Post infection Serosurveillance Study of Foot and Mouth Disease using NSP 3AB3 as Antigen by DIVA ELISA

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DEDICATED

TO

MY

BELOVED PARENTS

AND

MY FAMILY

TO WHOM IT MAY CONCERN

This is to certify that the research work embodying the results reported in this thesis entitled “Post infection Serosurveillance study of Foot and Mouth Disease using NSP 3AB3 as Antigen by DIVA ELISA” submitted by Sanjoy Chandra Bhattacherjee, has been carried out under co-supervision in the Central Disease Investigation Laboratory, Bangladesh.

It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka.

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Abstract

Foot and mouth disease (FMD) remains a tremendous problem in developing countries like Bangladesh where proper vaccination strategy was maintained haphazardly to protect the disease stature. Sometimes animals become infected but do not show any clinical symptoms and constantly shed virus and become the most important source of disease spread. Affected animals, through their mouth secretions, milk and semen secretions as well as from their infected hooves shed virus into the environment which can spread 300km in water course and 60km by air.

FMD vaccine is produced from structural protein of the FMD virus and but in case of live viral infection, antibodies produced in the cattle body are against structural and non-structural protein. DIVA ELISA test is a serological test, which has been devised to determine the immune status of animals form the basis of understanding that it could differentiate infected from vaccinated animals. Tests have been devised to use non-structural proteins (NSP) of FMDV since it is only on infection that antibodies are produced against such proteins. This study through DIVA ELISA testing was conducted to show the clinical status of different crossbreed cattle and native/indigenous cattle to determine carrier status and to observe the effect of vaccination against FMD in different organized and unorganized dairy farms in different districts of Bangladesh after a severe outbreak recorded at the end of 2011. For this purpose, 218 cattle sera were collected from various regions, where cattle are reared. Serum sample were divided into three groups depending on the vaccination history. Blood samples were collected from the infected animals, susceptible animals and disease free animals during or/after infection.

The study was conducted to find out if any, relevance between breed and vaccination status, to reveal antibody response against 3AB3 non-structural protein between native and crossbreed cattle and to explore any relevant significant relationship between breed and non-structural protein 3AB3. There was significant relevance found between vaccination and breed preference than other criteria set to conduct the test. People were more curious to vaccinate cross breed cattle than native cattle. Presently, such tests are at the heart of confirming whether an organized farm is free from FMD virus (replication) even where there is no observed clinical disease which is vital to trade issues, milk and meat production in live animals and animal products.

What is apparent from this work and other data is that the criteria for selecting cutoffs as to whether animals are positive or negative have to be re-assessed. Another finding is that the tests are very useful at the herd level, but due to the measured diagnostic uncertainly, are not so useful at the single animal level in tune with most serological testing. Single ELISA systems using NSP for screening are powerful tools for assessing infection in a herd since they only require a single test, but are best backed up by confirmatory tests.

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LIST OF ABBREVIATION

FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
NSP	Non structural Protein
ELISA	Enzyme linked Immunosorbent Assay
BLRI	Bangladesh Livestock Research Institute
CDIL	Central Disease Investigation Laboratory
DIVA	Differentiation of Infected from Vaccinated Animal
BLRI	Bangladesh Livestock Research Institute
CDIL	Central Disease Investigation Laboratory
FAO	Food and Agricultural Organization
OIE	Organization International Des Epizooties
Poy(A)	Poly adenosine
RGD	Arginine-Glycine-Aspartic acid
RNA	Ribonucleic Acid
SAT	South African Territory
VP	Viral Protein
WRL	World Reference Laboratory
µg	Microgram
µl	Microlitre
µM	Micromolar
^{0}C	Degree Celsius
nt	Nucleotide
G	Gram
M	Molar
IRES	Internal Ribosomal Entry Site
OPD	Orthophenylene-diamine dihydrochloride

HRP	Horse reddish peroxidase

CHAPTER 1

INTRODUCTION

1.1 Background

Foot and mouth disease(FMD) is a highly contagious and economically devastating disease of cloven-hoofed animals which hold a wide of the host spectrum such as cattle, pigs, sheep, goats, buffalo, deer, antelope and wild pigs and can severely constrain international trade of animals and animal products. It is highly contagious vesicular diseases known from centuries. Disease has been recognized as serious long term threat to the health and welfare of the domestic and wild ruminant animals, elephant and swine population of the world, with negative impacts on the livelihoods of animal keepers.

