification of FMD virus is epithelial tissue especially the linings of gums, dental pads, tongue and the interdigital space (**Figure 3**). The disease was initially confirmed on the basis of clinical picture with high fever and lesions in mouth and foot. Based on the clinical symptom samples (include total number) were collected for characterization and isolation of FMDVs circulating in Bangladesh; and study of molecular epidemiology of FMD in Bangladesh followed by complete genome analysis for the selecting appropriate representative of vaccine strain(s).

Figure 3. Clinical sign and symptoms of FMD. Outbreaks suspected for Cattle at Narail (Left) and for Pig at Gopalgonj (Right) on August 2015

3.1 Studies of the Outbreaks of Foot and Mouth Disease in Bangladesh

3.1.1 Questionnaire and Interviews

The data collected on the basis of questionnaire showed that FMD, which is locally known as Khura rog, is well known to herdsmen and they are well acquainted with the disease, its clinical signs, seasonality, duration and transmission. The husbandry systems practiced in the investigated herds were either intensive (12%), semi-intensive with free animal movement (43%) or extensive (45%). The questionnaire data showed that FMD clinical signs were observed only in cattle and pigs, and caused mild or no clinical signs in small ruminants, especially those intermingling with cattle. According to this study, it was predominantly encountered in the period of September to January. To reduce the effects of the disease, herdsmen add the powder of fried borax to the honey or molasses and rubbed on the tongue of infected animals, to cure mouth ulcers. After washing infected areas with Potassium permanganate (ppm 0.001%) solution or 4% sodium-bi-carbonate solution, herdsmen also apply turmeric powder or glycerin and antibiotics to protect infected animals from secondary bacterial infection.

3.1.2 Confirmation of FMDV in Clinically Suspected Animals

A total of 150 randomly selected samples (out of 283) collected from 39 suspected clinical outbreaks of FMD from Bangladesh in 2012 to 2016 (**Figure 3.1.2.1**) were initially tested using RT-PCR assays by employing three sets of universal primer pairs (VP1UF/NK61, 16F/16R and 16F/NK61) and finally 106 samples were found to be positive for FMDV RNA. Here, primer pair, 16F/16R targets a partial VP1 region of FMDV genome yielding an amplicon of about 426 bp identified the highest 106 samples as FMD positive (**Appendix II)**. On the other hand, 16F/NK61 primer pair (also targeting partial VP1 region and amplified about 716 bp amplicon) identified 66 samples as positive and another primer pair, VP1UF/ NK61 (targeting whole VP1 region of FMDV and amplified about 1141 bp amplicon) identified 36 samples as positive out of similar number (106) of filed samples. The rest of 44 samples which were found 16F/16R negative could not also be amplified by diagnostic RT-PCR assays with another two sets of primers, hence were not further processed. The representative picture of the amplicon is delineated in **Figure 3.1.2.2a-c.**

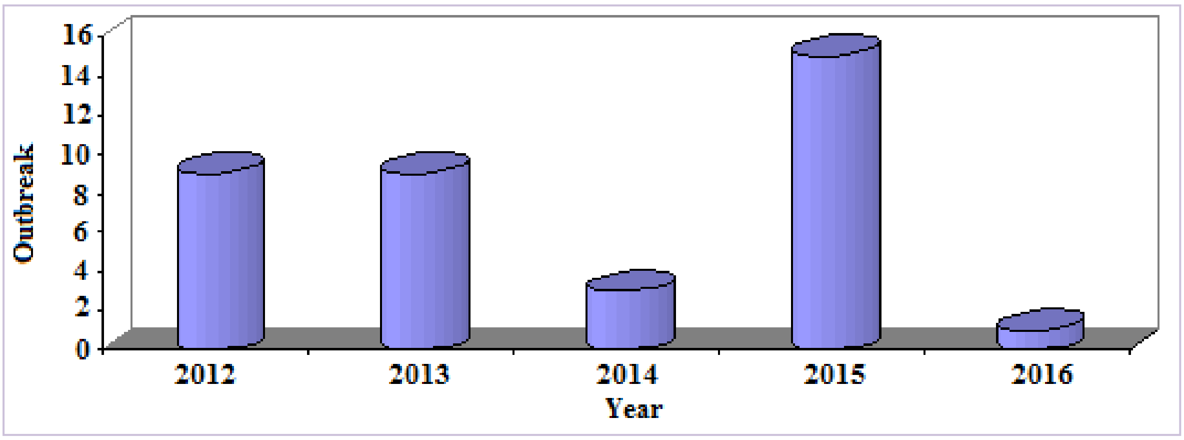


Figure 3.1.2.1 Distribution of foot and mouth disease outbreaks in Bangladesh, May 2012 to April 2016

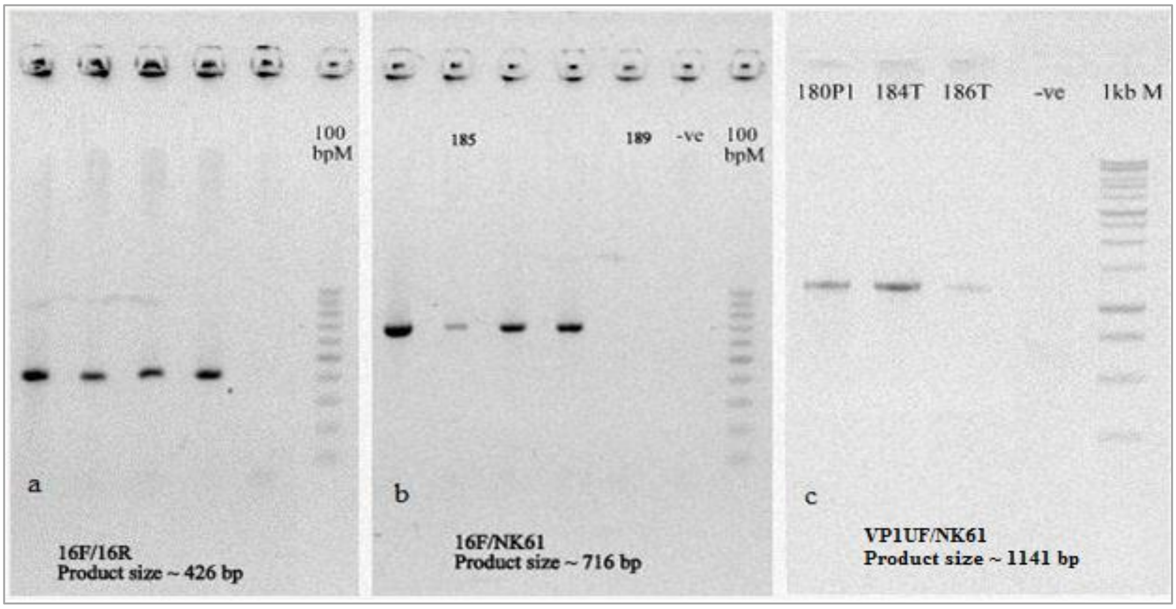


Figure 3.1.2.2a-c PCR amplification products of VP1 specific region (Representative). Amplicon size is about 426 bp (a), 716 (b) and 1141 bp (c). Here 100bp (Bioneer, USA) (a,b) and 1kb (Promega, USA) marker was used.

From these 106 sets, total 36 positive samples (not selected for sequencing of partial or whole VP1 gene) were further analyzed by amplifying the VP1 coding region using specific primers (for serotypes O, A and Asia-1) to determine the serotype and subtype of the virus to facilitate the study of epidemiology only. Moreover, 70 tissue samples from clinically infected animals, which also scored positive in the diagnostic assays for FMDV RNA, were amplified for complete VP1 region and sequenced to confirm the subtype of virus along with identification of virus lineage and topotype responsible for clinical FMD infections in Bangladesh.

3.1.3 Distribution of FMD in Bangladesh

Of the 106 VP1 positive samples determined, 77 in 34 (87%) outbreak areas were identified as serotype O, while 22 in 3 (8%) and 7 in 2 (5%) outbreak areas were found positive for serotypes A and Asia-1 respectively **(Figure 3.1.3a)**. The locations of outbreaks showing the presence of distinct FMDV serotypes included in this work are listed in **Figure 3.1.3b.**

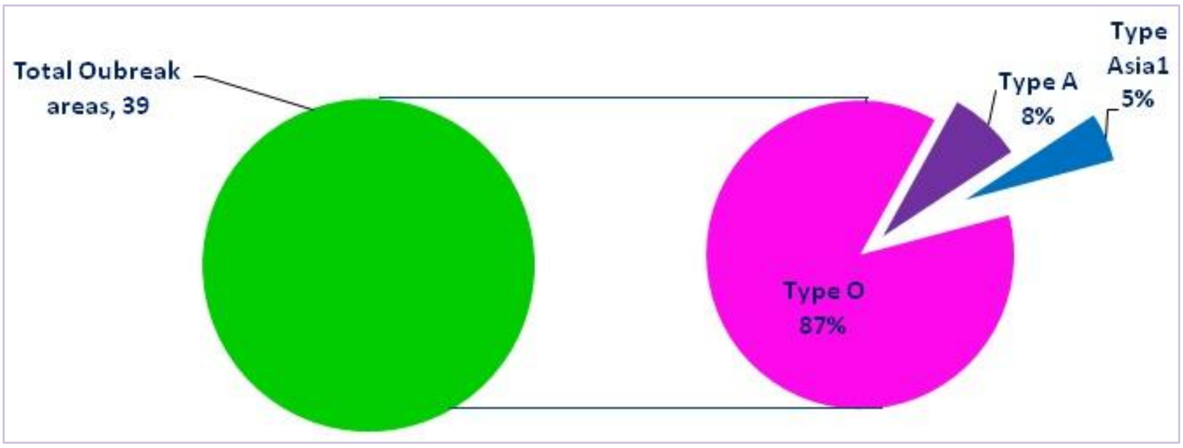


Figure 3.1.3a Percentage of foot and mouth disease virus type distribution in Bangladesh, 2012 to 2016

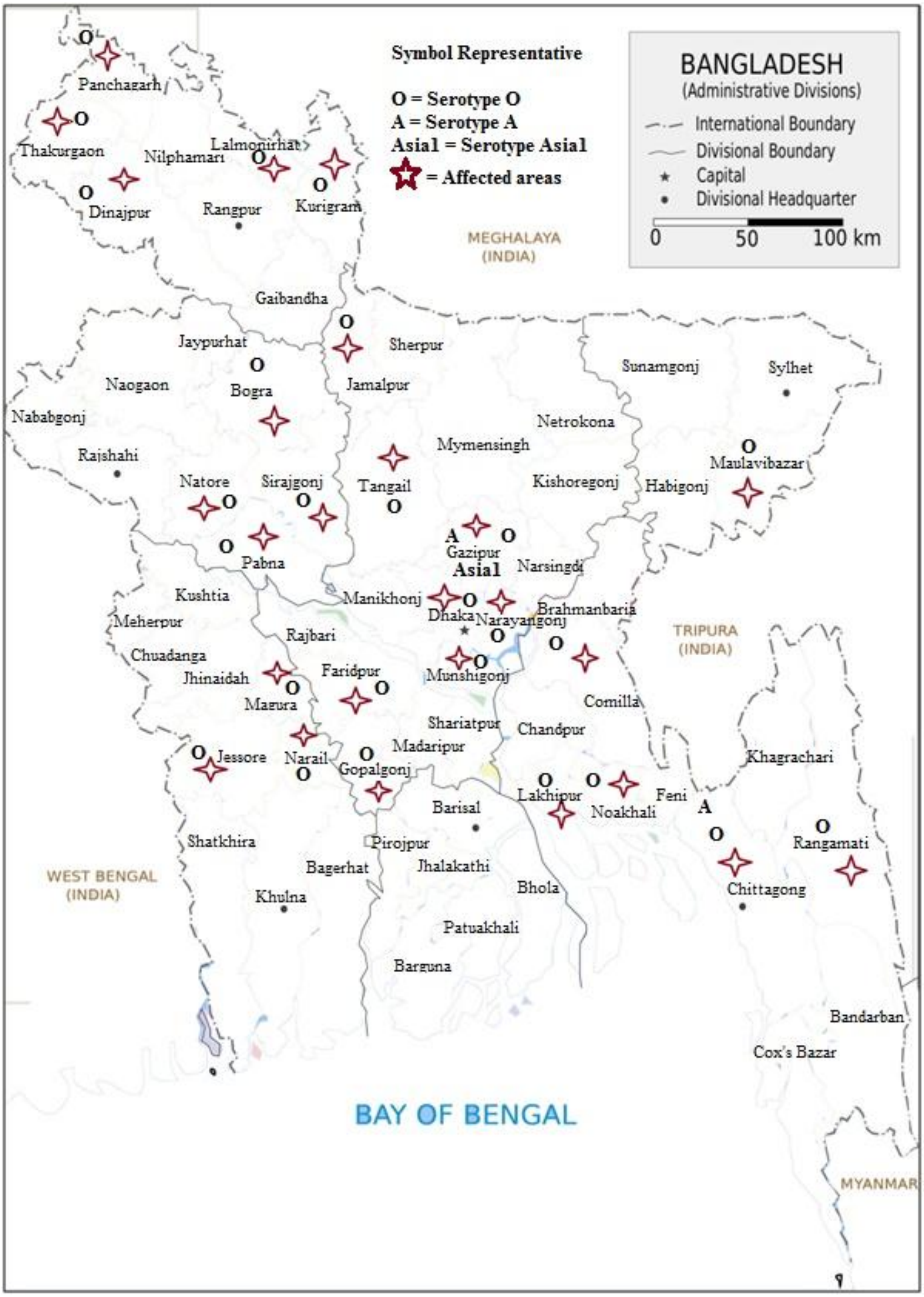


Figure 3.1.3b Geographical distribution of FMD outbreaks in Bangladesh during 2012-16 showing presence of distinct FMDV serotypes with VP1 positive cases.

For epidemiological findings, a total of 3129 cattle population in the 39 infected/outbreak areas were included. According to the study, it was found that the incidences of FMD outbreaks increased gradually following the late-monsoon period. The greatest number of outbreaks was observed during the pre-winter to post winter season, from September onwards. The monthly outbreak pattern of FMD during this period is shown in **Figure 3.1.3c.** The highest number of FMD outbreaks happened in the month of October (23.1%) followed by December (12.8%) and then March or August (10.3%) and the incidence decreased gradually up to mid-August, with the no number of FMDV cases observed in the month of April.

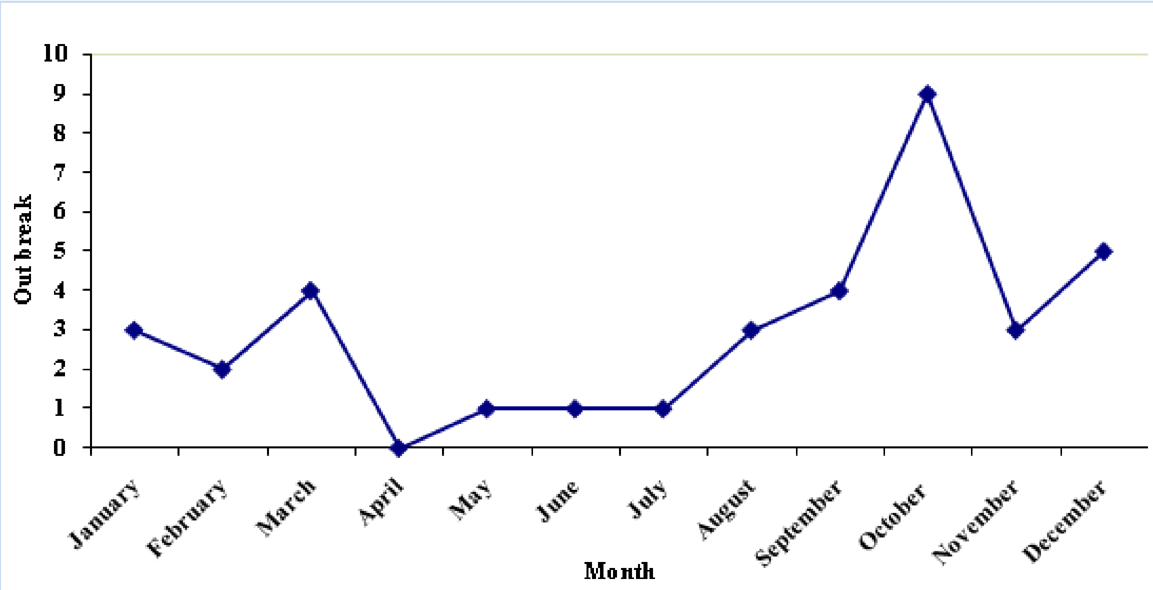


Figure 3.1.3c Seasonal distribution of laboratory-confirmed FMD outbreaks in Bangladesh taking place during 2012–2016 (n = 39)

In total population of cattle 3129, morbidity, mortality and case fatality rates were 53.8% (1684), 11.4% (356) and 21.1%, respectively. Among them, in young cattle (>1 year to before breeding) out of 1231, morbidity, mortality and case fatality rates were 53.85% (663), 9.1% (112) and 16.9% while in adult population 1521, it was 59.6% (912), 12.6% (192) and 21.1% respectively. In other age group (calves up to 1 year) out of 377, morbidity, mortality and case fatality rates were 28.9% (109), 13.8% (52) and 47.7% **(Figure 3.1.3d).**

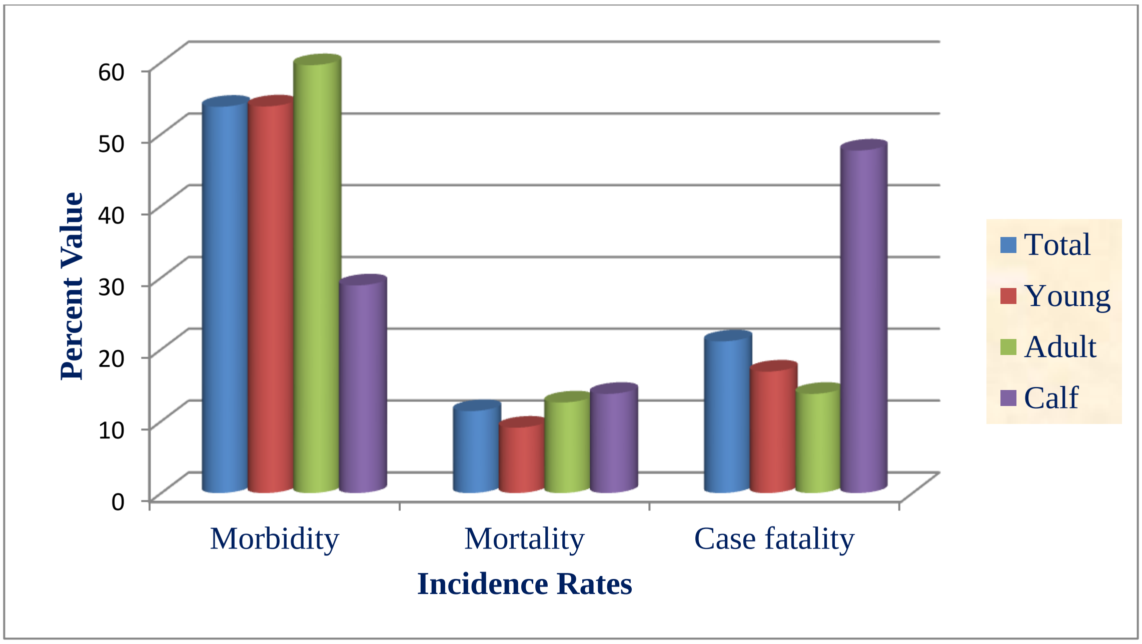


Figure 3.1.3d Morbidity, mortality and case fatality rates in cattle affected with FMD according to their age

The incidence of FMD showed higher in males 58.4% (833/1426) than that of females 49.9% (851/1703) indicating that the males were more susceptible to FMD than females. The susceptibility of various breeds of cattle to FMD revealed that the higher incidence in indigenous cattle 55.7% (1227/2201) than that of crossbred cattle 49.2% (457/928) indicating that cross breed is less susceptible than indigenous breed. FMD incidence in cattle varied on different feeding pattern. The animals on grazing (extensive) pattern had the highest FMD incidence 53.3% (791/1317) compared to those on manger (intensive) feeding 43.5% (143/329) and on combined manger and grazing pattern 50.9% (750/1483). Animals on grazing pattern are 1.2 times more at risk of getting FMD as compared to cattle on manger feeding. FMD was recorded in 43.2% (393/910) vaccinated cattle and 58.2% (1291/2219) in unknown of vaccination or non-vaccinated cattle **(Figure 3.1.3e)**. The survey revealed that non-vaccinated cattle are 1.3 times more at risk of infection due to FMD as compared to vaccinated cattle.

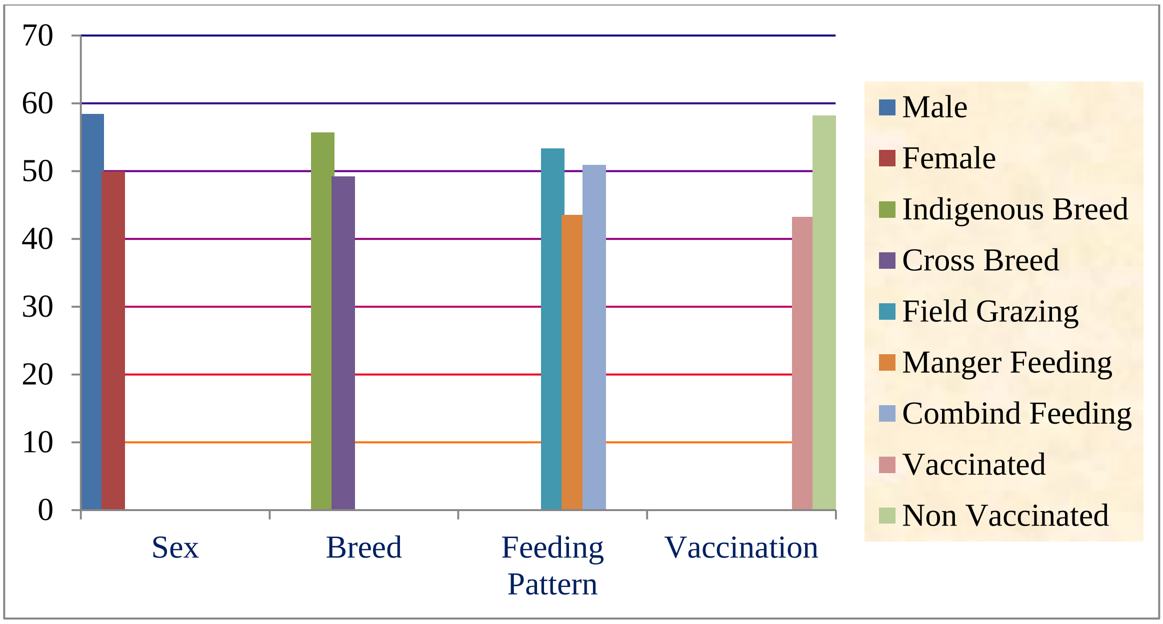


Figure 3.1.3e Morbidity rate in cattle according to their sex, breed, feeding pattern and vaccination status

3.1.4 Phylogenetic Relationships

Genetic characterization of FMDV serotypes prevalent in Bangladesh is one of the fundamental objectives in the molecular epidemiology of FMD virus. The results established the genetic identity of the virus, route of transmission of the FMDVs, and emergence and reemergence of virus strains.

3.1.4.1 Identification of serotypes

The complete VP1 coding sequences of FMDV serotypes O, A, C, Asia-1, SAT1, SAT2, SAT3 and the studied sample sequences were used to construct a sequence similarity tree **(Figure 3.1.4.1)**. This phylogenetic analysis showed that the 2012/2013 outbreak strains had the greatest sequence similarity to other FMDV serotype O, A and Asia-1 viruses and formed respective clade with them. On the other hand, the 2014/2015 outbreak strains had the greatest sequence similarity to the serotypes O only and formed a cluster with same strains.