The production performance and use of large ruminants for ploughing and traction are seriously diminished when infected with FMD. Though rarely fatal when complicated with pasteurellosis in adult animals it ranks first in the Office International des Epizootics list A diseases. (OIE terrestrial Manual, 2009) owing to nearly 100% percent morbidity, rapid spread, severe decrease in livestock production and mortality in young animals. Due to single stranded RNA nature, Foot-and-mouth disease virus is highly diverse which is shown by presence of 7 different serotype (O, A, C, Asia-1, South African Territories 1-3) and many different subgroups and lineages within each serotype. There is no interserotypic protection that means animal are susceptible to other serotypes after recovery or vaccination with one particular serotype. Within serotype protection is also highly variable.

Production and production efficiency is further diminished in terms of quality and quantity of dairy products and weight gain ratios. Apart from direct losses associated with disease, main impacts are due interference with International and regional movements of animals and animal products. As per OIE and FAO, Countries having FMD are more prone to food insecurity through reduced access to local, national and international markets and animal draught power for agriculture. In Bangladesh, the disease is most concerned in cattle, buffalo because of negative impact on rural economy principally based on these farm animals. As per an estimate there could be approximate 25% loss in livestock productivity (Rowlands, 2003) due to direct losses including reduced growth rate, decreased milk production, and crippled agricultural draught power. Indirect losses such as trade barrier will further add to this figure.

This study was done after an outbreak of Foot and mouth disease recorded in the month of November 2011. Sample was collected from different organized dairy farms and different parts of Jessore and Tangail where the disease causes significant impact. Most of the farms were maintaining regular vaccination strategies with FMD vaccine produced at Livestock Research Institute but heavily failed to give protection. Most of the farms, owner reared cross-breed cow along with native cows for dairy purpose as well as rear ox for fattening purpose.

A total of 218 blood sample were collected to draw a post-infection sero-surveilance study at Central Disease Investigation Laboratory, Bangladesh using a non-structural recombinant protein

3AB3 to differentiate infected and vaccinated animals. In Bangladesh FMD vaccination is still haphazardly maintained and so risk remains there always and to become FMD free country is always remaining an impossible task to accomplish.

In this practical ground, the study was aimed to determine relationship between crossbreed and native cows and their vaccination status with antibody response against recombinant non-structural protein 3AB3. The sole objective was not to differentiate infected from vaccinated animals because, I do not have any intention to refresh vaccination policy that is existing now rather to make a clear decision about some facts regarding to breed, vaccination and antibody response in them. In this manner, the cause of locally made FMD vaccine failure to give protection was not traced here as it requires long time and government infrastructural and logistic support, which was not possible in the speculated time frame.

Differentiating foot-and-mouth disease virus antibodies generated during a natural infection from those due to vaccination is crucial for proving freedom from disease after an outbreak and allowing resumption of trade in livestock products (Rweyemamu et al., 2008). The Office International des Epizooties recommends inactivated purified FMDV vaccines primarily induce antibodies to viral structural proteins, whereas replicating virus stimulates host antibodies specific for both structural and non-structural proteins. (Muller et al., 2010). Bangladesh is a highly populous country mostly dependent on agriculture. FMD is an endemic contagious disease in parts of the country declining production in this regard, for effective implementation of control policies, newer test which are rapid, highly sensitive and specific and are capable of handling large number of sample at low infrastructure cost needs to be developed and extended to the laboratories and to the field persons. DIVA ELISA for differentiating infected and vaccinated animals as well as trace a significant relationship between breed preference and vaccination status, which have been successfully validated in this series by Central Disease Investigation Laboratory, Bangladesh.

1.2 Epidemiology

1.2.1 Causative agent

FMD is caused by FMD virus (FMDV), a virus in the genus Aphthovirus within the family Picornaviridae .

1.2.2 Virus Etiology

Foot and mouth disease virus was the first recognized viral pathogen (Loefller and Frosch, 1898) and is the sole member of the genus Aphthovirus belonging to the Picornaviridae family. The history of FMD may be traced to era of Hieronymus Fracastorius, a monk who descreibed a disease outbreak in 1546 A.D. that occurred in cattle near Verona, Italy (Fracastorius, 1546).