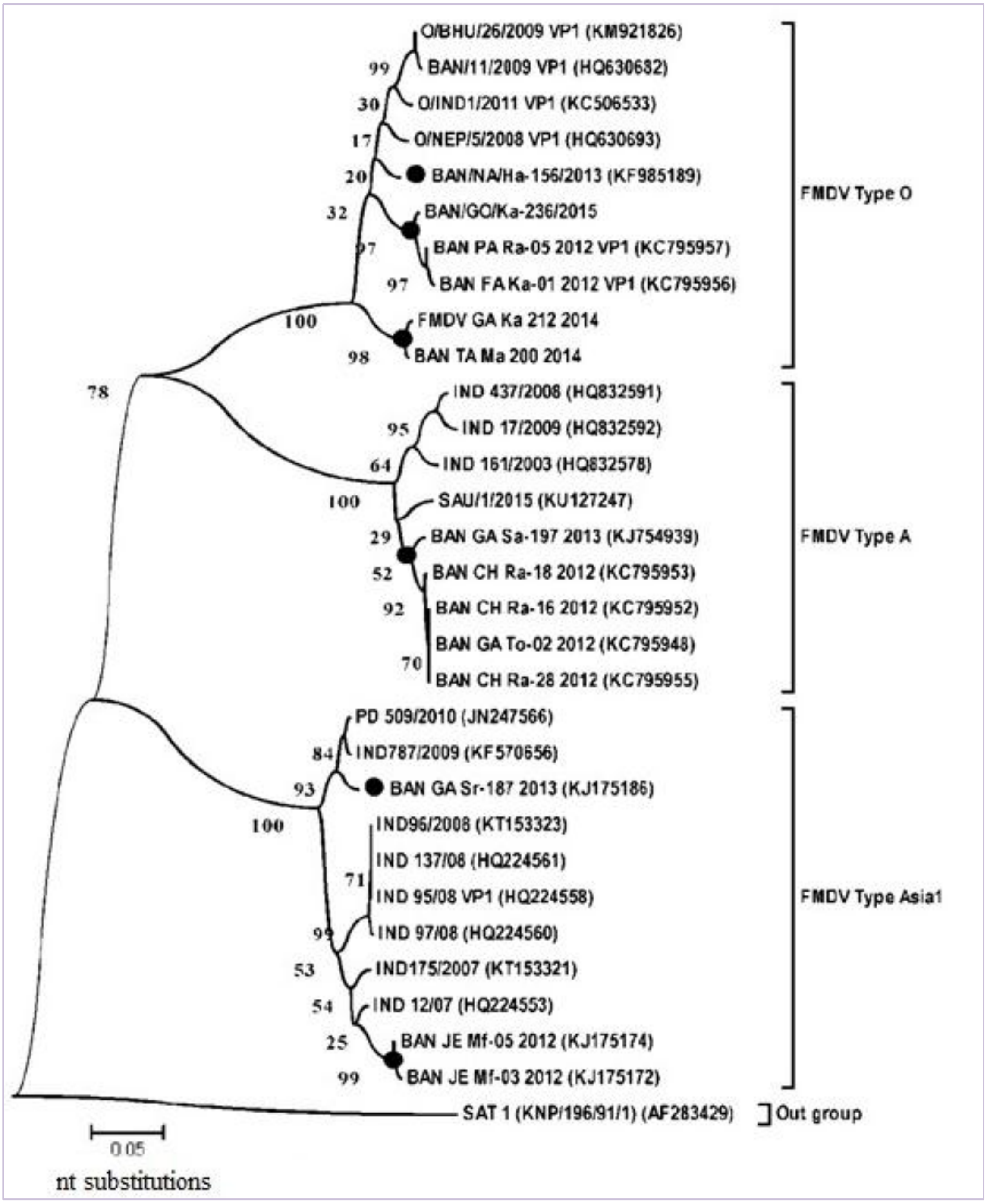


Figure 3.1.4.1 Phylogenetic tree constructed based on nucleotide sequence by neighbour joining method of the VP1 coding region of FMDV type O, A, C, SAT-1 and SAT-2 for the identification of viruses isolated during this study. FMDV type A of local origin was selected as appropriate out group. A trial number of 1000 was applied. The GenBank accession numbers of different FMDV subgroups are indicated by the brackets.

3.1.4.2. Detection of Genetic Lineages and Topotypes

3.1.4.2.1. FMDV Serotype O

Representative complete VP1 coding sequences of different topotypes for FMDV serotype O and the sequences of FMDV of current study were phylogenitically compared **(Figure 3.1.4.2.1)**.

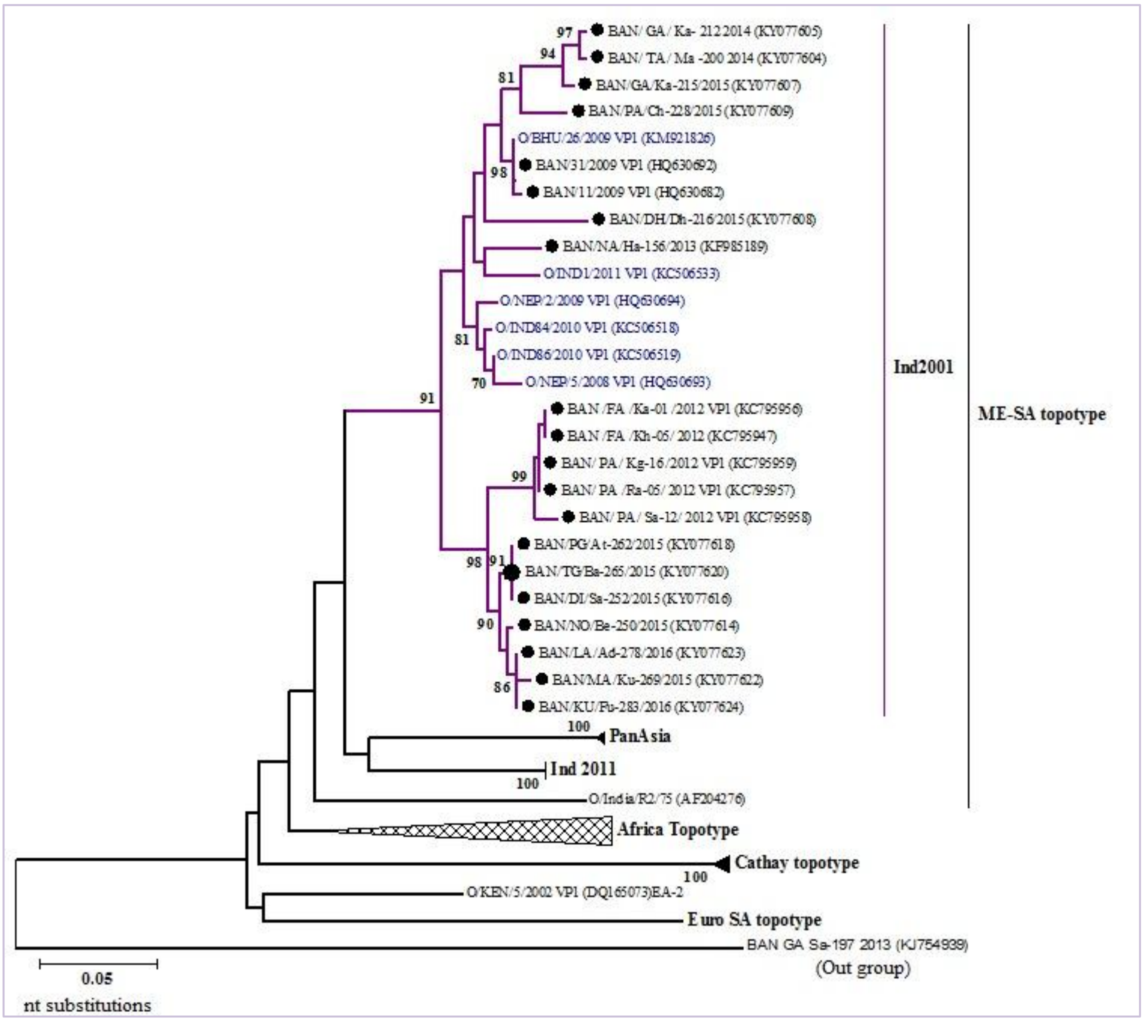


Figure 3.1.4.2.1 A neighbour-joining tree based on the nucleotide sequence of the VP1 structural-protein coding region FMDV type O, depicting the relationship of FMDV isolated originating from diverse geographical origin. FMDV type A of local origin was selected as appropriate out group. A trial number of 1000 was applied. Sequences with filled circle symbols are of local origin. The GenBank accession numbers of different FMDV subgroups are indicated by the brackets.

The serotype O outbreak strains during the study period had the greatest sequence similarity to the isolates from India (KC506518, KC506519 and KC506533), Nepal (HQ630693 and HQ630694) and Bhutan (KM921826) and formed a sub-clade with them. Viruses in this sub-clade belong to Ind2001 lineage within the ME-SA (Middle East South Asia) topotype which reemerged in late part of year 2008 in India. Therefore the study sequences belong to Ind2001 lineage of the ME-SA topotype.

3.1.4.2.2. FMDV Serotype A

The complete nucleotide sequence of VP1-coding region was determined for 14 FMDV type A viruses obtained from epithelium samples collected from three different outbreak areas in 2012-2013. A phylogenetic tree generated using the neighbor-joining method is shown in **Figure 3.1.4.2.2a**.All viruses studied from the outbreaks in Bangladesh showed a limited degree of variation in the VP1 gene, with values of over 97% genetic relatedness among them and clustered within the genotype VII of Asia topotype. From the results presented in **Figure 3.1.4.2.2b**, it became evident that all type A viruses sequenced from Bangladesh outbreaks belong to the genotype VII of Asia topotype.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Foot-and-mouth disease virus - type A isolate BAN GA Sa-197 2013, complete genome	15180	15180	100%	0.0	100%	KJ754939
Foot-and-mouth disease virus - type A isolate SAU/1/2015, complete genome	13572	13572	99%	0.0	97%	KU127247
Foot-and-mouth disease virus - type A isolate IND 245/2007, complete genome	12349	12349	99%	0.0	94%	HQ832590
Foot-and-mouth disease virus - type A isolate IND 437/2008, complete genome	12176	12176	99%	0.0	94%	HQ832591
Foot-and-mouth disease virus - type A isolate IND 17/2009, complete genome	11695	11961	99%	0.0	93%	HQ832592
Foot-and-mouth disease virus - type A isolate IND 447/2005, complete genome	11527	11527	99%	0.0	92%	HQ832583
Foot-and-mouth disease virus - type A isolate IND 281/2003, complete genome	11518	11518	99%	0.0	92%	HQ832579
Foot-and-mouth disease virus - type A isolate IND 161/2003, complete genome	11459	11459	99%	0.0	92%	HQ832578
Foot-and-mouth disease virus - type A isolate IND 818/2003, complete genome	11393	11393	99%	0.0	92%	HQ832580
Foot-and-mouth disease virus - type A isolate IND 43/2006, complete genome	11313	11313	99%	0.0	92%	HQ832586
Foot-and-mouth disease virus - type A isolate IND 249/2004, complete genome	11237	11237	99%	0.0	91%	HQ832582

Figure 3.1.4.2.2a FMDV type A sequences producing significant alignments using nucleotide BLAST analysis

When compared with the strains in the databank, they showed the closest relatedness with viruses SAU/1/2015(KU127247), IND245/2007(HQ832590), IND437/2008 (HQ832591), IND17/2009(HQ832592), IND447/2005(HQ832583), IND281/2003 (HQ832579), IND161/2003(HQ832578), IND818/2003(HQ832580), IND43/2006 (HQ832586) and IND 249/2004(HQ832582), with which they have approximately 91 to 97% nucleotide sequence identity as shown in **Figure 3.1.4.2.2a**.

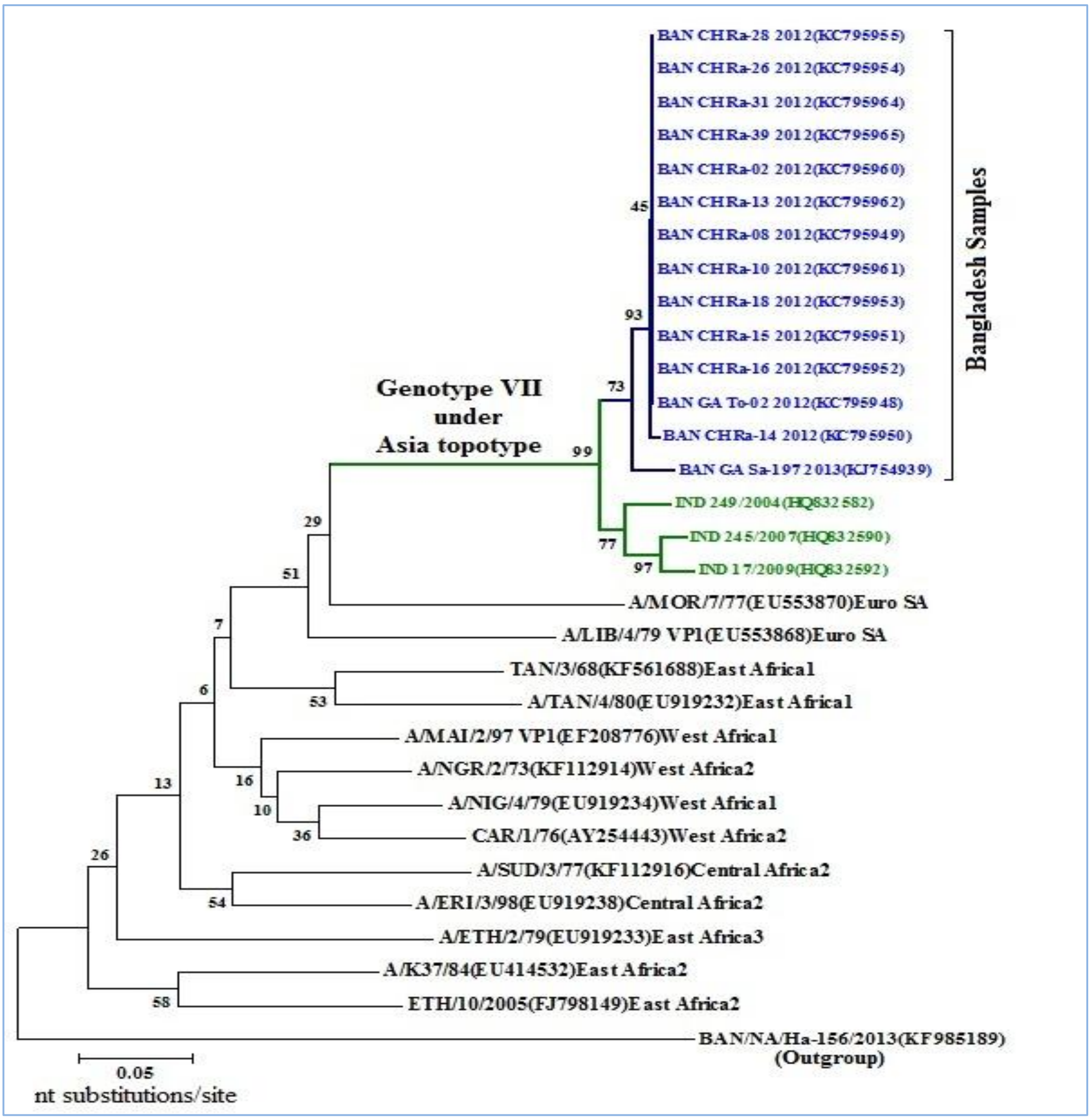


Figure 3.1.4.2.2b A neighbour-joining tree based on the nucleotide sequence of the VP1 structural-protein coding region FMDV type A, depicting the relationship of FMDV isolated originating from diverse geographical origin. FMDV type O of local origin was selected as appropriate out group. A trial number of 1000 was applied. The GenBank accession numbers of different FMDV subgroups are indicated by the brackets.

When compared with the vaccine strain IND 40/00[(HM854025), **Indian Immunologicals Ltd**], all detected viruses were recorded values of 10.4% nucleotide sequence difference and were placed in a different group as Shown in **Figure3.1.4.2.2b.**

3.1.4.2.3 FMDV Serotype Asia-1

A phylogenetic tree was constructed using the whole VP1 encoding regions of field isolates generated in this study with previously determined sequences of serotype Asia-1 viruses of different lineages collected from the NCBI GenBank database **(Figure 3.1.4.2.3)**.

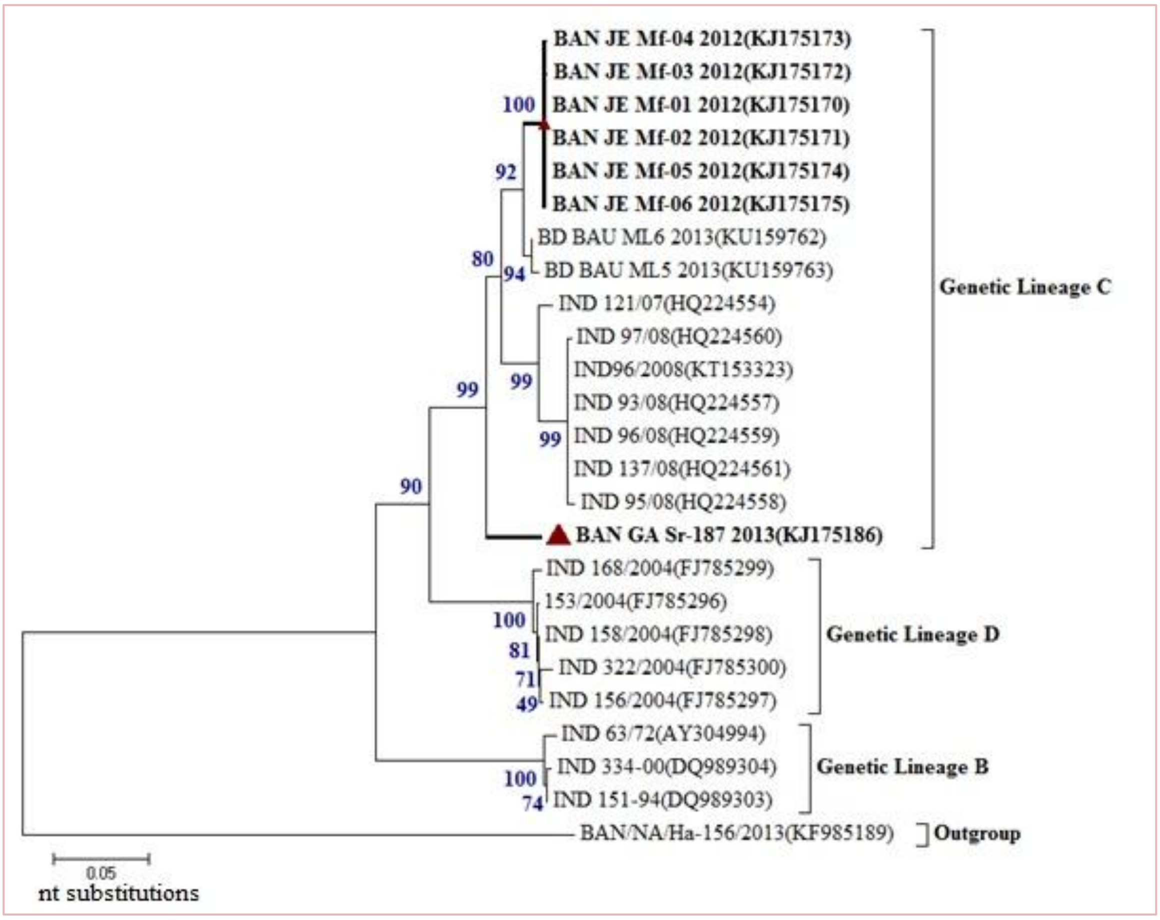


Figure 3.1.4.2.3 A neighbour-joining tree based on the nucleotide sequence of the VP1 structural-protein coding region of FMDV type Asia-1, depicting the relationship of FMDV isolates originating from diverse geographical origin. FMDV type O of local origin was selected as appropriate out group. Phylogeny reconstruction was carried out in MEGA 5.2. A trial number of 1000 was applied. Sequences in Bold with triangular symbols are of local origin. The GenBank accession numbers of different FMDV subgroups are indicated by the brackets.

The VP1 phylogeny showed that the sequences of local circulatory serotypes Asia-1 clustered within the genetic lineage C which was prominently circulating in India during the period 1993 to 2001 and re-emerged in 2005.

Lineage C has been responsible for all Asia-1 outbreaks in Bangladesh since 2012. During the period, outbreak due to Asia-1 serotype was recorded in Jessore and Gazipur districts of Bangladesh. The FMDV Asia-1 isolates, sequenced for molecular epidemiological studies, showed 94.42-100% identity at nucleotide level in the VP1 coding region to each other. These viruses are also closely related to viruses which circulated in India since 2008.

3.2 Molecular Characterization of Virus

3.2.1 Cell Line Establishment

Stock BHK-21 cell line was sub-passaged to establish suitable cell for virus inoculation. After three round of cell sub-passaging, a cell line was selected to inoculate virus (**Figure 3.2.1**).

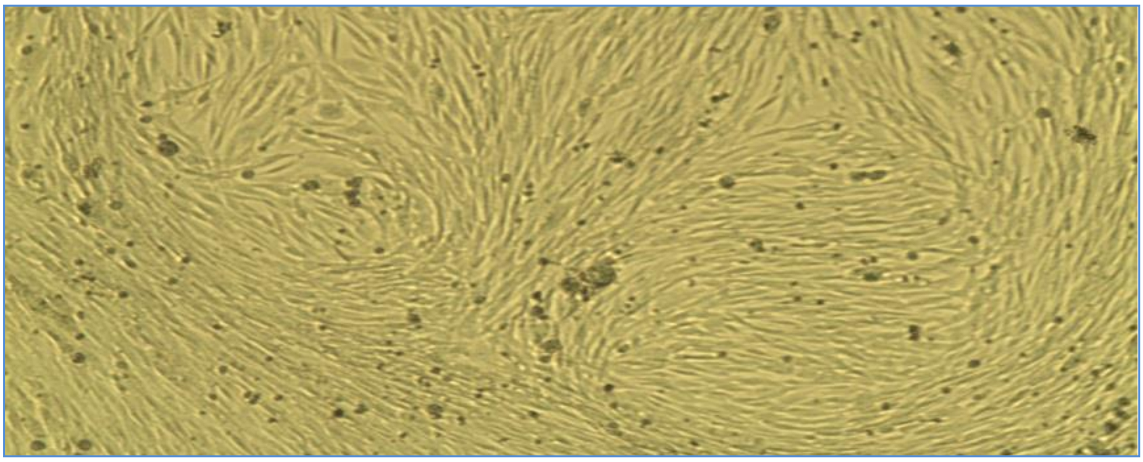


Figure 3.2.1 Monolayer of BHK-21 cell line with characteristic flattened shape.

3.2.2 Isolation of Virus

From a total of 106 positive samples, 12 selected samples were subjected for virus isolation using BHK-21 cell culture and CPE was developed after first passage or 2-3 blind passages (passage for virus adaptation) which were characterized by a fast destruction of BHK-21 mono layer cell and infected cells were round and formed singly as indicated in arrow **(Figure 3.2.2).** The results showed that 9 out of 12 selected tissue samples produced CPE in each of respective flasks with BHK-21 cell line as shown in **Table 3.2.3.1**. The BHK-21 adapted viral suspension in respective flasks was freeze-thawed and collected in 2 ml cryogenic vial and stored at -80^{0}C till further study.

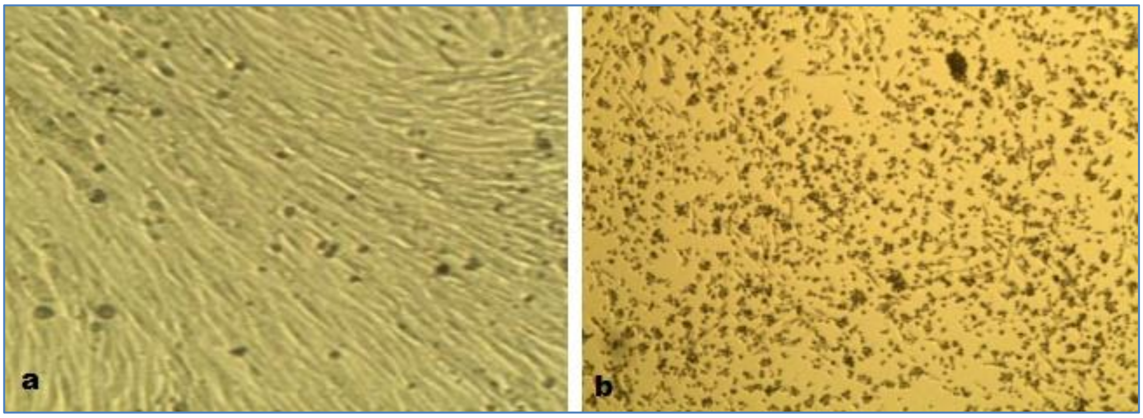


Figure 3.2.2 Monolayer of BHK-21 cell line with characteristic flattened shape. Cytopathic effect observed at passage level-2 of (b) virus inoculation with respect to (a) no virus control.

3.2.3 Screening of Samples and Detection of Serotype

3.2.3.1 VP1 Sequencing

For identification, RNA was extracted from 9 isolated FMD viruses, and each VP1-coding region was successfully amplified by RT-PCR by using at least 1 of the 3 described universal primer sets. The complete VP1 sequences were determined by directly sequencing the amplicons. For all these isolates, the VP1 gene consisted of 633 nt coding for 211 amino acids. The DNA sequences were aligned with the public database on NCBI homepage using BLAST algorithm and the VP1 sequences of isolated viruses identified as eight FMDV serotype O and one FMDV serotype A **Table 3.2.3.1.**

Table 3.2.3.1 Molecular Identification of FMDV with VP1 Sequencing

Serial No.	Sample Identification No.	Description of Sequence	Method of Identification	Serotype detected	Place 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

3.3. Entire Genome Amplification

Out of nine isolated FMD viruses, RNA was successfully amplified for the two representative viruses of serotype O (BAN/NA/Ha-156/2013) and serotype A (BAN/GA/Sa-197/2013) circulating in the cattle population of Bangladesh in order to complete genome sequencing and detail genome wide analysis accordingly.

3.3.1 Optimization of the Primer Pairs to Amplify Entire Genome

The optimization experiment was needed to access the optimum conditions to achieve the highest sensitivity over PCR reaction. The experiments were carried out according as described in the Material and Methods section. However, we evaluated the results directly from these experiments, choosing the best experiment as an optimum. Usually on the basis of these results and the sequence data of the primer binding regions of the amplifiable genes, we designed 8 forward and 6 reverse primers **(Appendix III)**. The priming efficiency of the primer set was optimized by the relative concentration of the primers and also by the design of the primer sequences. Introducing mismatches could help to reduce internal competition of the primers, but mismatches are kept at a minimum especially at the 3' end of the primers. This approach resulted in a smooth priming efficiency distribution. The optimized reaction has very high sensitivities and uniform amplification power.

3.3.2. Amplification of 5' Un-Translated Region (UTR)

20F (designed in this study): 1R (Reid *et al.*, 2000) and 1F:1OEXR primer pair targeting 5' UTR region amplified about 965 bp and 708 bp amplicon respectively (**Figure 3.3.2b**) for serotype O. A1F: A1R, A2F3:A2R and 682F:1293R primer pair targeting 5' UTR region of the isolated FMDV type A virus amplified about 374 bp, 622 bp and 626 bp amplicon respectively (**Figure 3.3.2a**).

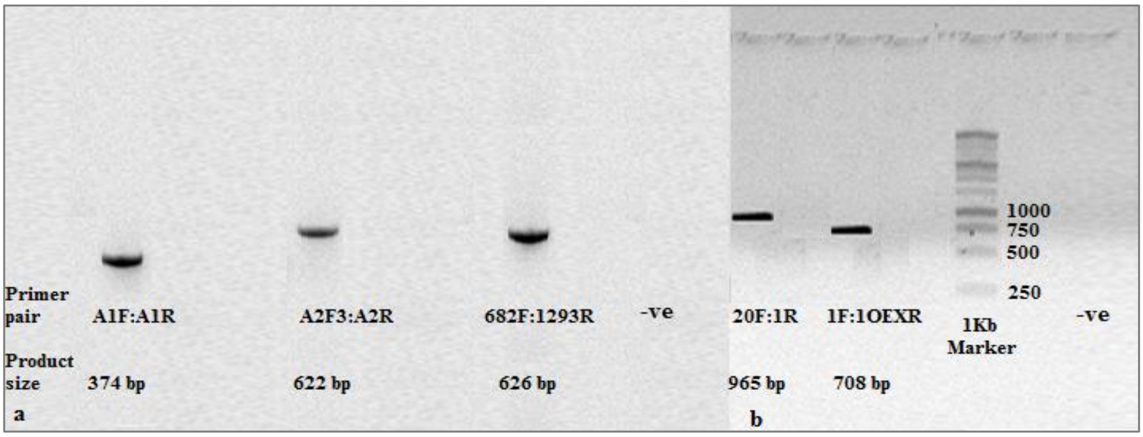


Figure 3.3.2a-b Primer combination for PCR amplification of the 5' UTR region.

3.3.3 Amplification of the Structural Region (VP4-VP1)

1F (Reid *et al.*, 2000): 1OR, 2OF:2OR, 3OF:3OR, 4OF:4OR, 5OF:NK61 (Samuel and Knowles, 2001) primer pairs were evaluated in this study to amplify structural region of the isolated FMDV type O virus. No amplification was found in case of primer pairs 1F:1OR and 5OF:NK61. Primer pairs 2OF:2OR, 3OF:3OR, 4OF:4OR amplified about 1006 bp, 861bp, and 890 bp amplicon respectively (**Fig. 3.3.3a**). Two back up primers were evaluated to amplify the target regions with 1F and NK61 primer. 1F:1OEXR primer pair amplified 708 bp amplicon whereas 5OEXF:NK61 primer pair amplified 718 bp amplicon (**Fig. 3.3.3b**). Five primer sets [1F:1OEXR, 2OF:2OR, 3OF:3OR, 4OF:4OR and 5OEXF:NK61] were selected finally to amplify complete structural region.

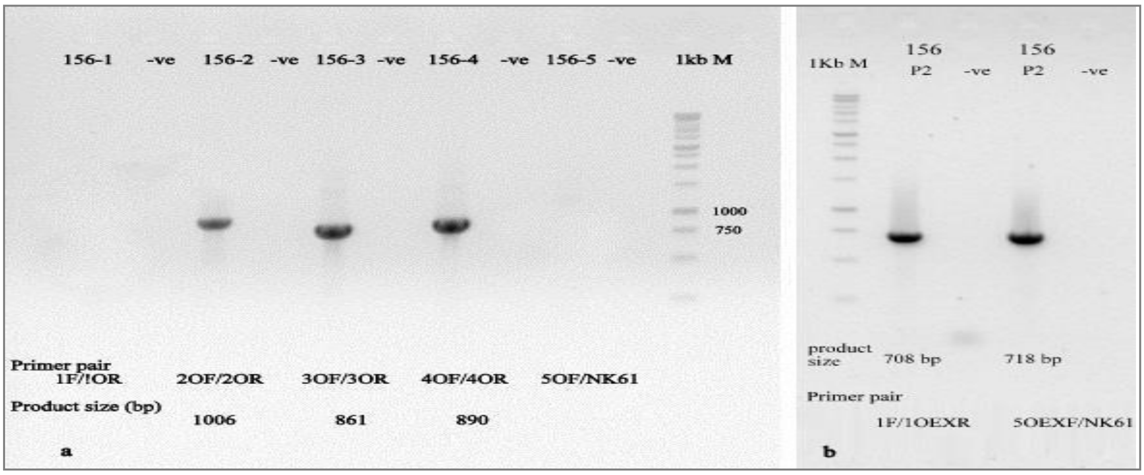


Figure 3.3.3a-b Primer combination for PCR amplification of the FMDV type O structural region.

Similarly, ten primer pairs 5'UTR-4F:5'UTR-4R, A4F:A4R, A5F:A5R, A6F:A6R, A7F:A7R, A8F:A8R, A9F:A9R, P1-3F3:NSP1R2 and A10F:A10R (Abdul-Hamid, Firat-Sarac *et al.*, 2011) were evaluated for the amplification of entire structural region of FMDV serotype A. All the primer pairs were found to amplify 620bp, 513bp, 714bp, 639bp, 501bp, 805bp, 629bp, 980bp and 606bp sized amplicons respectively.

3.3.4. Amplification of the Non-Structural Protein (NSP) Coding Region Plus 3' UTR

For both FMDV serotype O and A, 10 overlapping amplicons covered total non-structural region including 3' UTR were amplified using different sets of primers. Three combinations of primer sets (NSP 10 F/T21A, NSP 10F/T21C and NSP 10F/T21G, Abdul-Hamid, Firat-Sarac *et al.*, 2011) were optimized for 3' UTR. In this case, all the primer combinations were evaluated to assess the genotype spectrum of the reactions. The combination of NSP 10F/T21A and NSP 10F/T21C could not amplify the target gene, while the NSP 10F/T21G had a well-balanced and much more sensitive amplification and revealed a desired amplicon of 832 bp. In all primer combinations spurious band of 500 bp range was also observed (**Figure 3.3.4**). For this reason, gel purified product of desired amplicon was used for sequencing 3' UTR.

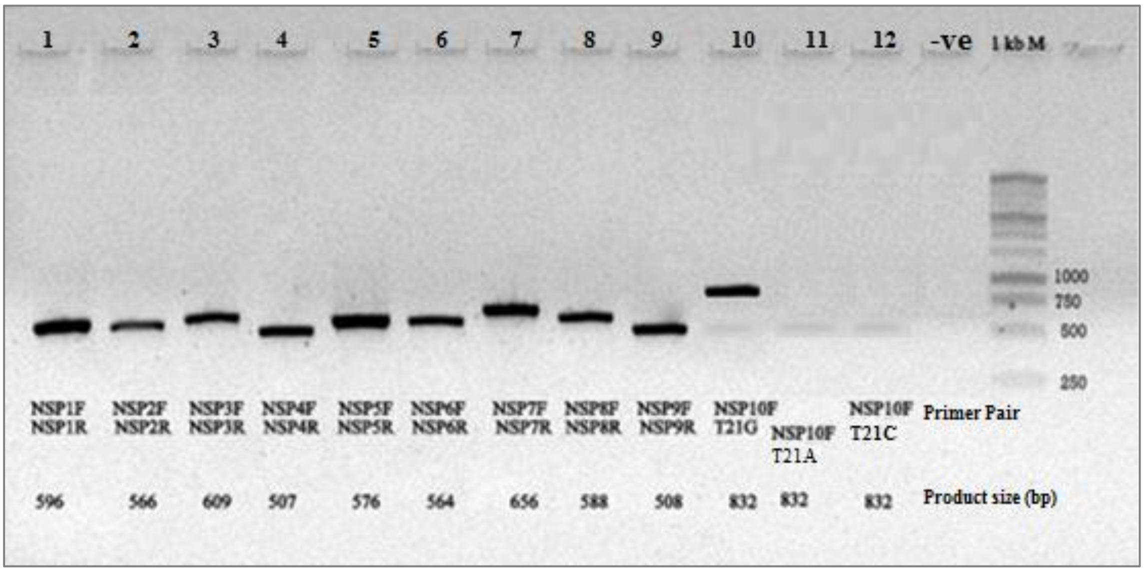


Figure 3.3.4 Comparison of amplification of NSP Region and 3'UTR with the different combination of primer sets.

3.4 Alignment of Sequences and Genome Annotation

The number of PCR products used for each genome ranged between 16 and 22 fragments, 16 fragments for BAN/NA/Ha-156/2013 and 22 fragments for BAN/GA/Sa-197/2013**.** These sequences were aligned together with previously published reference sequence (NC_004004) to identify the putative functional regions of the genomes **(Table 3.4)**. Complete genome sequences of serotype O and serotype A were 8131 and 8220 nucleotides in length respectively, containing an Open Reading Frame (ORF), a 5' UTR, and a 3' UTR.

The genome sequences were submitted to GenBank with the accession number of KF985189 (serotype O) and KJ754939 (serotype A). The detail genome annotation indicates putative functional regions for serotype O and A are shown in **Table 3.4.** Excluding stop codon ORF is 6996 nt (encoding 2332 aa) in length, which consists of L (603 nt), P1 (2202 nt), P2 (1470 nt), and P3 (2721 nt) genes. P1 protein is predicted to be cleaved into four structural proteins, including VP4 (85 aa), VP2 (218 aa), VP3 (220 aa), and VP1 (211 aa). There are three non-structural proteins in P2, including 2A (18 aa), 2B (154 aa), and 2C (318 aa). P3 protein contains 3A (153 aa), 3B (71 aa), 3C (213 aa), and 3D (470 aa) four proteins. The 5' UTR consists of S-fragment, polyC tract, multiple pseudoknots (PKs) and internal ribosome entry sites (IRES) structures, which are 291, 15, 157 and 557 nt in length for serotype O respectively and in serotype A, these segments are 369, 17, 157 and 557 nt in length respectively. On the other hand, the 3’ UTR were 91 nt and 93 nt in length, followed by a 21 nt and 28 nt polyA tail (at least) respectively.

Table 3.4 Different gene fragments of FMDV genome

Isolate	Type	Genome length	Annotate (Putative functional) region								
			5'UTR				Leader	Structural Protein coding region (P1)			
			S-fragment	Poly C	PK	IRES	L	VP4	VP2	VP3	VP1
BAN/NA/Ha-156/2013	O	8131	291	15	157	557	603	255	654	660	633
BAN/GA/Sa-197/2013	A	8220	369	17	157	557	603	255	654	660	633

Isolate	Type	Genome length	Annotate (Putative functional) region									
			Non-structural Protein coding region (P2+P3)									3'UTR + PolyA tail
			2A	2B	2C	3A	3B			3C	3D	
							3B1	3B2	3B3			
BAN/NA/Ha-156/2013	O	8131	54	462	954	459	69	72	72	639	1410	112
BAN/GA/Sa-197/2013	A	8220	54	462	954	459	69	72	72	639	1410	121

3.5 Comparison and Analysis of Genome Sequence

3.5.1. FMDV Genome

In order to undertake more detailed analysis, the whole genomes were divided into seventeen gene fragments as shown in **Table 3.4.**

3.5.2 5'-Untranslated Region (UTR)

Comparative genome wide analysis with reference sequence (NC_004004) revealed that within serotype O, there is an 82 nt deletion in S-fragment **Table 3.5.2a** and 43 nt consecutive insertion in the 5′ UTR was evident introducing an extra pseudoknot (PK) structure **Table 3.5.2a** and within the serotype A, there is an 84 nt insertion in the pseudoknot (PK) structure of 5' UTR and lengthened polyC tract were evident compare to vaccine strain (HM854025) **Table 3.5.2a**.

A

```
RefSeq     1 TTGAAAGGGGCACTAGGGTCTCATCTCTAGCACGCCAACGACGACTCCC     50
             |||||||||||.|||||||||||.|.||||||..|||.||||.|||||.
BAN_156    1 TTGAAAGGGGCGCTAGGGTCTCACCCCTAGCATACCACCGACAACTCCT     50

RefSeq    51 GCGTCGCACTCCACACTTACGTCTCTGCGAGTGTAGGAACCGACGGACTG    100
             ||||.||||||||||||||||.|..||||...|..|||||.||.||||||
BAN_156   51 GCGTTGCACTCCACACTTACGCCCGTGCGCTCGCGGGAACTGATGGACTG    100

RefSeq    01 TCGCTCACCCACCTACAGCTGAACTCACAACACCGCGTGGCCATTTTTAG    150
             |||.|||||||||||||||||.|||||||..|||||
BAN_156   01 TCGTTCACCCACCTACAGCTGGACTCACGGCACCG---------------    135

RefSeq    51 AGCTGGATTGTGCGGACAAACGCCGCTTCACGCACCTCGCGTGACCGGCC    200
                             |||
BAN_156   36 ----------------CAA-------------------------------    138

RefSeq    01 AGTACTCTTACCACTTTCCGCCTACTTGGTCGTCAGCGCTGTTTTGGGCA    250
                      ||||||          |||||| .|.|||||||..||||||
BAN_156   39 ---------ACCACT----------TTGGTC-ACTGCGCTGTCCTGGGCA    168

RefSeq    51 CTCCTGTTGGGGGCTGTTCGACGCTCCACGGTCTCCTCCGTACTGAC-AT    299
             |.|||||||||||||.|||||||||||.|||||||||.|||| .|||| ..
BAN_156   69 CCCCTGTTGGGGGCCGTTCGACGCTCTACGGTCTCCCCCGT-GTGACGGG    217

RefSeq    00 CTACGGTGTTGGGGCCGCCACGTGCGAGCCGCTCGCCTGGTGTGCTTCGA    349
             ||||||||.||||||||||.||.|||.|..|.|||||||||.|||||||.
BAN_156   18 CTACGGTGATGGGGCCGCCTCGCGCGGGTTGATCGCCTGGTCTGCTTCGG    267

RefSeq    50 CTGTCACCCGACGCCCGCCTTTCA 373
             |||||||.|||.||||.|||||||
BAN_156   68 CTGTCACTCGAAGCCCACCTTTCA 291
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B

```
RefSeq                                  386 TAGGTTCTACC      396
                                            ||.||| ||||
BAN_156                                 307 TAAGTT-TACC      316

RefSeq   397 GTCGTTCCCGACGTTTGAAGGGA----------------GGA----------   422
             ||||||||||||| ||.||||||                |||
BAN_156  317 GTCGTTCCCGACG-TTAAAGGGATGTAACCACAAACTTGGAACCGTCTTG     365

RefSeq   423 -------------------AACCACACGCTTGCAACACCACTCCCGGTGT     453
                                ||||||||||||.|.|.||..||||.|.||
BAN_156  366 CCCGACGTTAATGGGTTGTAACCACACGCTTGTACCGCCTTTCCCCGCGT     415

RefSeq   454 CAACGGGATGCAACCGCAAGATGGACCTTCGCCCGGAAGTAAAACGGC 501
             .||.||||.|.||||.||||||..|||||||||||||||||||||||
BAN_156  416 TAAAGGGAAGTAACCACAAGATAAACCTTCGCCCGGAAGTAAAACGGC 463
```

C

```
IND40_00 351 ACCCGGCGCCCGCCTTTCA----------TCCCCCCTAAGTTTTACCGTC   390
             |||||..||||.|||||||          .|||||||||| |||.|||||
BAN_197  351 ACCCGAAGCCCACCTTTCACCCCCCCCCCCCCCCCCCTAAG-TTTGCCGTC   399

IND40_00 391 G-------------------------------------------------   391
             |
BAN_197  400 GTTCCCGACGTTAAAGGGATGAAACCACAAGATTGAAGCCGTCTTACCCG   449

IND40_00 392 ------------------------------------TTCCCGGCGTTAAA   405
                                                 |||||||||||||.
BAN_197  450 ACGTCAACGGGTTGTGACCACACGCTTGTACCGCTTTTCCCGGCGTTAAT   499
```

Figure 3.5.2a Comparison of 5' UTR between Local strain and Reference sequence (NC_004004) for serotype O (A+B) and Local strain and Vaccine strain (HM854025) for serotype A (C)

The result showed that the IRES element of the FMDV strain was about 557 nt in length and had five domains **(Figure 3.5.2b)**, which participated in the viral protein translation in a cap independent manner. The ‘AAACA’ motif in the domain 1 or cre/bus stem loop just upstream of the IRES was found conserved for serotype O except native serotype A where it was AAGCA. Domain 2 to domain 5 containing four direct repeat motifs, GGTGACA, was located in IRES region.

The GNRA tetra loop was a thermo stable tetra loop which can exist within a RNA structure solely on its own, or take place in an interaction with a receptor. The ‘GNRA’ tetraloop in domain 3 was found to be ‘GTGA’ in the native FMDV type O strain and in native type A strain it was GTAA. The cleavage site for RNase P within the ‘GNRA’ stem-loop was found as ‘TCC’ motif. The ‘C’-rich loop, ACCCC, in domain 3 of FMDV was found conserved in both native serotype O and A. The conserved ‘motif A’ was found to be GCACA in serotype O and ‘GCACGA’ in serotype A in this analysis. On the other hand, motif B was found as AGCT and AACT in serotype O and A respectively. The eIF4G binding domain GCTAA, and the eIF4B interaction domain ACCGGAGG was shown to be conserved in both serotype O and A. Out of the three poly pyrimidine tract (Py tract) binding protein (PTB) binding sites mapped on FMDV IRES, the seven-nt long Py tract in the domain 2 loop (TCTTTCC), in the domain 4 loop (CTTCTTT) and the ten-nt long Py tract (CCTTTTCTTT) at the end of domain 5 just upstream of the initiation codon revealed conserved for serotype O and A respectively where the consensus common motif was found as CTTT.

```
1                    ←-----------PK 1---------------→                        ◄
CCCCCCTAAGTTTACCGTCGTTCCCGACGTTAAAGGGATGTAACCACAAACTTGGAAC

59----------PK II-----------------→                    ←----------PK III---------
CGTCTTGCCCGACGTTAATGGGTTGTAACCACACGCTTGTACCGCCTTTCCCCGCGTT

117----→                          ←-----------PK IV---------------→
AAAGGGAAGTAACCACAAGATAAACCTTCGCCCGGAAGTAAAACGGCAACCACACTC

174                                                              ←------------
AGTTTTGTCCGTTTTCATGAGAAACGGGACGTCTGCGCACGAAACGCGCCGTCGCTTG|

                  cre/bus
232---------------Domain 1-------------------------------------------→      ←----
AGGAAGACTTGTACAAACACGATCTATGCAGGTTTCCACAACTGACACAAACCGTG

                              Py Tract
288-------------------------------Domain 2--------------------------------→  ←--
CAACTTGAAACTCCGCCTGGTCTTTCCAGGTCTAGAGGGGTGACACTTTGTACTGTG

345-----------------------------------------Domain 3------------------------------------------
TTTGACTCCACGCTCGGCCCACTGGCGAGTGTTAGTAACAGCACTGTTGCTTCGTA

                                                GNRA
401--------------------------------------------------------------------------------------
GCGGAGCATGATGGCCGCGGGAATTCCCCCTTGGTGACAAGGACCCGCGGGGCCG

RAAA                                        'C-rich ACCC'  'motif A'   'motif B'

456--------------------------------------------------------------------------------------
AAAGCCACGTCCTAACGGACCCATCATGTGTGCAACCCCAGCACAGCAGCTTTACT

                                                                       eIF4G binding
512------------------------------------------------------------------→      ←--------
GTGAAAGCCACTTTAAGGTGACACTGATACTGGTACTCAAGCACTGGTGACAGGCTA

569-------------------------------------------------------------Domain 4-----------------------
AGGATGCCCTTCAGGTACCCCGAGGTAACAAGTGACACTCGGGATCTGAGAAGGGG