Picornavirus have played an important role in the history of Virology and veterinary medicine as in 1897, almost 400 years later, Friedrich Loeffler and Paul Frosch for the first time demonstrated

that FMD was caused by an agent that passed through filters that held back bacteria. (Loeffler and Frosch, 1897)

Seven immunologically different serotypes of the FMD virus are known namely A, O, C, Asial-1, South African Territories 1, 2, 3 which comprise more than 65 subtypes. Initially 2 types were named: type O for Oise in France and type A for Allmagne. (Vallee and Carre, 1922). Later type C was recognized as an additional type in Germany (Waldmann and Trautwein, 2003). Some 30 years later, work at Pirbright laboratory in England demonstrated 3 novel serotypes of FMDV in sample collected from the FMD outbreak in South Africa and Called SAT1, SAT2 and SAT 3. The seventh serotype that is Asial 1 was first recognized in a sample from Pakistan (Brooksby and Rogers, 1957).

1.2.3. Molecular Organization of FMD virus

The viral particle or virion contains a single stranded RNA of positive polarity approximately 8500 nucleotides long. It is an icosahedral particle with a smooth surface and a diameter of about 30nm (H.L. Bachrach, 1968). The fine structure of the virus has been described by X-ray crystallography [(R.Acarya et al.,1989) (W.M.Lee et al., 1993)(T.Jackson et al., 2003)].

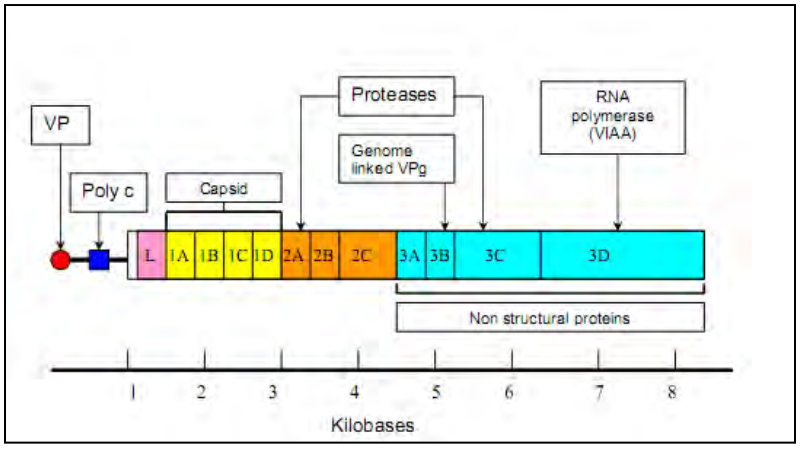
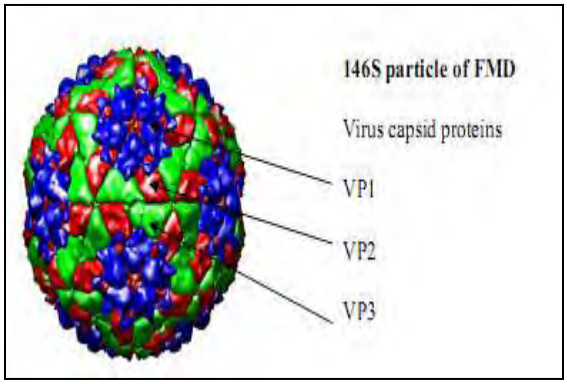


Fig 1.2: Genome of FMD and proteins produced on replication

1.2.3.1. Structural Protein



of FMD

There are 60 copies of each of the structural protein VP1, VP2, VP3 and VP4. While the first three structural proteins VPI, VP2, VP3 (MW≈ 24kDa) have surface components and the fourth VP4 (MW≈ 805kDa) is internal. The structural proteins like VP1, VP2, VP3, and VP4 are formed by post-translation cleavage of a precursor coded by 1D, 1B, 1C and 1A gene, respectively. The virion is also usually composed of one or two units of VP0, the precursor of VP2 and VP4.

The structural proteins VP1-3 fold into an eight stranded wedge shaped 13 barrel which fits together to form the majority of the capsid structure. The three- dimensional structure of FMDV has revealed a prominent surface feature formed by the loop between the G and H strands of VP1. The VP1 protein (1D gene) is mostly responsible for virus neutralization and contains a conserved arginine-glycine-aspartic acid sequence (RGD integrin binding motif) that binds virus to integrin cellular receptors. The RGD sequence is required for binding viruses to host cells by demonstrating that viruses with amino acid substitutions within the RGD sequence were unable to bind to or infect cells. Viruses created with a single point mutation in the sequences encoding the RGD segment reverted to virulence by regaining the RGD sequence (16), consistent with the well-known variability of the FMDV genome (11, 12).The VP4 protein is located inside the capsid. The strands of the 13- barrel of VP1-3 are connected by loops which form the outer surface of the virion.