               Py Tract                        A-bulge
625-------------------------------------------------------------------→      ←---------
ACAGGGGCTTCTTTAAAAGCGCCCTGTTTAAAAAGCTTCTATGCCTGGATAGGCGA

eIF4B interaction         Py Tract
681----Domain 5-------→                                       720
CCGGAGGCCGGCGCTTTTCCATTAAACTACTACTGACTTG
```

Figure 3.5.2b Architecture of the large fragment-5'UTR of FMDV transcriptional control region. Boundaries of different domains have been marked with dashed lines and arrows above the sequence. Conserved critical motifs were depicted in bold faced letter and underlined. eIF4G binding domain was GCTAA, and eIF4B interaction domain was ACCGGAGG.

3.5.3 Full Capsid Sequence Analysis

Except VP4 region (as the deduced amino acid residues of VP4 do not contribute to the virus antigenicity), an unbiased analysis of the capsid sequence (VP1-3) of the selected FMDV type O (KF985189) and type A (KJ754939) isolates generated in this study with their vaccine strains (AF204276 for serotype O and HM834025 for serotype A) revealed 12.17% (237/1947) and 10.32% (201/1947) nucleotide substitutions distributed across the region respectively. For serotype O, 83.12% (197) of total nucleotide substitutions were found to be synonymous (silent) and 16.88% (40) were non-synonymous (non-silent) that involved in various positions for codon formation and encoded thirty different amino acids. At the position of nucleotide 286-288 of VP1 region, all the three bases of the codon were mutated for encoding a different amino acid **(Table 3.5.3a).** The non-synonymous nt substitutions were not equally distributed across the capsid coding regions: there were eight substitutions present in VP2 and sixteen each for VP3 and VP1 respectively **(Table 3.5.3a).**

The analysis of the capsid amino acid residues of type O local virus revealed eight substitutions at VP2 and eleven each for VP3 and VP1 respectively. Compared to the vaccine serotype, critical amino acid substitutions (D138E, S140A and I114V) were determined in the VP1 G-H loop (residues 136~154), which are responsible for antigenic heterogeneity. In contrast, the RGDLXXL motif within VP1, involved in receptor binding (Fox *et al.*, 1989, Jackson *et al.* 1997), was found conserved and contained RGDLQVL motif.

Table 3.5.3a Full capsid sequence analysis of FMDV type O (KF985189)

Accession number	Amino acid position of VP2								Amino acid position of VP3						
	23	70	74	93	134	154	191	207	25	56	60	73	86	96	174
AF204276	T	A	S	S	N	V	T	N	V	R	G	V	I	H	T
KF985189	I	V	P	G	K	M	N	S	A	H	D	T	M	Q	A
	Non-synonymous (non-silent) nucleotide (nt) of VP2 (red)								Non-synonymous (non-silent) nt. of VP3 (red)						
AF204276	ACC	GCC	TCA	AGC	AAC	CTG	ACT	AAC	GTT	CGC	GGC	GTG	ATT	CAC	ACC
KF985189	ATC	GTC	CCA	GGC	AAG	ATG	AAT	AGC	GCC	CAC	GAC	ACG	ATG	CAG	GCT
					Amino acid position of VP1										
	195	201	219	220	13	82	96	110	123	138	140	144	154	197	198
AF204276	E	V	R	D	A	Y	N	A	Q	D	S	I	A	N	E
KF985189	D	I	T	Q	T	H	A	E	H	E	A	V	T	S	Q
					Non-synonymous (non-silent) nt. of VP1 (red)										
AF204276	GAA	GTG	AGA	GAC	GCC	TAC	AAC	GCA	CAA	GAT	TCG	ATA	GCG	AAC	CAG
KF985189	GAC	ATT	ACA	CAG	ACC	CAC	GCG	GAA	CAC	GAA	GCT	GTG	ACA	AGC	CAA

For serotype A, 70.65% (142) and 29.35% (59) of total nucleotide substitutions were found to be synonymous (silent) and non-synonymous (non-silent) respectively that involved in various positions for codon formation and encoded 37 different amino acids. At the position of nucleotide 193-195 of VP2 region, 247-249 and 508-510 of VP1 region, all the three bases of the codon were mutated for encoding three different amino acids (H~F, S~E and D~T, **Table 3.5.3b**). There were eighteen, thirteen and 27 non-synonymous nt substitutions found in VP2, VP3 and VP1 regions respectively (**Table 3.5.3b**).

Compared to vaccine strain (HM854025), the analysis of the capsid amino acid residues revealed eight, ten and fifteen substitutions at VP2, VP3 and VP1 regions respectively. Specifically, substitutions of four amino acids (T44N, T45A, N46S and T48I) in the VP1 B-C loop (residues 40 to 60) and two amino acids (T143V and I154V) in the VP1 G-H loop (residues 138 to 154) indicate antigenic heterogeneity. An amino acid deletion within the VP3 60 position was also observed for local FMDV type A isolate (**Table 3.5.3b)**.

Table 3.5.3b Full capsid sequence analysis of FMDV type A (KJ754939)

Accession Number	Amino acid position of VP2												Amino acid position of VP3						
	65	71	72	74	98	134	154	189	190	191	195	207	54	59	60	65	67	69	92
HM854025	H	A	D	A	F	T	T	N	A	G	T	Y	F	N	G	E	R	D	S
KJ754939	F	T	E	P	Y	P	M	T	T	S	P	H	L	D	-	V	N	G	A
	Non-synonymous (non-silent) nucleotide (nt.) of VP2 (red)												Non-synonymous (non-silent) nt. of VP3 (red)						
HM854025	CAC	GCG	GAC	GCA	TTT	ACG	ACG	AAT	GCC	CCG	ACT	TAC	TTC	AAC	GGG	GAG	AGA	GAT	TCA
KJ754939	TTT	ACG	GAG	CCA	TAC	CCG	ATG	ACC	ACC	CCA	CCC	CAT	CTT	GAC	- - -	GTG	AAT	GGT	GCA
				Amino acid position of VP1															
	94	139	204	3	4	33	35	44	45	46	48	83	143	154	170	171	190	196	
HM854025	I	K	A	T	A	S	I	T	T	N	T	S	T	I	D	A	L	S	
KJ754939	L	R	V	S	T	G	V	N	A	S	I	E	V	V	T	T	M	L	
				Non-synonymous (non-silent) nt. of VP1 (red)															
HM854025	ATA	AAA	GCC	ACG	GCC	AGC	ATA	ACC	ACT	AAC	ACA	AGC	ACA	ATT	GAC	GCC	CTG	TCG	
KJ754939	TTA	CGA	GTT	TCT	ACC	GGA	GTA	AAT	GCC	AGT	ATA	GAA	GTA	GTC	ACT	ACC	ATG	TTG	

3.5.4 NSP Sequence Analysis

3.5.4.1. Amino acid variation in the NSP

The nucleotide sequences for the non-structural protein-coding regions were translated and the deduced amino acid sequences aligned. A summary of the variability in the non-structural proteins for the local serotype O (KF985189) and serotype A (KF754939) along with 19 closely related (according to the BLAST search) sequences (Type O: KJ825802, KJ206908, KJ206909, KJ206910, KU291242,KM268895, HQ268524, HQ632770, AJ539138; Type A:KU127247, HQ832579, HQ832582, HQ832583, HQ832586, HQ832590, HQ832591, HQ832592, HM854025, KJ608371) and one reference sequence (RefSeq: NC_004004) of Asia continent is summarized in **Table 3.5.4.1** and discussed subsequently.

Table 3.5.4.1 Variability in the non-structural proteins of serotype O and serotype A with their related sequences.

Region in genome	No. of nucleotide positions aligned	No. of variant nucleotides	% of variant nucleotides	No. of residue positions aligned	No. of variant residues	% of variant residues
L^{pro}	603	208	**34.5**	201	48	**23.9**
2A	54	14	25.9	18	0	0
2B	462	121	26.2	154	23	14.9
2C	954	267	28.0	318	37	11.6
3A	459	182	**39.7**	153	55	**35.9**
$3B_{123}$	213	64	30.0	71	15	21.1
$3C^{pro}$	639	159	24.9	213	18	8.5
$3D^{pol}$	1410	385	27.3	470	60	12.8

3.5.4.2 Leader Protease (L^{pro})

The L^{pro}, 201 amino acids residues in length, was the second most variable of the non-structural proteins with 23.9% variable amino acid positions in a complete alignment of the local isolates of FMDV type O and A with the nineteen closely related and one reference sequences of Asia continent **(sub-section 3.5.4.1).** The critical residues used for autocatalysis of the L-VP_4, namely C51, H148 and D164 (Guarne *et al.*, 1998), were conserved in all the isolates. According to the numbering in this study **(Figure 3.5.4.2a)**, the catalytic triad is presented as C51, H148 and D163. Among the non-structural protein most of the variation was found in a hyper variable domain of the N-terminal half of the protein residues 12-28 **(Figure 3.5.4.2a and 3.5.4.2b).**

```
         1                           30 51        60 141      150 161      170
KF985189 MNTTDCFIAL LQALREVKAL FLSRTQGKME CWLNTILQLF IFLKGQEHAV AIDDEDFYPW
KJ754939 .......... .H....I.T. ..P..R.E.. .......... .......... ..........
KU127247 .......... .H....I... ..P..R.... .......... .......... ..........
HQ832590 .......... .H....I.T. .....R.... .......... .......... ..........
HQ832591 .......... .H....I.T. .....R.... .......... .......... ..........
HQ832592 .......... .R....I.T. .....R.... .......... .......... ..........
HQ832583 .......... .Y....I.T. ...K...... .......... ..M....... ..........
HQ832579 .......V.. .Y....I.T. ...K...... .......... ..M....... ..........
HQ832586 .S........ .Y.F..I... ....A..... .......... .......... ..........
HQ832582 .......... .H....I.T. ...K.R.... .......... ..M....... ..........
HM854025 .......... .Y.I..I.T. .R.....E.. .......... .......... ..........
KJ608371 .......... .H.I..I..R LF.K..E... .......... .......... ..........
KJ825802 .......... .H....F.T. .....R.... .......... .......... ..........
KJ206908 .......... .H....F.T. ....KR.... ....A..... .......... ..........
KJ206909 .......... .H....F.T. .....R.... .......... .......... ..........
KJ206910 .......... .H....F.T. .....R.... .......... .......... ..........
KU291242 ..A....... .H....F.T. .....R.... .......... .......... ..........
KM268895 .......... .H....I.T. .RT..R.... .......... .......... ..........
HQ268524 .......... .H....I.T. .RT....... .......... .......... ..........
HQ632770 .......... .H....I.T. .RT....... .......... .......... ..........
AJ539138 .S........ .Y.F..I.T. ....A..... .......... .......... ..........
NC004004 .......... .Y....I... .......... ....A..... ......D... ..........
```

Figure 3.5.4.2a Multiple sequence alignment of L^{pro} with MEGA 5.2 software was performed usingstudy sequences (n=1-2) and representative sequences (n=3-22) for each of FMDV serotypes collected from NCBI database. The sequences used were from serotypes O and A with the respective GenBank accession numbers.

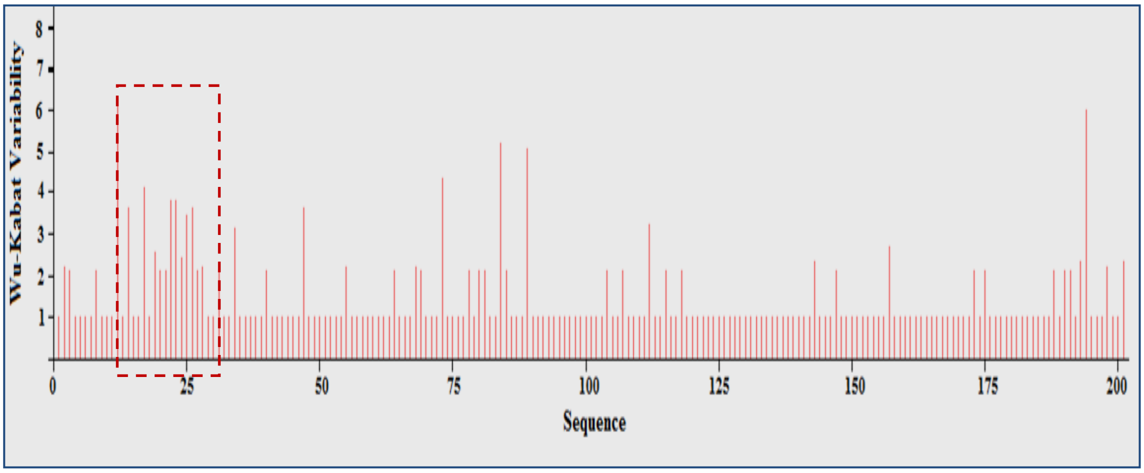


Figure 3.5.4.2b Protein variability plot of Leader protease. In the picture the highest variation is delineated in the N-terminal half of the protein residues 12-28.

3.5.4.3 Non-Structural Protein 2A

The 2A peptide of 18 amino acids in length was the most conserved of the three FMDV encoded proteases. All of the residues in a complete alignment of the 2A sequences of 22 FMDV type O and A viruses included in this study were found identical.

3.5.4.4 Non-Structural Protein 2B

The 2B protein (154 amino acids) contained no insertions or deletions and showed 85.1% conserved residues in a complete alignment of all FMDV sequences included in this study. Most of the variation was found in a hyper variable domain of the N-terminal half of the protein residues 5-23, while the sequence ^{69}RLSCMAAVAARSKD PVLVAIMLADTGL EIL^{98} was highly conserved among all the 22 FMDV sequences. Another conserved motif, located between residues 115 and 137 [FHVPAP(V/A) FSFG(A/V)PILLAG(L/F)VKVA] contain a hydrophobic domain**(Figure 3.5.4.4)**.

	69			98	115		137
KF985189	RLSC	MAAVAARSKD	PVLVAIMLAD	TGLEIL	FHVPAPVF	SFGAPILLAG	LVKVA
KJ754939	....	....	....	......	....	....	.....
KU127247	....	....	....	......	....	....	.....
HQ832590	....	....	....	......	....	....	.....
HQ832591	....	....	....	......	....	....	.....
HQ832592	....	....	....	......	....	....	.....
HQ832583	....	....	....	......	....	....	.....
HQ832579	....	....	....	......	....	....	.....
HQ832586	....	....	....	......	....	....	F....
HQ832582	....	....	....	......	....	....	.....
HM854025	....	....	....	......	....	....	.....
KJ608371	....	....	....	......	......A.	....	.....
KJ825802	....	....	....	......	....	....	.....
KJ206908	....	....	....	......	....	....	.....
KJ206909	....	....	....	......	....	....	.....
KJ206910	....	....	....	......	....	....	.....
KU291242	....	....	....	......	....	....	.....
KM268895	....	....	....	......	....	....	.....
HQ268524	....	....	....	......	....	....	...A.
HQ632770	....	....	....	......	....	...V......	.....
AJ539138	....	....	....	......	....	....	.....
NC004004	....	....	....	......	......A.	....	.....

Figure 3.5.4.4 Multiple sequence alignment NSP-2B with MEGA 5.2 software was performed using studied sequences (n=1-2) and representative sequences (n=3-22) for each of FMDV serotypes collected from NCBI database. The sequences used were from serotypes O and A with the respective GenBank accession numbers.

3.5.4.5 Non-Structural Protein 2C

The FMDV 2C protein is an AAA+ ATPase with RNA binding activity (Sweeney *et al.*, 2010) and was found 318 amino acids in length; mostly hydrophilic towards the C-terminus and contained 88.4% invariant residue positions when sequences of isolates in this study were compared. A highly conserved, hydrophobic motif was present between residues 17-34, i.e. ^{17}EWLVKLILAIRDWIKAWI^{34}. An exception is the 2C protein of IND142 (311)/2013 (n=13) that contained an I30V amino acid substitution. This aliphatic helix most probably involved in the attachment of 2C to the membrane (Echeverri and Dasgupta, 1995). Conserved residues at positions 110-117 (GKSGQGKS), 156-161 (VVVMDD) and 201-207 (VIIATTN) included in the characteristic Walker A, Walker B and C motifs of an AAA+ ATPase (Sweeney *et al.*, 2010). However, the Walker A motif for representative serotype A virus displayed a conservative K111R (n=3) substitution was observed **(Figure 3.5.4.5)**.

	17		34	110 117	156	161	201 207
KF985189	EW	LVKLILAIRD	WIKAWI	GKSGQGKS	VVV	MDD	VIIATTN
KJ754939	..	....	......	....	...	...	....
KU127247	..	....	......	.R......	...	...	....
HQ832590	..	....	......	....	...	...	....
HQ832591	..	....	......	....	...	...	....
HQ832592	..	....	......	....	...	...	....
HQ832583	..	....	......	....	...	...	....
HQ832579	..	....	......	....	...	...	....
HQ832586	..	....	......	....	...	...	....
HQ832582	..	....	......	....	...	...	....
HM854025	..	....	......	....	...	...	....
KJ608371	..	....	......	....	...	...	....
KJ825802	..	....	.V....	....	...	...	....
KJ206908	..	....	......	....	...	...	....
KJ206909	..	....	......	....	...	...	....
KJ206910	..	....	......	....	...	...	....
KU291242	..	....	......	....	...	...	....
KM268895	..	....	......	....	...	...	....
HQ268524	..	....	......	....	...	...	....
HQ632770	..	....	......	....	...	...	....
AJ539138	..	....	......	....	...	...	....
NC004004	..	....	......	....	...	...	....

Figure 3.5.4.5 Multiple sequence alignment of NSP-2C with MEGA 5.2 software was performed using studied sequences (n=1-2) and representative sequences (n=3-22) for each of FMDV serotypes collected from NCBI database. The sequences used were from serotypes O and A with the respective GenBank accession numbers.

3.5.4.6 Non-Structural Protein 3A

To determine the genetic heterogeneity of the 3A non-structural-protein-coding region, representatives of the twenty serotypes occurring on the Asia continent **(Sub section 3.5.4.1)** were selected and compared to Bangladesh isolates of FMDV type O and A. Comparative analysis of these sequences demonstrated that none of the 21 isolates of which the nucleotide sequences were determined in this study contained any insertions. However, ten amino acid deletions were observed in RefSeq 3A proteins, located 92-101 amino acids within the carboxyl terminus of the sequence **(Figure 3.5.4.6b)**.The nucleotide sequence variation calculated for all isolates amounted to 29.7%, while the amino acid variation was calculated as 35.9%. The amino acid sequence was found highly conserved within the N-terminus region of the protein. The most variable regions of the 3A protein for all the isolates were found to be located between residues 127 and 151. The N-terminal 41 amino acids were relatively conserved and contained two hydrophobic domains, i.e. ^{1}ISIPSQKSVLYFLIEK^{16} and ^{25}FYEGMV^{30}, while a third hydrophobic domains was located between residues 60 to 74 (^{60} EIVALCLTLLANIVI^{74}) **[Figure 3.5.4.6a]**.

```
             1               16 25   30  60            74  127                        151
KF985189 ISIPSQKSVL YFLIEK FFEGMV E IVALCLTLLA NIVI RTLP GHKVSDDVNS EPTEPVEEQP Q
KJ754939 .......... ...... ...... . .......... .... .... .Q.A..E... ..AK.T.... .
KU127247 .......... ...... ...... . .......... .... .... .Q.A...... ..AK.AG..S .
HQ832590 .......... ...... ...... . .......... .... .... .......M.. ...K...... .
HQ832591 .......... ...... ...... . .......... .... .... .......... ...K...... .
HQ832592 .......... ...... ...... . .......... .... .... .Q........ .....AGD.. .
HQ832583 .......... ...... ...... . .......... .... .... .Q........ ..AK...... .
HQ832579 .......... ...... ...... . .......... .... .... .Q........ ..AK...... .
HQ832586 .......... ...... ...... . .......... .... .... R........T ...K...... .
HQ832582 .......... ...... ...... . .......... .... .... .Q........ ..AK...... .
HM854025 .......... ...... ...... . .......... .... .... .Q..G...K. .....AR... .
KJ608371 .......... ...... ...... . .......... .... .S.. ..A....... ...K.A.... .
KJ825802 .......... ...... ...... . .......... .... .... .Q........ ...K...... .
KJ206908 .......... ...... ...... . .......... .... .... .Q........ ...K..G... R
KJ206909 .......... ...... ...... . .......... .... K... .Q........ ...K...... .
KJ206910 .......... ...... ...... . .......... .... .... .Q........ ...K....R. .
KU291242 .......... ...... ...... . .......... .... K... .Q..G..... ..?R...D.. .
KM268895 .......... ...... ...... . .......... .... .... .......... .......... .
HQ268524 .......... ...... ...... . .......... .... K... .......... ...K...... .
HQ632770 .......... ...... ...... . .......... .... .... .......... ...K...... .
AJ539138 .......A.. ...... ...... . .......... .... K... ...A...... ..AK...... .
NC004004 .......... ...... ...... . V......... .... .SPT EQGTRE.A.A ..VVFGR... R
```

Figure 3.5.4.6a Multiple sequence alignment NSP-3A with MEGA 5.2 software was performed using studied sequences (n=1-2) and representative sequences (n=3-22) for each of FMDV serotypes collected from NCBI database. The sequences used were from serotypes O and A with the respective GenBank accession numbers.

```
RefSeq_3A     1 ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKR  50
                ||||||||||||||||||||||||||||||||||||||||||||||||||
BAN_156_3A    1 ISIPSQKSVLYFLIEKGQHEAAIEFFEGMVHDSIKEELRPLIQQTSFVKR  50

RefSeq_3A    51 AFKRLKENFEVVALCLTLLANIVIMLRQARKRYQSVDDPLD---------  91
                ||||||||||:||||||||||||||:|:.|||.|.|||.::
BAN_156_3A   51 AFKRLKENFEIVALCLTLLANIVIMIRETRKRQQMVDDAVNEYIEKANIT 100

RefSeq_3A    92 -GDVTLGDAEKNPLETSGASAVGFRERSPTEQGTREDANAEPVVFGREQP 140
                 .|.||.:||||||||||||.||||||:.......:|.|:||.....|||
BAN_156_3A  101 TDDKTLDEAEKNPLETSGASTVGFRERTLPGHKVSDDVNSEPTEPVEEQP 150

RefSeq_3A   141 RAE 143
                :||
BAN_156_3A  151 QAE 153
```

Figure 3.5.4.6b Comparison of 3A protein region between Reference sequence and Local strain for serotype O. In the figure, RefSeq is NCBI Reference Sequence (NC_004004) and BAN_156_3A is the local isolate (KF985189).

3.5.4.7 Non-Structural Protein 3B

The three copies of 3B varied in length between 23 ($3B_1$) to 24 ($3B_2$ and $3B_3$) residues demonstrating 21.1% overall variability, whilst each copy varied by 34.7%, 16.6% and 12.5% respectively. The N-terminal motif, GPYXGP (where X is any amino acid), was conserved for all the 22 serotypes included in this study **(Figure 3.5.4.7).**

```
            1                                                                          71
KF985189 GPYAGPL ERQKPLKVRA KLPQQEGPYA GPMERQKPLK VKAKAPVVKE GPYEGPVKKP VALKVKAKNL IVT
KJ754939 ....... .......... .......... .......... .......... .......... .......... ...
KU127247 ....... .........T .......... .......... .......... .......... .......... ...
HQ832590 ....... .......... .......... .......... .......... .......... .......R.. ...
HQ832591 ....... .........V .......... .......... .......... .......... .......... ...
HQ832592 ...T... .......... .......... ..L....... .......... .......... .......... ...
HQ832583 ....... .......... .......... .......... ..V....... .......... ........T. ...
HQ832579 ....... .......... .......... .......... .......... .......... .......... ...
HQ832586 ....... .........T .......... .......... .......... .......... .......... ...
HQ832582 ....... .......... .......... .......... .R........ .......... A......... ...
HM854025 ....... .......... .......... .......... .......... .......... .......... ...
KJ608371 ....... ...R....K. .......... .......... .......... .......... .......... ...
KJ825802 ....... ......R... T......... .......... .......... .......... .......... ...
KJ206908 ....... ..R....... .......... .......... ..V....... .......... .......... ...
KJ206909 ....... .H........ .......... .......... .......... .......... .......... ...
KJ206910 ....... .......... .......... .......... .......... .......... .......... ...
KU291242 ....... .H........ .......... .......... ....V..... .......... .......... ...
KM268895 ....... .......... .......... .......... .......... .......... .......... ...
HQ268524 ....... .......... .......... .......... .......... .......... .......... ...
HQ632770 ....... .......... .......... .......... .......... .......... .......... ...
AJ539138 ...T... .......... .......... .......... ..V....... .......... .......... ...
NC004004 ....... ........K. E......... .......... .......... .......... .......... ...
```

Figure 3.5.4.7 Multiple sequence alignment NSP-3B with MEGA 5.2 software was performed using studied sequences (n=1-2) and representative sequences (n=3-22) for each of FMDV serotypes collected from NCBI database. The sequences used were from serotypes O and A with the respective GenBank accession numbers.

3.5.4.8 Non-Structural Protein 3C ^{pro}

The 3C ^{pro} coding region translated into 213 amino acids with 8.5% variable positions in an overall alignment. The variation was not random, but defined in hyper-variable regions separated by highly conserved residues. The conserved residues point towards the significant contribution of these residues to structural and/or non structural constraints. The N-terminal 60 amino acids of 3C ^{pro}, especially 12-48 (^{12}VMGNTKPVELILDGKTVAICCATGVFGTAYLVPRHLF^{48}) were found conserved and constituted a hydrophobic domain ^{27}TVAICCATGVFGTAYLVP^{44}. Other conserved domains included ^{66}DYRVFEFEIKVKGQDMLSDAALMVLH^{91}, ^{129}FSGEALTYKDIV VCMDGDTMPGLFAY(K/R)A^{156} and ^{161}GYCG^{164}. The active triad of 3C ^{pro}, consisting of H46, D84 and C163 (Birtley *et al.*, 2005), showed complete conservation **(Figure 3.5.4.8)**.

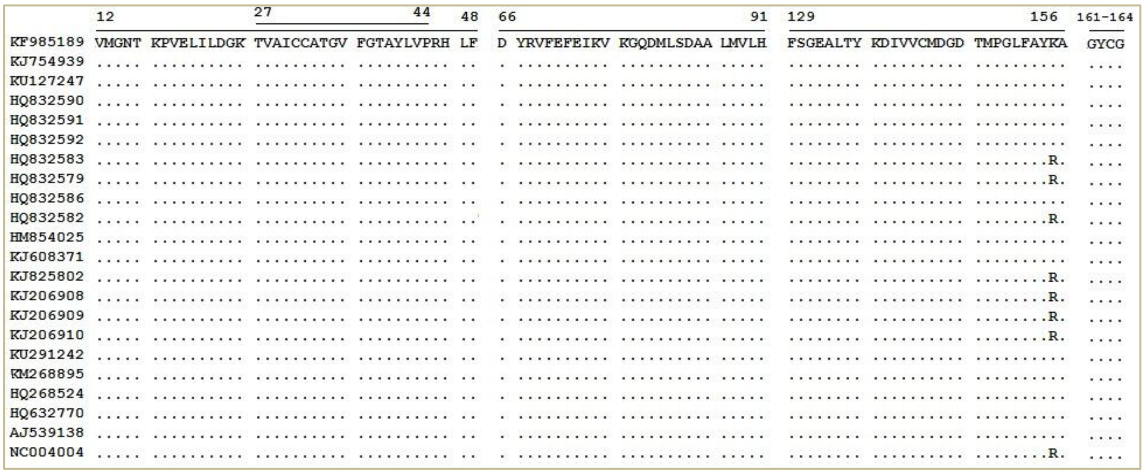


Figure 3.5.4.8 Multiple sequence alignment NSP-3C pro with MEGA 5.2 software was performed using studied sequences (n=1-2) and representative sequences (n=3-22) for each of FMDV serotypes collected from NCBI database. The sequences used were from serotypes O and A with the respective GenBank accession numbers.

3.5.4.9 Non-Structural Protein $3D^{pol}$

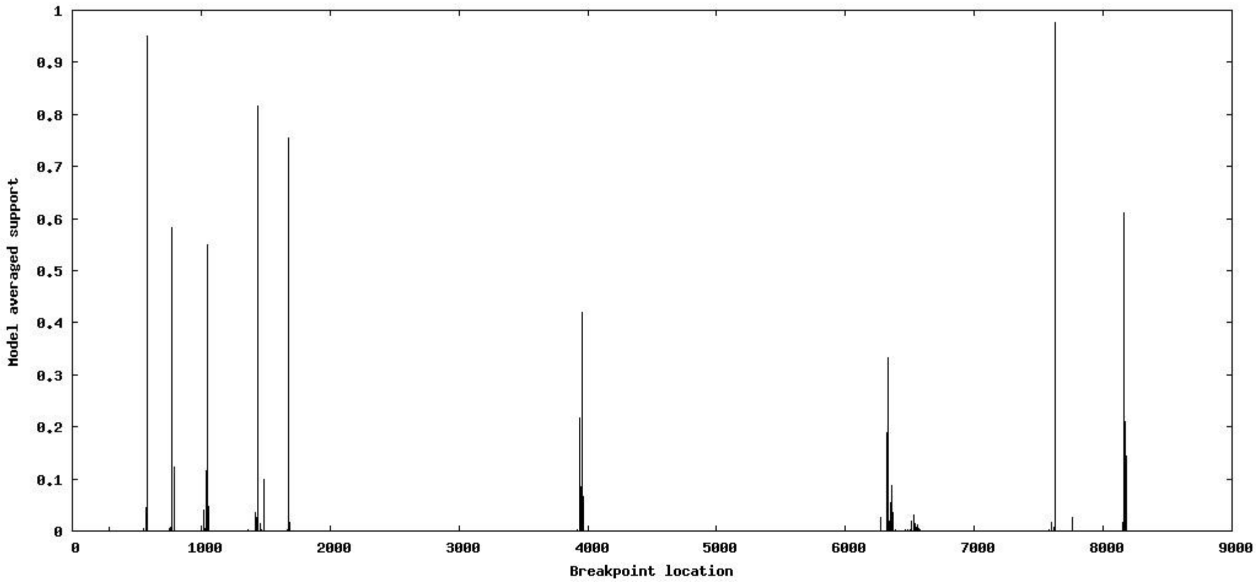
The $3D^{pol}$ (470 amino acid peptide), the longest of non-structural proteins, demonstrated 12.8% variable residues. The $3D^{pol}$ variation was not limited to certain areas as seen for $3C^{pro}$. Previously five conserved motifs were described for $3D^{pol}$ FMDV (Doherty *et al.*, 1999; Ferrer-Orta *et al.*, 2004). The ^{240}DYSAFD^{245}, ^{297}PSG^{299}, ^{336}YGDD^{339} and ^{385}FLKR^{388} motifs were conserved in all FMDV sequences considered in this study. However, the ^{164}KDELR^{168} motifs present in the O and A sequences either as KDEIR or KDEVR **(Figure 3.5.4.9)**.

	164 168	240 245	297 299	336 339	385 388
KF985189	KDEIR	DYSA FD	PSG	YGDD	FLKR
KJ754939	.....	.... ..	...	....	....
KU127247	.....	.... ..	...	....	....
HQ832590	.....	.... ..	...	....	....
HQ832591	.....	.... ..	...	....	....
HQ832592	.....	.... ..	...	....	....
HQ832583	.....	.... ..	...	....	....
HQ832579	.....	.... ..	...	....	....
HQ832586	.....	.... ..	...	....	....
HQ832582	.....	.... ..	...	....	....
HM854025	.....	.... ..	...	....	....
KJ608371	.....	.... ..	...	....	....
KJ825802	...V.	.... ..	...	....	....
KJ206908	.....	.... ..	...	....	....
KJ206909	.....	.... ..	...	....	....
KJ206910	.....	.... ..	...	....	....
KU291242	.....	.... ..	...	....	....
KM268895	.....	.... ..	...	....	....
HQ268524	.....	.... ..	...	....	....
HQ632770	.....	.... ..	...	....	....
AJ539138	.....	.... ..	...	....	....
NC004004	.....	.... ..	...	....	....

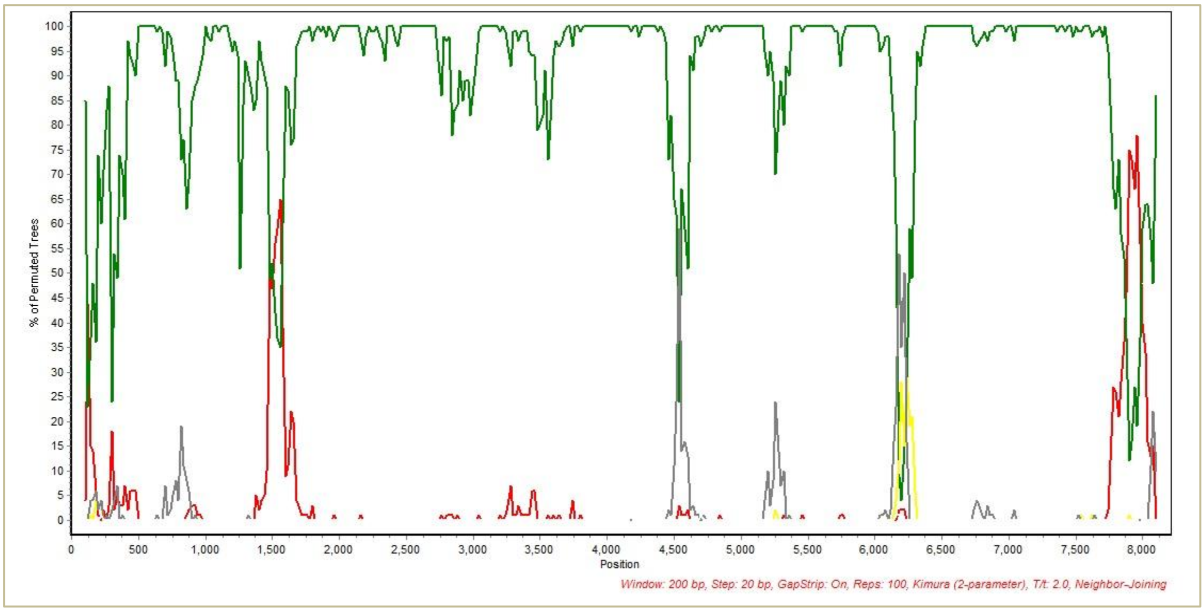
Figure 3.5.4.9 Multiple sequence alignment NSP-3D^{pol} with MEGA 5.2 software was performed using studied sequences (n=1-2) and representative sequences (n=3-22) for each of FMDV serotypes collected from NCBI database. The sequences used were from serotypes O and A with the respective GenBank accession numbers.

3.6 Recombination Analysis for Evolutionary Genomics

The complete genome of local FMDV serotype O (BAN/NA/Ha-156/2013, BAN/GO/Ka-236/2015), A (BAN/GA/Sa-197/2013) and other related complete genome sequences including reference strain plus vaccine strains were checked for possible recombination breakpoints. Using GARD (Genetic Algorithm Recombination Detection), 9 breakpoints by comparing Akaike information criteria score of best fitting model (**Figure 3.6a**) were found. The result was also supported by boot-scan analysis using default parameters in **SimPlot software version 3.5.1**, which detected probable evidence of recombination when BAN/NA/Ha-156/2013 was used as query sequence (**Figure 3.6b**). By examining the points at which the similarities between query and reference sequences increased or decreased, we could tentatively identify recombination breakpoints.



a)



b)

Figure 3.6 a) Breakpoint graph generated in GARD b) Bootscan analysis result in SimPlot using BAN/NA/Ha-156/2013 as query sequence, BAN/GO/Ka-236/2015 (green), BAN/GA/Sa-197/2013 (red) as reference sequence and other stains plus vaccine strain (gray) as an out group.

3.7. Phylogeography of FMDV

To uncover the route of transmission, phylogeography history of FMDV type O (as it is the most prevalent strain) in Bangladesh was studied. From the evidence of study, it is found that the FMDV type O viruses circulating in India represent similar genetic lineages and according to the phylogeography figure, Bangladesh appear to experience a much greater exchange of same viruses across the northern borders through the trans-boundary livestock movements (a common feature in this area) **Figure 3.7.**

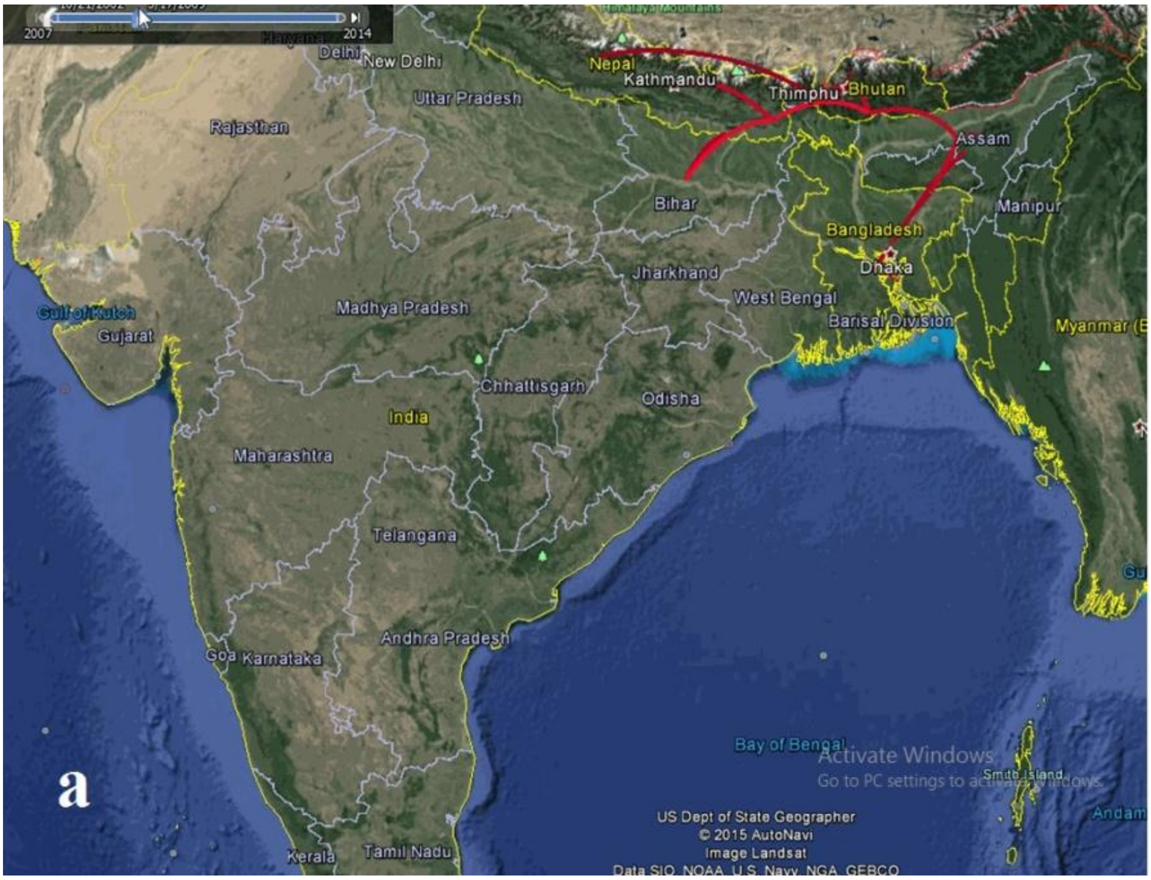


Figure 3.7 Route of Transmission of FMDV type O virus

3.8 Study of Structural Genomics

3.8.1 Secondary Structure of Pseudoknot

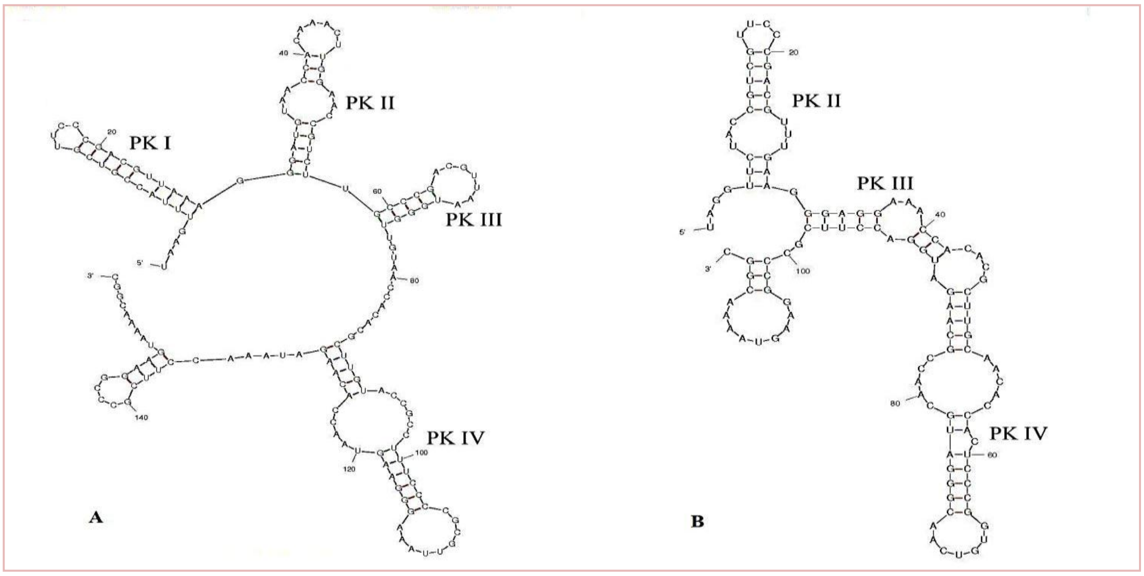


Figure 3.8.1 Secondary Structure of Pseudoknot of 5'UTR of BAN/NA/Ha-156/2013 (A) and NCBI RefSeq (B)

The free energy changes for the predicted secondary structure of Pseudoknot region of BAN/NA/Ha-156/2013 and NCBI RefSeq was -29.80Kcal/mol and -28.70Kcal/mol respectively. An extra Pseudoknot loop (PK I) is evident in the BAN/NA/Ha-156/2013 compared to NCBI RefSeq (**Figure 3.8.1A**).

3.8.2 Three Dimensional (3D) Structure of Leader Protease (Lb^{pro})

The PyMOL view of the complete **Leader Protease (Lb^{pro})** is delineated in **Figure 3.8.2** with different motifs.

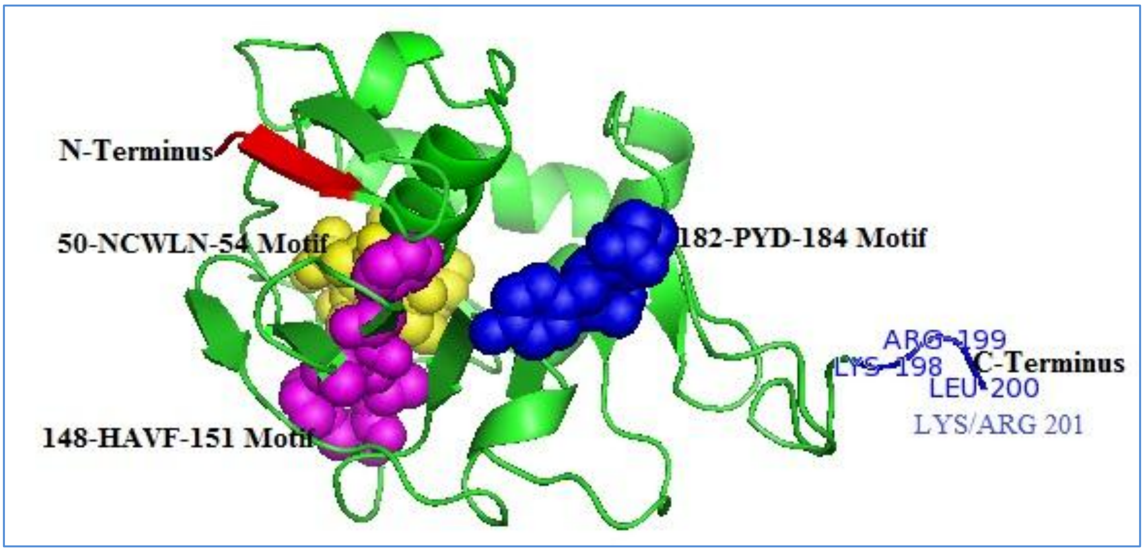


Figure 3.8.2 PyMoL view of of the three Dimensional (3D) Structure of FMDV Lb^{pro}

3.8.3 Prediction of the Secondary Structure of 3' UTR

The predicted secondary structure of the 3' UTR is given in **Figure 3.8.3**. The free energy of the thermodynamics ensemble is -37.22Kcal/mol. The frequency of the Minimum Free Energy (MFE) structure in the ensemble is 8.54% and ensemble diversity is 7.37 for serotype O. On the other hand, serotype A showed the free energyof the thermodynamics ensemble is -24.42Kcal/mol. The frequency of the Minimum Free Energy (MFE) structure in the ensemble is 3.2% and ensemble diversity is 22.18.

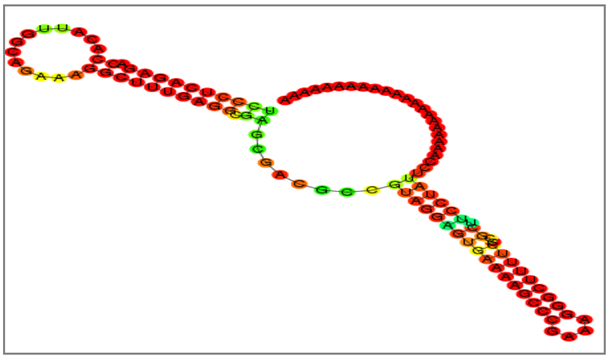


Figure 3.8.3 Secondary structure of the 3' UTR.

3.8.4 Three Dimensional (3D) Structure of VP1

The 3D structure of the VP1 region of BAN/NA/Ha-156/2013 is predicted in **Figure 3.8.4** the 3D structure of the VP1 is shown. The FMDV VP1 gene with the position of B-C loop, G-H loop and RGD motif is shown as grey, red and green spherical shape.

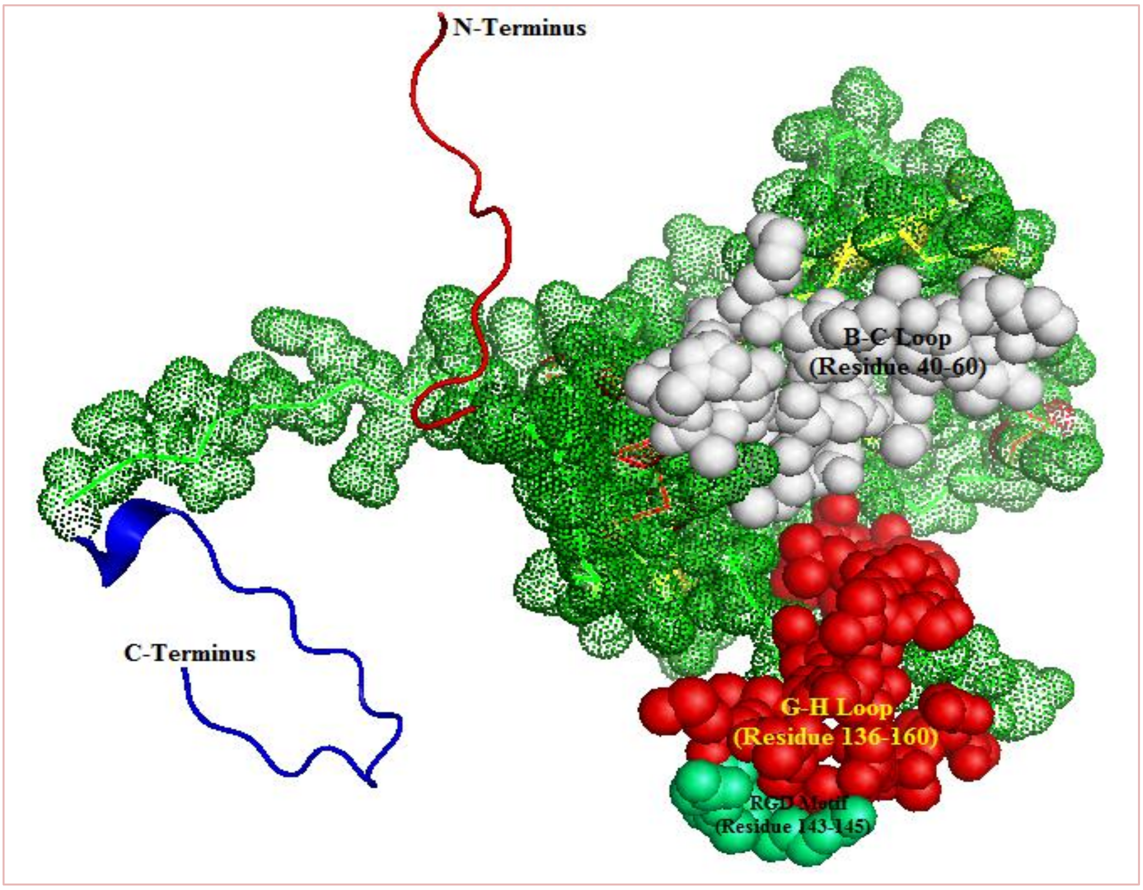


Figure 3.8.4 PyMoL view of the three Dimensional (3D) Structure of VP1

3.9 Selection of Vaccine candidate

3.9.1 Quantification of virus with $TCID_{50}$ titer calculation

The BHK-21 cell line was grown in each well of the 96 well plates and virus dilution followed by inoculation was done according to the protocol described in materials and methods section. $TCID_{50}$ titer was determined by adapting the method of Reed and Munch and it was calculated as $10^{8.5}$ per ml **(Table 3.9).** The end-point dilution assay was used to measure virus titer and also be used to determine the virulence of virus in animals as because less pathogenic and highly immunogenic candidate stains are suitable for vaccine preparation.

Table 3.9 $TCID_{50}$ Calculation

Serial no.	Dilution factor	Wells with CPE	Wells without CPE	Cumulative value		Proportion CPE/Total
				CPE	No CPE	
1	10^5	8	0	23	0	100
2	10^6	6	2	15	2	75
3	10^7	5	3	9	5	62.5
4	10^8	3	5	4	10	37.5
5	10^9	1	7	1	17	12.5

$$\text{Proportion distance (P.D)} = \frac{\text{CPE next above 50\%-50\%}}{\text{CPE next above 50\%-CPE next below 50\%}}$$

$$\text{Proportion distance (P.D)} = \frac{62\text{-}50}{62\text{-}37} = 0.5 \text{ approx.}$$

$$TCID_{50} = 10^{7.5} \text{ per 0.1ml}$$

$$\mathbf{TCID_{50} = 10^{8.5} \text{ per ml}}$$

3.9.2 Analyses of FMDV for the Selection of Candidate Vaccine Strain

A quality foot and mouth disease (FMD) vaccine is a prerequisite for effective control measure in disease endemic countries like Bangladesh. Based on geographic location and topotype/ subtype within the country a total of twenty nine VP1 amino acid sequences (twenty one for serotype O and eight for serotype A) were selected for sequence similarity matching test. In this study the local isolates from bovine origin BAN/NA/Ha-156/2013 and BAN/GA/Sa-197/2013 were selected as candidate vaccine strains for serotype O and A respectively. On alignment the selected representative field strains revealed high antigenic similarities with the proposed candidate vaccine strains tested (Figure 3.9.2 a-b) which suggest a close relationship between field strains and vaccine strains.

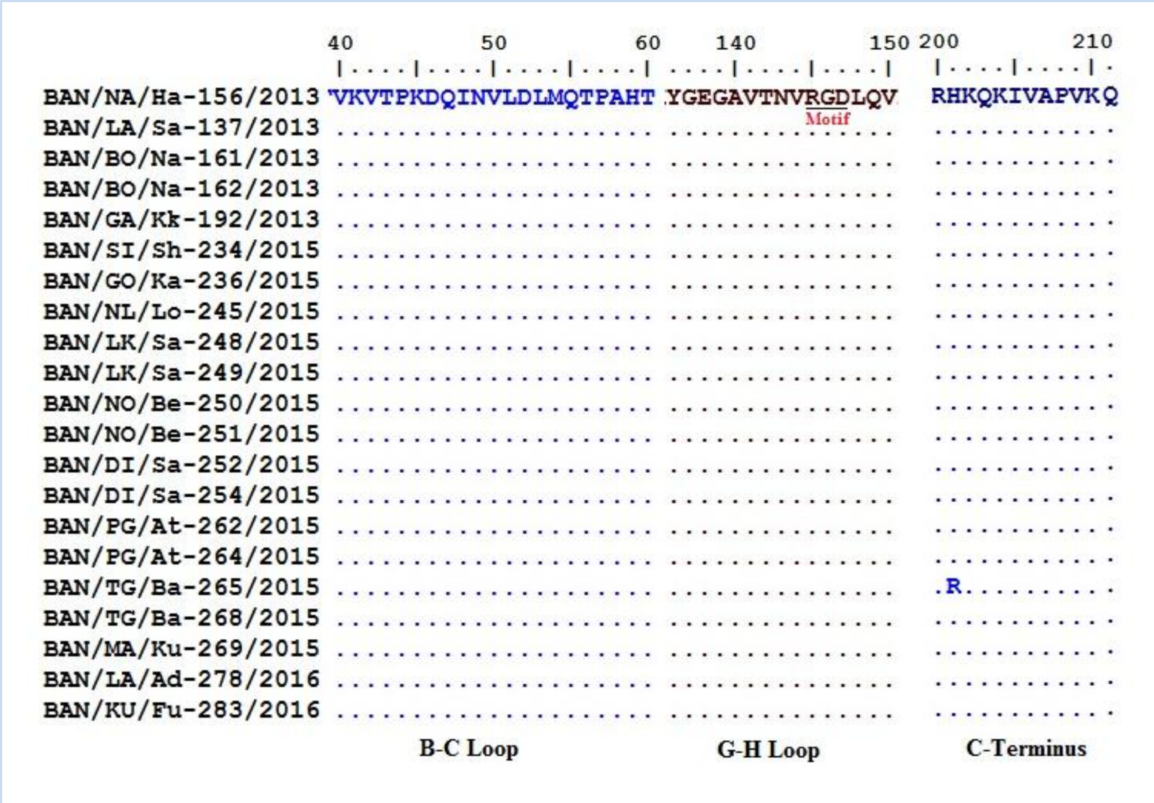


Figure 3.9.2a Comparison of three major antigenic sites of FMDV type O VP1 coding region of candidate vaccine strain and 20 local field strains.

Note: The number represents the amino acid position; the conserved residues are indicated by dots.

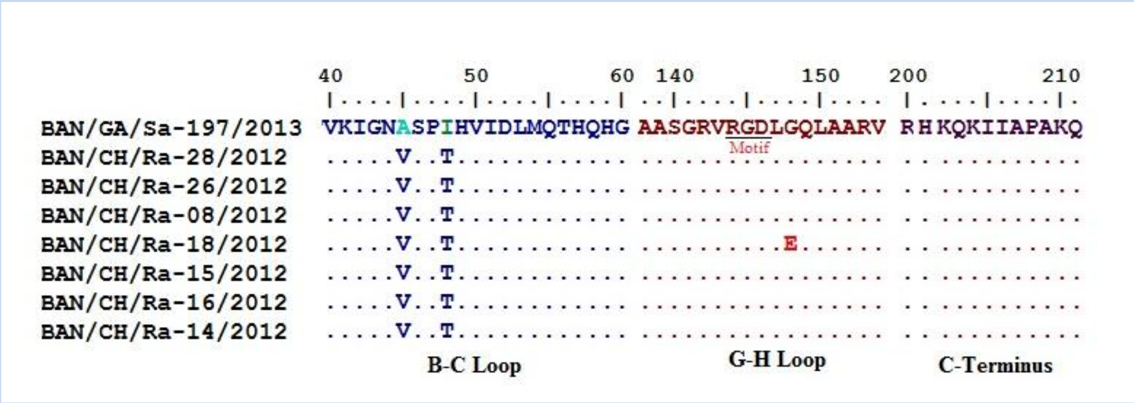
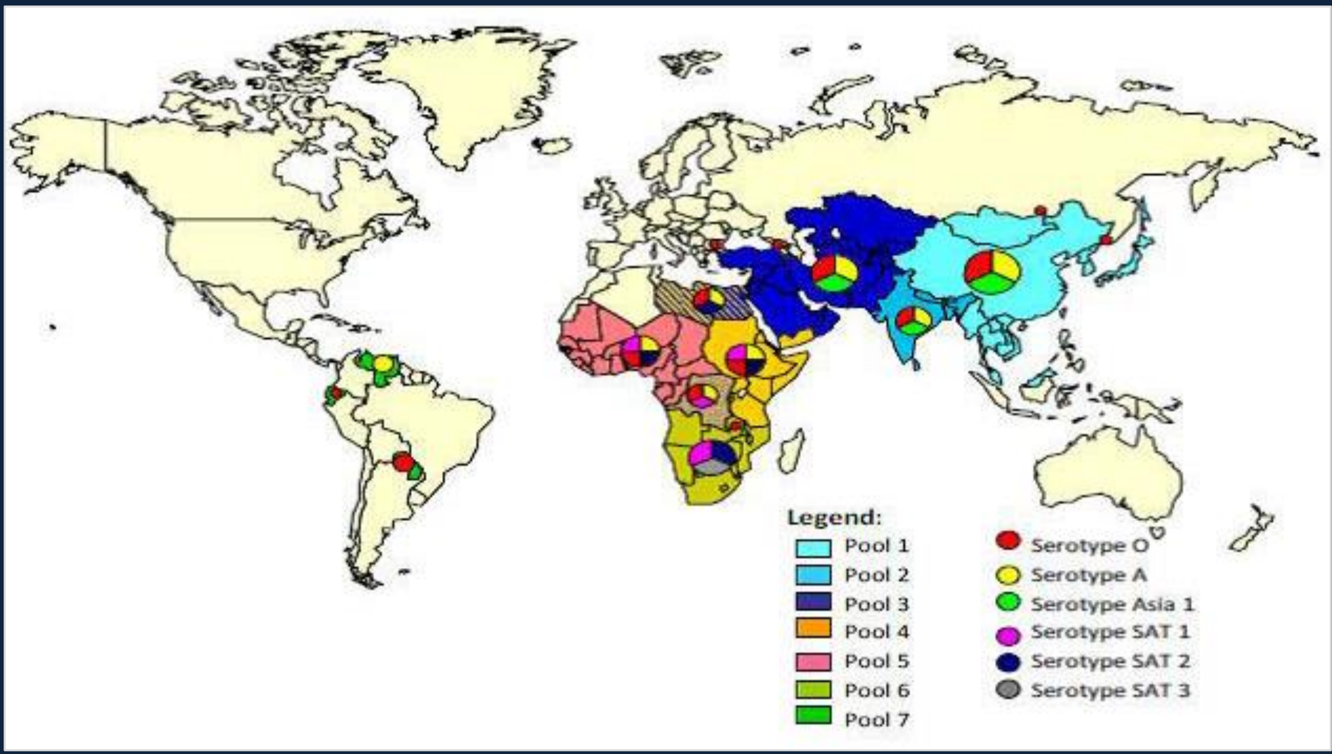
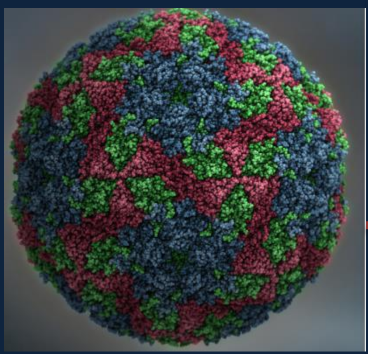


Figure 3.9.2b Comparison of three major antigenic sites of FMDV type A VP1 coding region of candidate vaccine strain and 7 local field strains.

Note: The number represents the amino acid position; the conserved residues are indicated by dots.

4





Chapter 4

Discussion

4. Discussion

Foot and mouth disease (FMD) is one of the highly contagious diseases of domestic animals. Economy of FMD control cannot be estimated without the basis of FMD epidemiology. Molecular epidemiological studies help in planning control strategies by elucidating current disease transmission patterns within and between countries. One of the main limitations to FMD eradication is lack of effective vaccines designed with appropriate circulatory FMDV strains or strain(s) that immunological close to circulatory strain(s) or question of quality of the vaccine also recently raised in India too. High levels of genetic diversity will most likely be reflected in antigenic differences and it has been shown that for vaccination to be effective, the viruses incorporated into the vaccines need to be antigenically related to viruses circulating in the field. For this purpose, before development of vaccine to combat FMD, epidemiological study and characterization of vaccine strain(s) at genomic level is essential. Furthermore, there is a need for better integrated strategies that fit the specific needs of endemic regions. The present investigation conclusively inferred the demography, epidemiology, characterization of FMDVs circulating in Bangladesh at the genomic level and phylogeography of the circulating FMDV strains.

4.1 Demography and Local treatment

According to the study, most data in the questionnaires returned were similar, but it is recognized that the responses of herdsmen to the questionnaire did not take into account the economic effect of the disease on their animals, as they do not pay great attention to this, or do not record it.

The effect of environmental factors might play a major role in the spread of FMD. During the rainy season (mild outbreaks) between June and August, heavy rain, high relative humidity and moist winds may inhibit aerosol transmission of the disease. During this season also, the movement and transport of animals from one place to another is hindered in some areas by heavy rain or floods. In the winter months, from October onwards, however, the number of reported outbreaks increased due to favorable climatic conditions of dry weather and dry winds with low temperatures and moderate relative humidity. Such favorable climatic factors might cause more rapid propagation of the viral disease among the susceptible animal population. In the summer, month of April to May, due to extremely hot weather (with average temperatures of 38ºC to 40ºC), the number of FMD outbreaks was greatly reduced. The effect of environmental factors on the incidence of FMD had also been considered in earlier reports (Bhattacharya *et al.*, 1984 and Chowdhury *et al.*, 1986).

The age-specific prevalence study revealed an increasing prevalence as the age increases, which is in agreement with the report of Gelaye *et al.*, 2009. This may be attributable to the young cattle being herded in homestead areas and hence having less chance of exposure. The old cattle may have acquired the infection from multiple serotypes and/or infections. Higher prevalence in old cattle is likely due to constant re-exposure to FMD (Mackay *et al.*, 1998).

The association between the sex with an FMD incidence of cattle was also observed where male cattle showed a higher incidence rate which supports the agreement with the reports of Remond *et al.*, 2002. The breed specific incidence study depicted that the FMD was observed affecting mostly indigenous cattle which is in agreement with the report of Samuel and Knowles, 2001. The higher incidence of the disease in indigenous cattle compared to cross breeds might be due to the sub-optimum level of management practices implemented on indigenous cattle, as they were supplemented with minimum inputs due to their low production and body weight gain, which increase the degree of acquiring FMD on contact with sick animals (James and Rushton, 2002; Rufael *et al.*, 2008).

The results from a descriptive analysis of grazing pattern found that the semi-intensive (combined manger and field grazing pattern) or field grazing smallholder livestock in developing countries are prone to FMD. The reasons might stem from either the increased contact between animal infected and animal susceptible to the transmission or from higher virus survival in the more humid microclimate around water sources (Geering and Lubroth, 2002). Once an animal is infected, the virus can be disseminated into the environment, including field pastures, water resources and soil. Sharing of pasture and water source is common in Bangladesh because the majority of smallholders feed their animals by letting the animals freely roam in public pastures. This promotes the spread and infection of FMD. Moreover, FMDV infection in cattle is mainly transmitted via infected animal and susceptible animals in the same area by aerosol, because cattle are sensitive to respiratory infections (Kitching, 2005).

For the treatment of FMD locally, it is realized that the application of glycerin or turmeric powder by herdsmen reduces significantly secondary bacterial infections as a consequence of FMD lesions in the mouth or feet of infected cattle. Schlievert *et al.,* 1992 have been mentioned that glycerin acts as an antibacterial agent to inhibit the growth and toxins of potentially pathogenic bacteria associated with wounds. Turmeric and isolated compounds from turmeric have demonstrated a remarkable variety of beneficial pharmacological activities. These include antibacterial (Negi *et al.,* 1999), antifungal (Lutomski *et al.,* l974) and antiviral (Mazumder *et al.,* 1995) activities.

4.2 Epidemiology of etiological agents of FMDVs in Bangladesh from 2012-2015

In line with this study, nucleotide sequence comparisons conducted using the BLAST search indicated that all the sequences obtained from the 2012/2013 Bangladesh FMD outbreaks had the greatest sequence similarity to FMDV isolates of serotype O, A and Asia-1 with their respective Genbank sequences included in this study. Moreover, sequences obtained from the 2014 to 2016 outbreaks had the greatest sequence similarity to FMDV isolates of serotype O only. This confirmed that serotype O, A and Asia-1 were responsible for the FMD outbreaks of Bangladesh in recent years. This is consistent with previous studies reported by Nandi *et al.,* 2015.

From the evolutionary history, based on VP1 gene sequence, it is found that the FMDV identified from 2012 to 2016 in Bangladesh was more related to India serotypes. The finding of a similar strain in this study indicates transcend of FMDV strain from the neighboring country specially India and may be vice versa due to unrestricted unidirectional cross-border movement of herds. This study further confirmed that serotype O was solely responsible for FMD outbreaks in Bangladesh between 2012 and

2016 and serotype A and Asia-1 were responsible FMD outbreaks between 2012 and 2013 (Sultana *et al.*, 2014; Ullah *et al.*, 2014 and 2015).

This study also demonstrated the presence of single lineage and topotype of each serotype O (Ind2001 of ME-SA topotype), A (Genotype VII of Asia topotype) and Asia-1 (Genetic lineage C) of FMDV in Bangladesh which is in contrast with previous studies where a limited number of isolates from the region were included (Loth et al, 2011; Sultana *et al.*, 2014; Ullah *et al.*, 2014 and 2015). It illustrates the importance of performing comprehensive studies for molecular epidemiology and to include representative samples from all regions in the analysis to reach correct conclusion.
Present study results indicated that similar strains of viruses can be confined to a certain country and evolved within that country over time while other strains can transcend country boundaries. One could speculate that the sharing of genotypes between countries or the confinement of certain genotypes to a specific country could be largely influenced by the social, economic, climatic and political situation in that specific area at any given point in time.

4.3 Comparative genomics of circulatory FMDVs in Bangladesh

Knowledge of Genome sequence of a virus, particularly which continuously evolving is most fundamental and extremely important for its characterization. This investigation isolated the circulatory FMDVs from infected cattle and completed whole genome of the circulatory FMDV type O and A. The results demonstrated that the FMDV 5' UTR contains a short fragment called S-fragment, a poly (C) tract of variable length, followed by a large fragment (LF) of over 700 bases in length (LF-5' UTR) that can form a number of highly conserved secondary structures that include randomly repeated pseudoknots (PKs), a *cis* acting replication element (*cre*) and an internal ribosome entry site (IRES) similar to reports found in literatures (Mohapatra *et al.*, 2009; He *et al.*, 2011). The total number of PKs predicted in this study varied from 3 to 4 even after deletions or without deletions. The majority of the mutations either by being non-disruptive or by being accompanied by compensatory mutations preserved stems base pairing and overall structure of PK mutations disrupted prediction of PK I and PK II structure. The PK IV domain was observed to be the most stable among all the PKs in terms of sequence conservation in agreement with an earlier observation for type Asia-1 (Mohapatra *et al.*, 2008). The fact that no natural isolate with less than two PKs have been detected so far suggests that two complete PK domains are essential for certain aspects of virus biology, considering their probable role in replication (Mason *et al.*, 2003). The *cre* region was essential for RNA genome replication (Marvin and Barry, 2004) and a conserved 'AAACA' motif in the *cre/bus* region has been recently shown to be involved in VPg uridylylation (Lopez *et al.*, 2001; He *et al.*, 2011). But it is noteworthy that in case of poliovirus, only the first three 'A' residues were proven to be essential for uridylylation reaction by ''slideback'' mechanism (Paul *et al.*, 2003). Domain 4 followed by domain 5 in the IRES displayed the highest degree of conservation, whereas domain 3 followed by domain 1 revealed maximum variability.

The 'GNRA' tetraloop in domain 3 played a critical role in determining the tertiary structural conformation of the IRES element (Fernandez-Miragall and Martinez-Salas, 2003). The 'TCC' motif in the conserved bulge within the 'GNRA' stem-loop, identified as the cleavage site for RNase P, a ribozyme (Serrano *et al.*, 2007). The conserved 'C'-rich loop in domain 3 of FMDV, which is a candidate for PCBP-2 binding in other picornaviruses (Walter *et al.*, 1999) had significant impact on aphthovirus IRES activity (Stassinopoulos and Belsham 2001; Martinez-salas *et al.*, 2002). The conserved 'motif A' (GCACA) that makes distant reciprocal interaction with 'GNRA' motif to maintain structural organization of the central domain of IRES (Fernandez-Miragall *et al.*, 2006). Mutations in 'motif A' causes decrease in IRES activity as severe as mutations in 'GNRA' motif. But the mutation in 'motif A' observed here did not have an impact on overall virus infectious titer. Mutations, in particular transversions in 'motif B' (GACT) were shown to reduce IRES activity minimally (Fernandez-Miragall *et al.*, 2006) and here this motif was found to tolerate only transitions at each position. Hence, motif B may be better represented as 'RRYY' (where 'R' is a purine and 'Y' is a pyrimidine).

The 'A'-rich bulge in domain 4 spanning from position 654 to 658 residues present in a conserved internal loop at the base of domain 4 thought to form eIF4G interaction site (Lopez de Quinto and Martinez-Salas, 2000).

Extensive studies of the structural capsid coding region have shown that the G–H loop of VP1 region is the major immunodominant site (Baxt *et al.*, 1989 and Stave *et al.*, 1988*)* and it can induce a strong antibody response against the virus, which is known to play a major role in protection induced by the current FMDV vaccines (Mateu *et al.*, 1995). The G-H loop near the C-terminal region and the B-C loop near the N-terminal region of the VP1 region have been reported as the antigenic regions for the FMD viruses. Alignment of the deduced amino acid sequence revealed that there were 11 and 15 distinct substitutions in the serotype O and A field isolates respectively, compared to the VP1 region of the vaccine strain. In addition, FMDV antigenic variations within other antigenic sites are implicated in the full, complete immunologic response (Mateu *et al.*, 1995; Grubman and Baxt., 2004). Among these substitutions, three were located in the G-H loop of VP1 for serotype O. On the other hand, two were located in the G-H loop and four were located in the B-C loop of VP1 region for serotype A. Any alteration of critical residues would confer antigenic specificity to the FMD viral variants (Carrefio *et al.*, 1992; Martinez *et al.*, 1991 and Mateu *et al.*, 1990). Therefore, we presumed that the antigenic differences between the variants of native strain and the current vaccine strain might account for incomplete protection.

We observed variation in the deduced amino acid sequence alignments for the eight FMDV non-structural proteins (L^{pro}, 2A, 2B, 2C, 3A, $3B_{123}$, $3C^{pro}$ and $3D^{pol}$), that ranged from 35.9% for 3A, 23.9% for L^{pro}, 21.1% for $3B_{123}$, 14.9% for 2B, 12.8% for $3D^{pol}$, 11.6% for 3C, 8.5% for $3C^{pro}$ and no variation for 2A.

L^{pro}, which is the first protein to be synthesized, cleaves itself from the rest of the growing polypeptide (Strebel and Beck, 1986) before cleaving the eukaryotic translation initiation factor eIF4G (Piccone *et al.*, 1995; Guarne *et al.*, 1998). Despite the high variability observed (23.9%), residue conservation in the Bangladesh FMDV is maintained amongst the essential auto-catalytic residues (C52, H149 and D165). Residues involved in substrate specificity were mapped to D50, D164, D165 were conserved for all isolates investigated.

The 2A protein induces a modification of the cellular translation apparatus resulting in 2A release (Donnelly *et al.*, 2001a). This is achieved by modifying the activity of the ribosome to promote hydrolysis of the peptidyl (2A)-tRNAGly ester linkage and the release of the P1-2A precursor in the translational complex (Donnelly *et al.*, 2001b). We observed all out of 18 amino acids as invariable in a complete alignment; however, the conserved functional domain of ^{12}DVEXNPG^{18} was indicating structural and functional constraints associated with this domain. 2A is cleaved from the P1 polypeptide by the $3C^{pro}$ in the later stage of processing and its function as an independent protein is not known.

The small hydrophobic 2B protein of FMDV associates with the ER may cause rearrangement of the ER membrane (Moffat *et al.*, 2005). The hydrophobic motif at residue positions 115-117 is likely to be responsible for positioning 2BC complexes to allow its membrane bound activities at sites of FMDV replication in the ER-derived vesicles in the host cytoplasm (Grubman and Baxt, 2004; Moffat *et al.*, 2005). The hydrophobic character of this domain was highly conserved in the Bangladesh viruses.

The FMDV 2C protein is an AAA+ ATPase that affects initiation of minus strand RNA synthesis (Sweeney *et al.*, 2010), and localizes the Golgi-derived membrane structures. The three ATPase binding motifs were highly conserved in the Bangladesh viruses. The interaction of 3C, 3A and a cellular polyA-binding protein with the RNA helicase A (RHA) leads to a ribonucleoprotein complex formation at the 5' end of the genome and has been shown to play an important role in FMDV replication (Lawrence and Rieder, 2009). The 2C protein and its precursor, 2BC, induce vesicle formation in the cytoplasm (Moffat *et al.*, 2005).

The 3A protein is proposed to be the membrane anchor for the picornavirus replication complex (Weber *et al.*, 1996). It is associated with viral-induced membrane vesicles and contributes to the cytopathic effect and the inhibition of protein secretion (Doedens and Kirkegaard, 1995). Earlier literature indicated that 3A peptide has been associated with virulence in picornaviruses (Pacheco *et al.*, 2003), and ten amino acid insertion in the peptide at the C-terminus (92-101) in study virus type O (isolated from cattle) compare to the RefSeq (isolated from pig) correlated to altered host range (Knowles *et al.*, 2001; Pacheco *et al.*, 2003). This evidence is contrasting with previous studies and showed intact 3A peptide can infect pigs and cattle with similar virulence (Ali *et al.*, 2016). So, 3A protein might not be the sole determinant of host specificity and there can also be probable roles of pseudoknot, IRES and L^{Pro} regions in host specificity.

Although all three copies of the 3B/VPg protein were present in the study isolates, they were highly variable in the complete alignments. However, the N-terminal motif ^{1}GPYXGP^{6} was conserved in all the viruses. The VPg protein participates in the initiation of RNA replication and plays a role in the encapsidation of viral RNA. Each of the VPg proteins contains 3Y, which is known to be involved in phophodiester linkage to the viral RNA (Forss and Schaller, 1982) and was conserved in the Bangladesh viruses in this investigation.

The $3C^{pro}$ is a cysteine protease (Birtley and Curry, 2005) responsible for catalyzing 10 of the 13 proteolytic cleavage events necessary for polyprotein processing (Clarke and Sanger, 1988). In the $3C^{pro}$, there were notably two changes to neutral residue substitutions in a conserved motif of Bangladesh type O and type A viruses (residue R97-S and D98-V). However, there is conservation of the active triad for the $3C^{pro}$, residue H46, D84 and C163 as well as the substrate pocket. The pocket contains a H181 hydrogen bonded with Y154 and T158, which donate hydrogen bonds to the P1 peptide substrate (Birtley and Curry, 2005), all of which are invariable in the Bangladesh viruses. Residues located on the surface of the $3C^{pro}$, opposite from the catalytic site of the protease, have been shown to be essential for VPg uridylation, which is the first stage in the replication of viral RNA, by binding RNA (Nayak *et al.*, 2005).

As in other picornaviruses, protein $3D^{pol}$ is the RdRp responsible for the replication of the RNA genome via negative strand intermediates (Doherty *et al.*, 1999; Ferrer-Orta *et al.*, 2004). The $3D^{pol}$ was most resistant to variation indicating the importance of conserving the structural and functional integrity of the RdRp.

The 3' UTR, composed of two stem-loops and a poly(A) tract, was required for viral infectivity and stimulates IRES activity (Serrano *et al.*, 2006). The 3' end established two distinct strand-specific, long-range RNA-RNA interactions, one with the S-region and another with the IRES element (Serrano *et al.*, 2006). The S-region was recognized by each of the separate stem-loops. S-3' UTR interaction was dependent on a structural conformation induced by the presence of the poly(A) tract (Serrano *et al.*, 2006).

4.4 Phylogeography of FMDVs circulating in Bangladesh

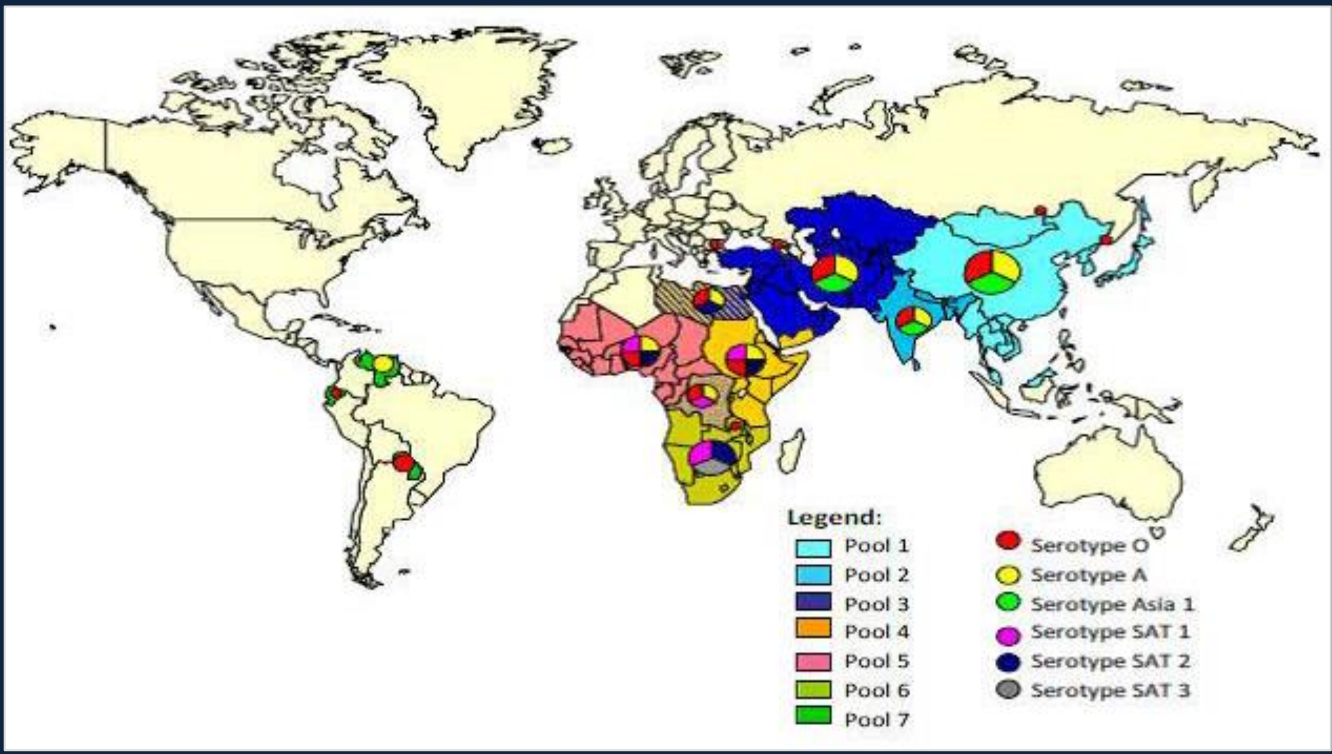
Several interesting aspects about the history of FMDV type O viruses emerge from our sub-continental phylogeography approach. According to the phygeographic evidence, it is found that the most likely root location of FMDV type O is in Bihar, India and in agreement with our results suggests that the route of entry of the virus has been along the Assam to the Bangladesh. Moreover, this topotype virus was detected to be homogenously distributed across the regions according to our previous study. The circulation of the same lineage in Bangladesh and India at the same time is only due to the unrestricted unidirectional transboundary animal movement from our neighboring country as chief meet demand in Bangladesh. This highlights the importance of a regional approach to trans-boundary animal disease control. It is apparent from the FMDV analysis presented here that monitoring of the emerging strains in the region is required for the success of vaccination strategies.

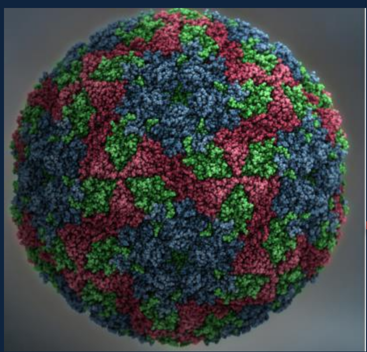
4.5 Selection of Vaccine Candidate

From the result it is presumed that FMDV with the titer $10^{8.5}$ $TCID_{50}$/ml can be used as vaccine preparation for the immunization of guinea pig, although virus neutralization test (VNT) and protection test are necessary for the validation of vaccine candidate selection. According to the terrestrial manual for FMD (OIE, 2009), it is clear that the minimal protective antigenic 146S content of the used FMDV serotypes should be around $10^{6.0}$$TCID_{50}$/dose from each serotype to ensure the highest protection rate either in guinea pig or in cattle (OIE, 2009). The result also supported by Sedeh *et al.*, 2008 where it was observed that the dose range for FMDV inactivation with virus titration $10^{7.5}$ $TCID_{50}$/ml showed unaltered antigenicity.

Candidate vaccine strains were selected to close antigenic match to the field isolates. The result of this study has indicated that the selected field isolates could be used as candidate vaccine strains for the production of FMD vaccine in Bangladesh. Though the data described in sub-section 3.9.2 indicated an apparent variation VP1 protein coding region (antigenic region of FMDV) for serotype A and underscored the need to continue further molecular epidemiological investigations to substantiate this findings.

5





Chapter 5

Conclusion

5. Conclusion

In summary, it is revealed that FMD severely constrains the development of competitive livestock enterprises in developing countries like Bangladesh. The points emerging from the study are outlined as under:

- From the epidemiological study for FMD the results demonstrated that the disease is endemic in Bangladesh and FMDV type O, A and Asia-1 are widely circulating throughout the year. Single lineage and topotype of FMDV serotypes O (Ind2001 lineage and O/ME-SA topotype), A (genotype VII of Asia topotype) and Asia-1 (genetic lineage C) are circulating in Bangladesh.
- The findings provided evidence for the porous nature of borders between Bangladesh and neighboring countries and highlight the continued threat posed by FMD as a transboundary disease in the region.
- The viruses showed genetic heterogeneity and differed significantly from other viruses on the continent.
- Local circulatory FMDV serotypes would help in the selection of proper strains for incorporation into vaccines.
- Occurrence of FMD in vaccinated animal conclusively demonstrated that the current vaccine is less effective might be due mismatches with the circulatory strains, but other reason like instability of the vaccine could not be ruled out.

Recommendations

- Effective control and prevention of FMD relies largely on the implementation of strategies such as control of animal movements within and between the neighboring countries, careful assessment of the risk of FMDV introduction and repeated vaccination of animals susceptible to the disease.

- Proper disease reporting to OIE/FAO office is essential. As Bangladesh crosses the stage-1 of the PCP-FMD road map at the moment, but due to an unplanned official reporting policy of the disease to the OIE/FAO is the main obstacle for the recognition. A national FMD control initiative should be adopted targeting the aim of moving Bangladesh to a higher level on the PCP. Using an epidemiological network, the system might be streamlined.

- As inconsistent and misinterpretations data about FMDVs among different laboratories were common scenarios in Bangladesh. So integrated research and official approach is important to establish a strong epidemiological network throughout the country.

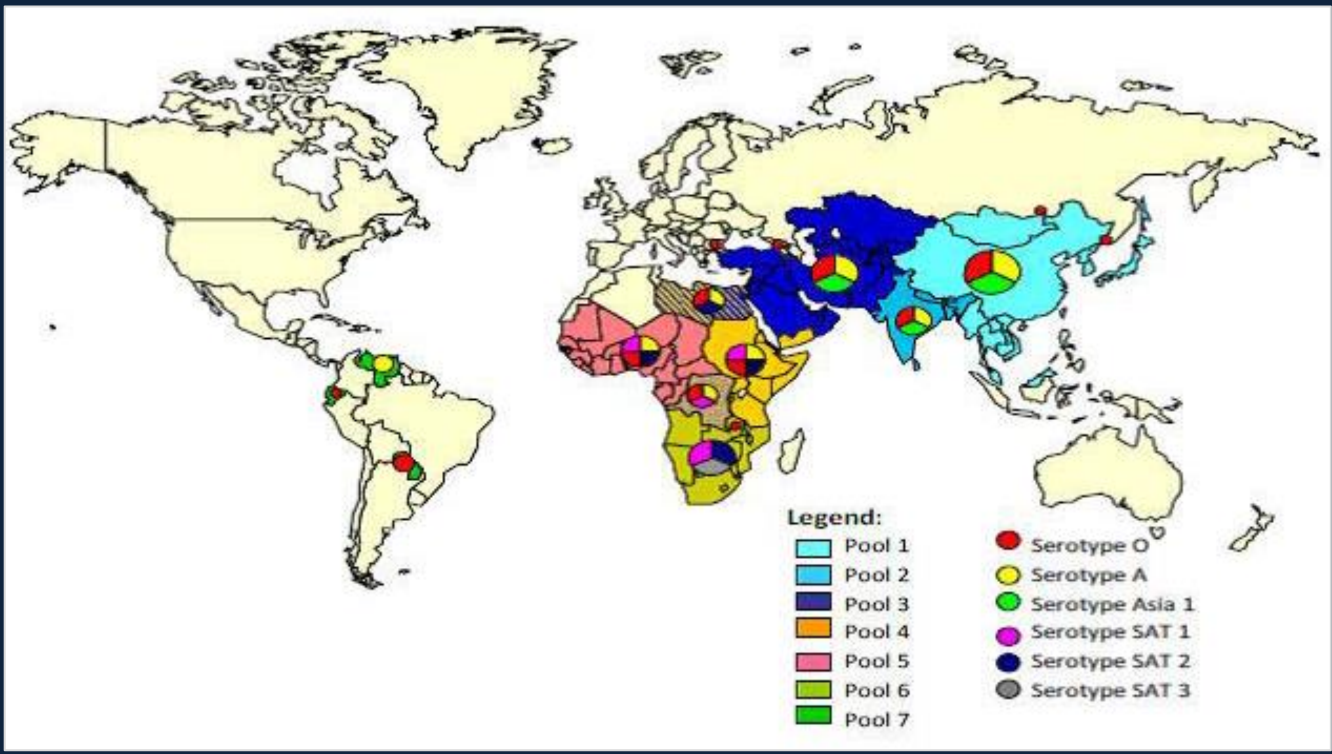
- Government and Research Stakeholders must work together and prepare a plan to eradicate the disease within a prescribed time frame like PCP-FMD road map.

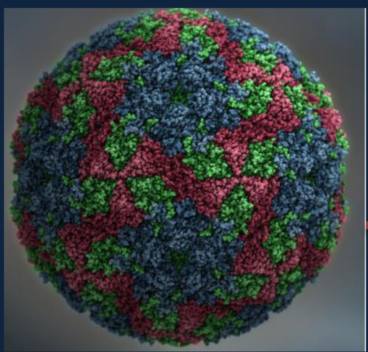
- An updated 'Animal disease control Act' is to be implemented carefully to prevent animal movement, management and restriction of animal products. In the context of fundamental cooperation must be established with the neighboring countries, especially restriction of animal movement, animal products and by-products through country-border areas.

- Continued studies on the characterization of virus types responsible for field outbreaks from different geographical areas would help in the selection of proper strains for incorporation into vaccines.

- In addition, continuous monitoring is necessary to find out any lineage turnover in Bangladesh as both genetic lineage C and genetic lineage D is reported to be circulating in India.

6





Chapter 6

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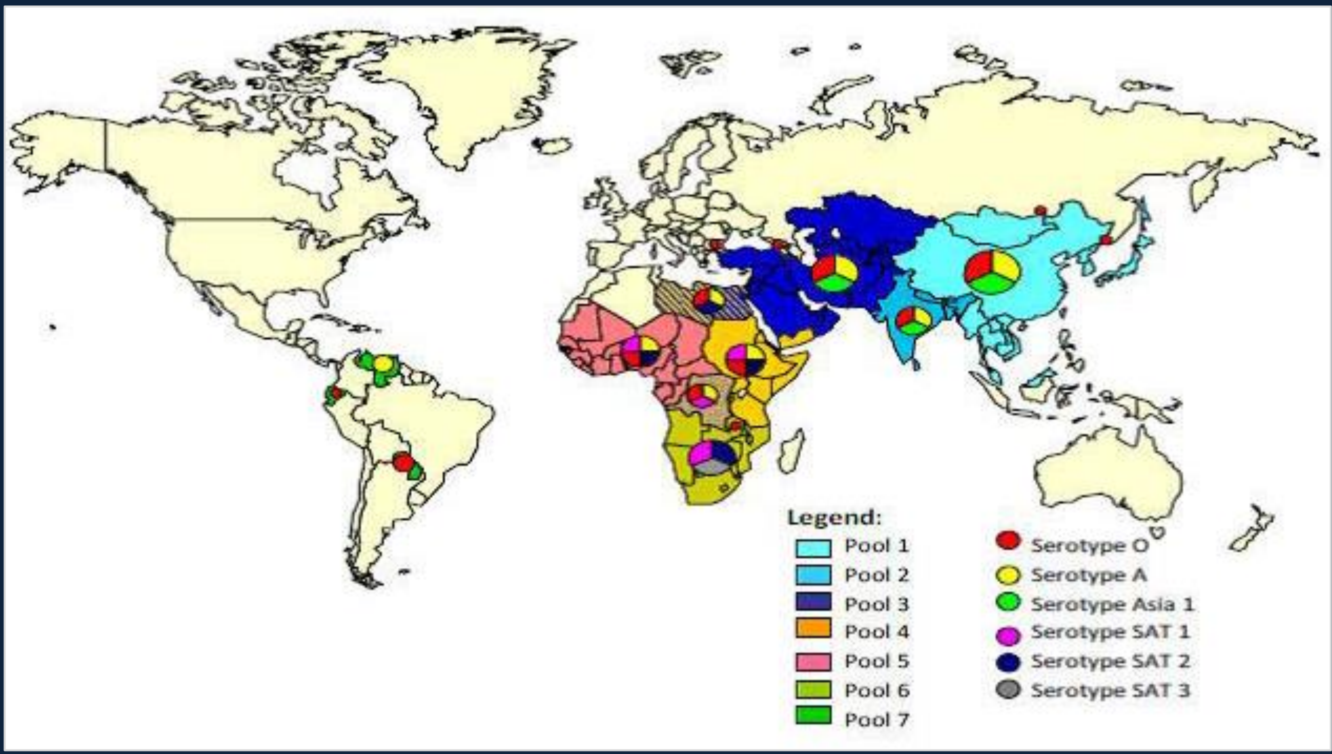
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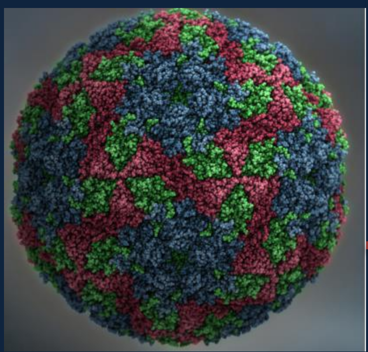
Pool 2
Pool 3
Pool 4
Pool 5
Pool 6
Pool 7
Serotype O
Serotype A
Serotype Asia 1
Serotype SAT 1
Serotype SAT 2
Serotype SAT 3



Appendices

Appendix-I

Part-1

Recipes and Preparation Notes for Media

1X TAE Buffer

First preparation of a 50X stock 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 mMTris-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
ImPro-II™ Reverse Transcriptase	1.2 Kanamycin Positive Control RNA
ImPro-II™ 5X Reaction Buffer	Upstream Control Primers
$MgCl_2$	Downstream Control Primers
dNTP Mix	Nuclease-Free Water
Oligo(dT)15 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		

Part-2

Standard Supply of Instrument and Equipments 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^{0}C	Vestfrost, Denmark
Horizontal Gel Elctrophoresis Apparatus Hl-SET	CBS Scientific, UK	Water bath, Model:SUM	England
Microcentrifuge	Mikro20, Germany	-80° 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 II

Sample ID	Description of Samples			PCR Results	Serotype	Accession Number
	Species	Collected Sample	Collection date			
Military Firm, Jessore (February 2012)						
BAN/JE/Mf-01/2012	Bovine	Epithelium	19.02.2012	FMDV	Asia1	KJ175170
BAN/JE/Mf-01/2012	Bovine	Epithelium	19.02.2012	FMDV	Asia1	KJ175171
BAN/JE/Mf-01/2012	Bovine	Epithelium	19.02.2012	FMDV	Asia1	KJ175172
BAN/JE/Mf-01/2012	Bovine	Epithelium	19.02.2012	FMDV	Asia1	KJ175173
BAN/JE/Mf-01/2012	Bovine	Epithelium	19.02.2012	FMDV	Asia1	KJ175174
BAN/JE/Mf-01/2012	Bovine	Epithelium	19.02.2012	FMDV	Asia1	KJ175175
Ramgarh, Chittagong (May 2012)						
BAN/CH/Ra-02/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795960
BAN/CH/Ra-08/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795949
BAN/CH/Ra-10/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795961
BAN/CH/Ra-13/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795962
BAN/CH/Ra-14/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795950
BAN/CH/Ra-15/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795951
BAN/CH/Ra-16/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795952
BAN/CH/Ra-18/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795953
BAN/CH/Ra-26/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795954
BAN/CH/Ra-28/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795955
BAN/CH/Ra-31/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795964
BAN/CH/Ra-39/2012	Bovine	Epithelium	28.05.2012	FMDV	A	KC795965
Lohjong, Munshigonj (June 2012)						
BAN/MU/Lo-02/2012	Bovine	Epithelium	25.06.2012	FMDV	O	-
BAN/MU/Lo-04/2012	Bovine	Epithelium	25.06.2012	FMDV	O	-
BAN/MU/Ra-07/2012	Bovine	Epithelium	25.06.2012	FMDV	O	-
Ghatail, Tangail (July 2012)						
BAN/TA/Gh-01/2012	Bovine	Epithelium	20.07.2012	FMDV	O	-
BAN/TA/Gh-02/2012	Bovine	Epithelium	20.07.2012	FMDV	-	-
Bhanga, Faridpur (July 2012)						
BAN/FA/Bh-01/2012	Bovine	Epithelium	22.07.2012	FMDV	O	-
BAN/FA/Kh-05/2012	Bovine	Epithelium	22.07.2012	FMDV	O	KC795947
Sadar, Chittagong (July 2012)						
BAN/CH/Sa-01/2012	Bovine	Epithelium	28.07.2012	FMDV	O	-
BAN/CH/Sa-02/2012	Bovine	Epithelium	28.07.2012	FMDV	O	-
Sakhipur, Tangail (September 2012)						
BAN/TA/Sa-02/2012	Bovine	Epithelium	06.09.2012	FMDV	O	-
BAN/TA/Sa-03/2012	Bovine	Epithelium	06.09.2012	FMDV	O	-
Sadar, Faridpur (October 2012)						
BAN/FA/Ka-01/2012	Bovine	Epithelium	01.10.2012	FMDV	O	KC795956
BAN/FA/Ka-02/2012	Bovine	Epithelium	01.10.2012	FMDV	O	KC795947
BAN/FA/Do-11/2012	Bovine	Epithelium	02.10.2012	FMDV	O	KJ175178
BAN/FA/Do-12/2012	Bovine	Epithelium	02.10.2012	FMDV	O	KJ175179
Sadar, Pabna (October 2012)						
BAN/PA/Ra-05/2012	Bovine	Epithelium	14.10.2012	FMDV	O	KC795957
BAN/PA/Sa-12/2012	Bovine	Epithelium	14.10.2012	FMDV	O	KC795958
BAN/PA/Kg-16/2012	Bovine	Epithelium	14.10.2012	FMDV	O	KC795959
BAN/PA/Kg-20/2012	Bovine	Epithelium	14.10.2012	FMDV	O	KJ175180

Sample ID	Description of Samples			PCR Results	Serotype	Accession Number
	Species	Collected Sample	Collection Date			
Sadar, Gazipur (November 2012)						
BAN/GA/To-01/2012	Bovine	Epithelium	08.11.2012	FMDV	A	-
BAN/GA/To-02/2012	Bovine	Epithelium	08.11.2012	FMDV	A	KC795948
BAN/GA/To-03/2012	Bovine	Epithelium	08.11.2012	FMDV	A	-
BAN/GA/To-04/2012	Bovine	Epithelium	08.11.2012	FMDV	A	-
Sakhipur/Mirzapur, Tangail (November 2012)						
BAN/TA/Sa-01/2012	Bovine	Epithelium	17.11.2012	FMDV	O	-
BAN/TA/Mi-04/2012	Bovine	Epithelium	17.11.2012	FMDV	O	-
BAN/TA/Mi-05/2012	Bovine	Epithelium	17.11.2012	FMDV	O	-
BAN/TA/Mi-06/2012	Bovine	Epithelium	17.11.2012	FMDV	O	-
Sadar, Lalmonirhat (March 2013)						
BAN/LA/Ch-129/2013	Bovine	Epithelium	25.03.2013	FMDV	O	-
BAN/LA/Du-135/2013	Bovine	Epithelium	25.03.2013	FMDV	O	KJ175181
BAN/LA/Sa-137/2013	Bovine	Epithelium	26.03.2013	FMDV	O	KJ175182
BAN/LA/Ch-141/2013	Bovine	Epithelium	26.03.2013	FMDV	O	-
Singra, Natore (July 2013)						
BAN/NA/Ra-151/2013	Bovine	Epithelium	06.07.2013	FMDV	O	-
BAN/NA/Ha-156/2013	Bovine	Epithelium	06.07.2013	FMDV	O	KF985189
BAN/NA/Pa-157/2013	Bovine	Epithelium	06.07.2013	FMDV	O	-
Nandigram, Bogra (July 2013)						
BAN/BO/Na-161/2013	Bovine	Epithelium	08.07.2013	FMDV	O	KY077600
BAN/NA/Na-162/2013	Bovine	Epithelium	08.07.2013	FMDV	O	KY077601
Sadar/Melandah, Jamalpur (September 2013)						
BAN/JA/Sa-173/2013	Bovine	Epithelium	26.09.2013	FMDV	O	-
BAN/JA/Me-180/2013	Bovine	Epithelium	27.09.2013	FMDV	O	KJ175183
Dhanbari, Tangail (October 2013)						
BAN/TA/Dh-184/2013	Bovine	Epithelium	14.10.2013	FMDV	O	KJ175184
BAN/TA/Dh-185/2013	Bovine	Epithelium	14.10.2013	FMDV	O	KJ175176
BAN/TA/Dh-186/2013	Bovine	Epithelium	14.10.2013	FMDV	O	KJ175185
Sreepur, Gazipur (October 2013)						
BAN/GA/Sr-187/2013	Bovine	Epithelium	14.10.2013	FMDV	Asia1	KJ175186
Rangamati (October 2013)						
BAN/RA/Sa-189/2013	Pig	Epithelium	25.10.2013	FMDV	O	KJ175177
Kaliakoir, Gazipur (October 2013)						
BAN/GA/Kk-190/2013	Bovine	Epithelium	28.10.2013	FMDV	O	-
BAN/GA/Kk-191/2013	Bovine	Epithelium	28.10.2013	FMDV	O	KY077602
BAN/GA/Kk-192/2013	Bovine	Epithelium	28.10.2013	FMDV	O	KY077603
Sadar, Gazipur (December 2013)						
BAN/GA/Sa-193/2013	Bovine	Epithelium	25.12.2013	FMDV	A	-
BAN/GA/Sa-194/2013	Bovine	Epithelium	25.12.2013	FMDV	A	-
BAN/GA/Sa-195/2013	Bovine	Epithelium	25.12.2013	FMDV	A	-
BAN/GA/Sa-196/2013	Bovine	Epithelium	25.12.2013	FMDV	A	-
BAN/GA/Sa-197/2013	Bovine	Epithelium	25.12.2013	FMDV	A	KJ754939
Madhupur, Tangail (March 2014)						
BAN/TA/Ma-198/2014	Bovine	Epithelium	16.03.2014	FMDV	O	-
BAN/TA/Ma-199/2014	Bovine	Epithelium	16.03.2014	FMDV	O	-
BAN/TA/Ma-200/2014	Bovine	Epithelium	16.03.2014	FMDV	O	KY077604
Rupgonj, Narayangonj (March 2014)						
BAN/NA/Ru-202/2014	Bovine	Epithelium	25.03.2014	FMDV	O	-
BAN/NA/Ru-203/2014	Bovine	Epithelium	25.03.2014	FMDV	O	-

Sample ID	Description of Samples			PCR Results	Serotype	Accession Number
	Species	Collected Sample	Collection Date			
Kaligonj, Gazipur (September 2014)						
BAN/GA/Ka-204/2014	Bovine	Epithelium	18.09.2014	FMDV	O	-
BAN/GA/Ka-205/2014	Bovine	Epithelium	18.09.2014	FMDV	O	-
BAN/GA/Ka-212/2014	Bovine	Epithelium	18.09.2014	FMDV	O	KY077605
BAN/GA/Ka-213/2014	Bovine	Epithelium	18.09.2014	FMDV	O	KY077606
Kaligonj, Gazipur (March 2015)						
BAN/GA/Ka-215/2015	Bovine	Epithelium	16.03.2015	FMDV	O	KY077607
Dhamrai, Dhaka (June 2015)						
BAN/DH/Dh-216/2015	Bovine	Epithelium	15.06.2015	FMDV	O	KY077608
BAN/DH/Dh-217/2015	Bovine	Epithelium	15.06.2015	FMDV	O	-
Chatmohor, Pabna (August 2015)						
BAN/PA/Ch-220/2015	Bovine	Epithelium	02.08.2015	FMDV	O	
BAN/PA/Ch-228/2015	Bovine	Epithelium	02.08.2015	FMDV	O	KY077609
Shajadpur, Sirajgonj (August 2015)						
BAN/SI/Sh-233/2015	Bovine	Epithelium	08.08.2015	FMDV	O	-
BAN/SI/Sh-234/2015	Bovine	Epithelium	08.08.2015	FMDV	O	KY077610
Kashiani, Gopalgonj (August 2015)						
BAN/GO/Ka-236/2015	Pig	Epithelium	26.08.2015	FMDV	O	KX712091
BAN/GO/Ka-237/2015	Pig	Epithelium	26.08.2015	FMDV	O	-
BAN/GO/Ka-239/2015	Pig	Epithelium	26.08.2015	FMDV	O	-
Lohogorah, Narail (August 2015)						
BAN/NL/Lo-241/2015	Bovine	Epithelium	27.08.2015	FMDV	O	-
BAN/NL/Lo-245/2015	Bovine	Epithelium	27.08.2015	FMDV	O	KY077611
Sadar, Lakshmipur (October 2015)						
BAN/LK/Sa-248/2015	Bovine	Epithelium	06.10.2015	FMDV	O	KY077612
BAN/LK/Sa-248/2015	Bovine	Epithelium	06.10.2015	FMDV	O	KY077613
Begumgonj, Noakhali (October 2015)						
BAN/NO/Be-250/2015	Bovine	Epithelium	07.10.2015	FMDV	O	KY077614
BAN/NO/Be-251/2015	Bovine	Epithelium	07.10.2015	FMDV	O	KY077615
Sadar, Dinajpur (October 2015)						
BAN/DI/Sa-252/2015	Bovine	Epithelium	26.10.2015	FMDV	O	KY077616
BAN/DI/Sa-254/2015	Bovine	Epithelium	26.10.2015	FMDV	O	KY077617
Atowari, Panchagarh (October 2015)						
BAN/PG/At-262/2015	Bovine	Epithelium	27.10.2015	FMDV	O	KY077618
BAN/PG/At-264/2015	Bovine	Epithelium	27.10.2015	FMDV	O	KY077619
Baliadangi, Tagoregaon (October 2015)						
BAN/TG/Ba-268/2015	Bovine	Epithelium	27.10.2015	FMDV	O	KY077620
BAN/TG/Ba-268/2015	Bovine	Epithelium	27.10.2015	FMDV	O	KY077621
Kulaura, Maulovi Bazar (December 2015)						
BAN/MA/Ku-269/2015	Bovine	Epithelium	16.12.2015	FMDV	O	KY077622
Sadar, Siragonj (December 2015)						
BAN/SI/Sa-273/2015	Bovine	Epithelium	20.12.2015	FMDV	O	-
Sadar, Magura (December 2015)						
BAN/MG/Sa-275/2015	Bovine	Epithelium	25.12.2015	FMDV	O	-
Aditmari, Lalmonirhat (January 2016)						
BAN/LA/Ad-278/2015	Bovine	Epithelium	21.01.2016	FMDV	O	KY077623
Fulbari, Kurigram (January 2016)						
BAN/KU/Fu-280/2015	Bovine	Epithelium	23.01.2016	FMDV	O	-
BAN/KU/Fu-283/2015	Bovine	Epithelium	23.01.2016	FMDV	O	KY077624

Figure: List of the positive samples included in the study

Appendix III

Table 1. 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**	**965**	**Designedinthis study**
1R	**CCAGTCCCCTTCTCAGATC**	**5'UTR**	**948-965**		**Reid *et al.*, 2000**
1F	**GCCTGGTCTTTCCAGGTCT**	**5'UTR**	**640–658**	**708**	**Reid *et al.*, 2000**
1OEXR	**CCCTCGTGYAGYTCAAGACC**	**VP4**	**1329–1348**		**Designed in this study**
2OF	**CCMTTCYTCGAMTGGGTCTA**	**VP4**	**1254–1273**	**1006**	
2OR	**TGGTTWCCCACTGCRGTGAC**	**VP2**	**2241–2260**		
3OF	**ARGACTTYGTGAGYGGGCC**	**VP2**	**2035–2053**	**861**	
3OR	**AAGTGCAGGTTRATGGTGCC**	**VP3**	**2877–2896**		
4OF	**CAAGGTSTATGCCAACATCG**	**VP3**	**2507–2526**	**890**	
4OR	**RTYTGCATCAGGTCCAACAC**	**VP1**	**3378–3397**		
5OEXF	**GAGAACTACGGTGGTGAGAC**	**VP1**	**3276–3295**	**718**	
NK61	**GACATGTCCTCCTGCATCTG**	**2B**	**3971–3994**		**Samuel and Knowles, 2001**
NSP1F	**GAGACGTYGAGTCCAACCC**	**2B**	**3939-3958**	**596**	**Abdul-Hamid, Firat-Sarac *et al.*, 2011**
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**	
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**		

Table 2. Primer pairs used to amplify the complete genome of FMDV serotype A

Primers	5'-3' Sequence	Location	Position	Amplicon size (bp)	Reference
A1F	TTGAAAGGGGGCGCTAGGG	5'UTR	1-19	374	Abdul-Hamid, Firat-Sarac *et al.*, 2011
A1R	GGGTGAAAGGCGGRCTTCG	5'UTR	355-373		
A2F3	CCCCCCTAAGTTTTACCGTCR	5'UTR	374-394	622	
A2R	CCTTCTCAGATCCCGAGTGTCG	5'UTR	974-995		
682F	CCAGGTCTAGAGGRGTGA	5'UTR	687-704	626	
1293R	CGAGTCGTAGACCCAGTC	L^{Pro}	1296-1313		
5' UTR-4F	GCCTGAATAGGYGACCGGAG	5'UTR	1040-1059	620	
5' UTR-4R	CCGTTGAGYGGTTCTTGATCG	L^{Pro}	1640-1660		
A4F	GAGCCTTTCTTCGACTGGGTC	L^{Pro}	1284-1304	513	
A4R	CCATGGAGTTCTGGTACTGRTGC	VP4	1775-1797		
A5F	GGGTGGTARGCGATCGACG	L^{Pro}	1563-2276	714	
A5R	WCACCTCCACGTCCCAGC	VP2	2645-2668		
A6F	AYTCGAGTGTGGGAGTCACS	VP2	2029-2048	639	
A6R	GCTGTTTTRGGGTCTGTTGTCACC	VP3	2645-2668		
A7F	CTGGACYCTGGTRGTGATGG	VP2	2477-2496	501	
A7R	GTACGCCACCATGTASCGG	VP3	2960-2978		
A8F2	GGYYTGGTGACAACAGACCC	VP3	2640-2659	805	
A8R2	SCGTGYTGGTGKGTTTGC	VP1	3428-3445		
A9F	GTACAGGGYTGGGTCTGC	VP3	3144-3161	629	
A9R	CGTGGCRRGAATTGCACC	VP1	3756-3773		

Primers	5'-3' Sequence	Location	Position	Amplicon size (bp)	Reference
P1-3F3	**CACCTGAGGCAGCCTTG**	**VP1**	**3544-3560**	**980**	**Abdul-Hamid, Firat-Sarac*et al.*, 2011**
PCR NSP 1R2	**CTTCTGAGGCGATCCATG**	**2C**	**4507-4524**		
A10F	**GGTYCCCAATGGAGCACC**	**VP1**	**3530-3547**	**606**	
A10R	**CTTGTACCAGGGRTTGGCC**	**2B**	**4118-4136**		
PCR NSP 1F	**GAGACGTYGAGTCCAACCC**	**2B**	**3939-3958**	**596**	
PCR NSP 1R2	**CTTCTGAGGCGATCCATG**	**2C**	**4517-4535**		
PCR NSP 2F	**CAGCTCARAGCACGTGACAT**	**2C**	**4423-4443**	**566**	
PCR NSP 2R	**GCCATRGGCGGGATRAA**	**2C**	**4972-4989**		
PCR NSP 3F	**TGACCACTTYGACGGTTA**	**2C**	**4860-4878**	**609**	
PCR NSP 3R	**ACCATCCCCTCRAAGAAY TC**	**3A**	**5449-5469**		
PCR NSP 4F	**CGRAGGTTYCACTTTGAC**	**3A**	**5098-5116**	**507**	
PCR NSP 4R	**CATRATCACTATGTTTGCCA**	**3A**	**5585-5605**		
PCR NSP 5F	**GAATTCTTTGAGGGGATGGT**	**3A**	**5449-5469**	**576**	
PCR NSP 5R	**CACTTTCAAAGCGACAGG**	**3C**	**6007-6025**		
PCR NSP 6F	**CRAGCTGAAGGACCCTAC**	**3B**	**5831-5849**	**564**	
PCR NSP 6R	**GGGGGTKCCYTTCTTCAT**	**3C**	**6377-6395**		
PCR NSP 7F	**GGACAGGACATGCTCTCAG**	**3C**	**6283-6302**	**656**	
PCR NSP 7R	**GACGCGTAGTCRGCAGC**	**3D**	**6922-6939**		
PCR NSP 8F	**ATGCGCAAAACCAAGCT**	**3D**	**6736-6753**	**588**	
PCR NSP 8R	**AATTTGCGGTCCGTTGT**	**3D**	**7307-7324**		
PCR NSP 9F	**RACCTTCCTGAAGGACGAR**	**3D**	**7170-7189**	**508**	
PCR NSP 9R	**GTCCAGCTCRACTCCCTC**	**3D**	**7660-7678**		
PCR NSP 10F	**AACGTGTGGGATGTGGA**	**3D**	**7393-7410**	**832**	
RACE-T21G*	**CAGGAAACAGCTATGACTTTTT TTTTTTTTTTTTTTTTG**	**3'UTR**	**8186-8225**		
PCR NSP 10F	**AACGTGTGGGATGTGGA**	**3D**	**7393-7410**	**832**	
RACE-T21C*	**CAGGAAACAGCTATGACTTTTT TTTTTTTTTTTTTTTTC**	**3'UTR**	**8186-8225**		
PCR NSP 10F	**AACGTGTGGGATGTGGA**	**3D**	**7393-7410**	**832**	
RACE-T21A*	**CAGGAAACAGCTATGACTTTTT TTTTTTTTTTTTTTTTA**	**3'UTR**	**8186-8225**		
PCR NSP 10F	**AACGTGTGGGATGTGGA**	**3D**	**7393-7410**	**832**	
RACE-T21R*	**CAGGAAACAGCTATGACTTTTT TTTTTTTTTTTTTTTTR**	**3'UTR**	**8186-8225**		

Table 3. Reference strains used from GenBank for phylogenetic analysis

GenBank Accession no.	Strain	Serotype	GenBank Accession no.	Strain	Serotype
VP1 Sequence Based Phylogeny for the Detection of Serotypes					
KM921826	**O/BHU/26/2009**	FMDV-O	KF570656	IND787/2009	FMDV-Asia1
KC506533	**O/IND/1/2011**		KT153323	IND96/2008	
HQ530693	**O/NEP/5/2008**		HQ224561	IND137/08	
HQ832591	IND437/2008	FMDV-A	HQ224558	IND95/08	
HQ832592	IND17/2009		HQ224560	IND97/08	
HQ832578	**IND161/2003**		KT153321	IND175/2007	
KU127/247	**SAU/1/2015**		HQ224553	IND12/07	
PD509/2010	**JN247566**	FMDV-Asia1	AF283429	KNP/196/91/1	FMDV-SAT1
Lineage and Topotypes Detection of FMDV Seroptype O					
Genbank Accession no.	Strain	Topotype	Genbank Accession no.	Strain	Topotype
KC506519	**O/IND86/2010**	Ind2001 of ME-SA Topotype	KC506439	**O/IND23/2012**	**PanAsia of ME-SA Topotype**
HQ630693	**O/NEP/5/2008**		GU566054	O/SUD/26/2004	Africa Topotype
KC506518	**O/IND84/2010**		FJ798143	O/ETH/60/2005	
HQ630694	**O/NEP/2/2009**		AJ296327	O/UGA/5/96	
KC506533	**O/IND1/2011**		AJ303485	O/CIV/8/99	
KM921826	**O/BHU/26/2009**		HM230709	O/ALG/2/99	
KC506554	**O/IND179/2011**	Ind2011 of ME-SA Topotype	AJ294921	O/HKN/12/91	Cathay Topotype
KC506583	**O/IND182/2011**		KM243068	O/TAW/81/97	
KC506547	**O/IND189/2011**		AJ294928	O/TAW/4/99	
KC506443	**O/IND56/2011**	**PanAsia of ME-SA Topotype**	X00871	**O1/Kaufbeuren/FRG/66**	**Euro-SA Topotype**

Genbank Accession no.	Strain	Topotype	Genbank Accession no.	Strain	Topotype
Lineage and Topotypes Detection of FMDV Seroptype A					
HQ832582	IND249/2004	Genotype VII under Asia Topotype	**EF208776**	A/MAI/297	**West Africa1**
HQ832592	IND17/2009		**EU919234**	A/NIG/4/79	
HQ832590	IND245/2007		**KF112914**	A/NGR/2/73	**West Africa 2**
EU553870	A/MOR/7/77	Euro-SA	**AY254443**	CAR/1/76	
EU553868	A/LIB/4/79		**EU919238**	A/ERI/3/98	**Central Africa 2**
KF561688	TAN/3/68	East Africa1	**KF112916**	A/SUD/3/77	
EU919232	A/TAN/4/80		**EU414532**	A/K37/84	East Africa 2
EU919233	A/ETH/2/79	East Africa 3	**FJ798149**	**ETH/10/2005**	
Lineage and Topotypes Detection of FMDV Seroptype Asia1					
KT153323	IND96/2008	Genetic Lineage C	**FJ785296**	IND153/2004	Genetic Lineage D
HQ224561	IND137/08		**DQ101239**	IND114-04	
HQ224558	IND95/08		**FJ785300**	IND322/2004	
HQ224560	IND97/08		**FJ785298**	IND158/2004	
KT153321	**IND177/2007**		**FJ785297**	IND156/2004	
HQ224553	IND12/07		**AY304994**	IND63/72	Genetic Lineage B
KF570656	IND787/2009		**KP822943**	IND63/1972	
JN247566	PD509/2010		**DQ989304**	IND334-00	
GQ220868	IND149/01	Genetic Lineage D	**DQ989303**	IND151-94	
FJ785299	IND168/2004				

Appendix IV

Foot-and-mouth disease virus-type O isolate BAN/NA/Ha-156/2013, complete genome

>KF985189

**TTGAAAGGGGGCGCTAGGGTCTCACCCCTAGCATACCACCGACAACTCCTGCGTTGCAC
TCCACACTTACGCCCGTGCGCTCGCGGGAACTGATGGACTGTCGTTCACCCACCTACAG
CTGGACTCACGGCACCGCAAACCACTTTGGTCACTGCGCTGTCCTGGGCACCCCTGTTG
GGGGCCGTTCGACGCTCTACGGTCTCCCCCGTGTGACGGGCTACGGTGATGGGGCCGC
CTCGCGCGGGTTGATCGCCTGGTCTGCTTCGGCTGTCACTCGAAGCCCACCTTTCACCC
CCCCCCCCCCCCTAAGTTTACCGTCGTTCCCGACGTTAAAGGGATGTAACCACAAACTT
GGAACCGTCTTGCCCGACGTTAATGGGTTGTAACCACACGCTTGTACCGCCTTTCCCCG
CGTTAAAGGGAAGTAACCACAAGATAAACCTTCGCCCGGAAGTAAAACGGCAACCACAC
TCAGTTTTGTCCGTTTTCATGAGAAACGGGACGTCTGCGCACGAAACGCGCCGTCGCTT
GAGGAAGACTTGTACAAACACGATCTATGCAGGTTTCCACAACTGACACAAACCGTGCA
ACTTGAAACTCCGCCTGGTCTTTCCAGGTCTAGAGGGGTGACACTTTGTACTGTGTTTG
ACTCCACGCTCGGCCCACTGGCGAGTGTTAGTAACAGCACTGTTGCTTCGTAGCGGAGC
ATGATGGCCGCGGGAATTCCCCCTTGGTGACAAGGACCCGCGGGGCCGAAAGCCACGT
CCTAACGGACCCATCATGTGTGCAACCCCAGCACAGCAGCTTTACTGTGAAAGCCACTT
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AGCGCCCTGTTTAAAAAGCTTCTATGCCTGGATAGGCGACCGGAGGCCGGCGCTTTTCC
ATTAAACTACTACTGACTTGATGAACACAACTGACTGTTTTATCGCTCTGTTACAAGCTC
TCAGAGAGGTTAAAGCATTGTTTCTTTCACGAACACAAGGAAAGATGGAATTCACACTT
CACAACGGTGAGAAAAAGACCTTCTATTCTAGGCCCAACAGCCACGACAATTGTTGGTT
GAACACCATCCTTCAGTTGTTTAGGTACGTCGACGAACCTTTCTTCGACTGGGTCTATG
AGTCGCCTGAAAACCTCACCCTTGAGGCGATTAGGCAACTAGAAGAAGTTACTGGTCTT
GAGCTGCACGAGGGTGGACCGCCCGCTCTCGTCATTTGGAACATCAAGCACTTGCTCCA
CACCGGAGTCGGCACTGCTTCGCGACCCAGCGAGGTGTGCATGGTTGATGGCACGGAC
ATGTGTTTGGCCGACTTCCACGCTGGCATCTTCCTGAAAGGGCAAGAACACGCTGTGTT
CGCCTGCGTCACCTCCAACGGGTGGTACGCGATCGACGACGAGGACTTCTACCCCTGG
ACGCCGGACCCGTCCGACGTTCTGGTGTTTGTCCCGTACGATCAAGAACCACTTAATGG
AGAGTGGAAAACAAAGGTTCAAAGACGACTCAAAGGAGCCGGGCAATCCAGCCCGGCG
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GCAGTACCAGAACTCTATGGACACACAACTTGGAGACAACGCCATCAGCGGAGGCTCCA
ACGAGGGGTCCACAGATACCACCTCCACCCACGCAACCAATACCCAAAACAATGATTGG
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GAAAACCGAGGAGACCACTCTCCTCGAGGACCGCATCCTCACTACCCGCAACGGGCAC
ACGATCTCGACAACCCAGTCGAGCGTCGGAGTCACCTACGGGTACGCAACAGCTGAGG
ACTTTGTGAGTGGGCCAAACACATCCGGTCTCGAGACCAGGGTTGTGCAGGCAGAGCG
GTTCTTCAAAACCCACTTGTTCGACTGGGTCACCAGTGATCCATTCGGGCGGTGCCACC
TGCTGGAACTTCCAACTGACCACAAAGGGGTCTACGGCGGCTTGACCGACTCGTATGCT
TATATGAGAAACGGTTGGGACGTTGAGGTCACTGCGGTGGGAAATCAGTTCAACGGAG
GTTGCCTGCTGGTGGCCATGGTGCCAGAACTTTGCTCCATCCAAAAGAGAGAGCTATAC
CAACTCACGCTCTTTCCCCACCAGTTCATCAACCCTCGCACGAACATGACGGCACACAT
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CCCTTGTGGTCATGGTCGTGGCCCCACTGACTGTCAACAATGAAGGTGCCCCACAGATC
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AGAAGGGATCTTCCCCGTGGCATGCAGCGATGGTTACGGCGGTTTGGTGACCACTGAC
CCGAAGACGGCTGACCCCGCCTACGGGAAAGTGTTCAACCCCCCTCGCAACATGTTGCC
CGGGCGGTTCACCAACTTCCTTGATGTGGCTGAGGCGTGTCCCACGTTTCTGCACTTTG**

AAGGTGACGTGCCGTACGTGACCACAAAGACAGATTCGGACAGGACGCTCGCTCAGTT
CGACCTGTCTTTGGCAGCAAAGCACATGTCAAACACCTTTCTGGCAGGCCTCGCCCAGT
ACTACACACAGTACAGCGGCACCATCAACCTGCATTTCATGTTCACAGGACCCACAGAC
GCGAAAGCGCGTTACATGATTGCATATGCCCCACCAGGCATGGAGCCGCCTAAGACACC
CGAGGCGGCTGCTCACTGCATTCATGCGGAATGGGACACAGGGTTGAACTCAAAATTCA
CATTTTCAATCCCTTACCTTTCGGCGGCTGATTACGCGTACACCGCGTCTGACGCTGCC
GAAACCACAAATGTACAGGGATGGGTTTGCTTGTTTCAGATAACACACGGGAAAGCTGA
CGGTGACGCACTGGTCATTCTGGCTAGCGCCGGTAAGGACTTTGAGCTGCGTTTGCCGG
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CCTGTTGGCCATCCACCCGAGCCAAGCCAGACACAAACAAAAGATTGTGGCACCCGTGA
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CCGGTCGTTAAGGAAGGACCTTACGAGGGACCGGTGAAGAAGCCTGTCGCTTTGAAAG
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AAAAAAAAAAAAA

Foot-and-mouth disease virus - type A isolate BAN_GA_Sa-197_2013, complete genome

>KJ754939
TTGAAAGGGGGCGCTAGGGTCTCACCCCTAGCATGCCATCGGCAGCTCCTGCGCTGCA

CTCCACACTTACGCCCGCGTACTCGCGGGAACCGATGGACTGTCGTTCACCCACCTACA

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CGCATCTCGCGTGACCGGTTAGTACTCTTACCACCTTCCGCCTACTTGGTCGTTAGCGC

TGTCTTGGGCACTCCTGTTGGGGGCCGTTCGACGCTCCACGGTCTCCCCCGTGTATCGG

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TCCTTATTCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA**

Appendix V (List of Journal Publications)

Publications from this research work are mentioned below:

Towhid, S. T., Siddique, M. A., **Ullah, H.,** Sultana, M. and Hossain, M. A. (2016). Foot-and-Mouth Disease: Current Scenario in Asia and Bangladesh. *Malaysian Journal of Microbiology* (accepted).

Ali, M. R., **Ullah, H.,** Siddique, M. A., Sultana, M. and Hossain, M. A. (2016). Complete genome sequence of pig-originated foot-and-mouth disease virus serotype O from Bangladesh. *Genome Announcements* **4(5):e01150-16**. doi:10.1128/genomeA. 01150-16. **Received** 22 August 2016, **Accepted** 2 September 2016, **Published** 27 October 2016.

Siddique, M. A., **Ullah, H.**, Nandi, S. P., Chakma, D., Sultana, M. and Hossain, M. A. (2014). Molecular Characterization of Foot-and-Mouth Disease Virus Type O from Wild Pig in Bangladesh. *Bangladesh Journal of Microbiology* **31, 41-45**

Ullah, H., Siddique, M.A., Al Amin, M., Das, B.C., Sultana, M. and Hossain, M.A. (2015). Re-emergence of circulatory foot-and-mouth disease virus serotypes Asia1 in Bangladesh and VP1 protein heterogeneity with vaccine strain IND 63/72. *Letters in Applied Microbiology* **60, 168-173.**

Ullah, H., Siddique, M. A., Sultana, M. and Hossain, M. A. (2014). Complete Genome Sequence of Foot-and-Mouth Disease Virus Type A Circulating in Bangladesh. *Genome Announcements* **2:e0050614**; doi: 10.1128/ genomeA.00506-14.

Sultana, M., Siddique, M. A., Momtaz, S., Rahman, A., **Ullah, H.,** Nandi, S. P., & Hossain, M. A. (2014). Complete genome sequence of foot-and-mouth disease virus serotype O isolated from Bangladesh. *Genome announcements*, ***2*(1), e01253-13**.