Unlike other picornaviruses FMDV lacks a surface canyon or pit which is the receptor binding site for entero- and caridoviurses . Another feature of the virion is the channel at the fivefold axis which permits the entry of small molecules such as Cscl into the capsid resulting in FMDV having the highest buoyant density of the picornaviuruses. The genome of FMDV is about 8.5 kb in length enclosed within a protein capsid. It has four major parts: 5'Untranslated region, Coding region , 3'Untranslated region , and a poly A tail. The 5'UTR is linked with Vpg "which serves as primer in replication. 5'UTR contains an S fragment at its 5' end which encompasses 360 bases folds into long stem loop and plays a role in genome stability and the binding of protein involved in genome replication (*T.Bunch et al.,1994*) . Following S fragment there is poly C tract comprising over 90 % C residues. The length of this tract is extremely variable (*M.P.Costa Giomi et al., 1984*) associated with virulence and hence persistence (*T.J.R.Harris and F.Brown,1977*) "After poly C tract there is a series of RNA pseudo-knot structures of unknown function (*M.J Grubman and B.Baxt, 2004*)". Downstream to the pseudo-knot there is cis-acting replicative element (cre) (*P.W.Mason et al., 2002*) ". The cre has conserved AAA sequences and is essential for genome replication ". Downstream to cre is internal ribosomal entering sites (IRES). The IRES has role in initiation of viral protein synthesis and shutting down of host cell protein synthesis (*M.J Grubman and B.Baxt, 2004*) ". Due to the presence of IRES, picornaviruses are able to bind to ribosomeds to initiate the protein synteshesis. The picornaviruses have no 5' cap (7 -methyguanosine), so IRES serves as ribosomal entering site (*S.K.Jang et al., 1988*)". The 3' UTR follows the ORF termination codon and contains a short stretch of RNA which folds into specific stem loop structures (*E.V.Pilipenko et al ., 1992*)" followed by a poly A tract of variable length (*K.Dorsch Hasler et al., 1975*)" . This 3'UTR also serves as some functions in genome replication."

1.2.3.2. Non-structural Protein

FMDV genome contains a total of ten mature non-structural proteins (NSPs)(L, 2A, 2B, 2C, 3A, 3B, 3C, 3D; or some complex, such as 3AB or 3ABC) in form of a unique polyprotein from which the different viral polypeptides are cleaved by viral proteases. It has a high spontaneous mutation rate. There is also extensive genetic heterogeneity in individual serotypes with many distinct virus subtypes/ variants occurring within each serotype.

After translation the four primary cleavage products are formed: (i) the amino terminal L protease which cleaves at its own carboxy terminus,P1-2A, (ii) the precursor of the capsid proteins (iii) 2BC , and (iv) P3 which is cleaved to make the NSP's (*G.J.Belsham et al.,1993*). FMDV has two proteinases, The NSP leader proteinses (Lpro)located in the N-terminal region of the polyprotein acts both intra and intermolecularly. This protease initiates cleavage by separating itself from P1, the precursor of the capsid protein and the remainder of the growing polypeptide chain (*M.D.Ryan et al., 1991*). The 3C protease is responsible for the cleavage of P1 into 1AB (VP0), and 1D (VP1), the 1A/1B (VP4/VP2) cleavage occurs at the late stage in a virus morphogenesis and is associated with maturation of capsid. The 2C/3A primary cleavage is cis and subsequent cleavage is also mediated by the 3C pro and processing intermediates and mature proteins (*J.F.E. Newman et al.,1994*). In addition to viral protein processing, the 3C pro cleave the host cell protein histone H3 and may be involved with the shutting down of host cell transcription (*A.V.E.Capozzo et al., 2002*). The cleavage between 2A/2B junctions is mediated by 2A polypeptide separating itself and P1 away from 2BC/P3.This change is independent of both L and 3C. The FMDV 2A region is very short (18 amino acid) and together with the N-terminal residues of protein 2B, represents an autonomous element capable of mediating cleavage at its own C terminus (*M.L.L.Donnelly et al., 1997*).

1.4. Economic impact in Bangladesh

Foot and mouth disease is a very important transboundary animal disease endemic in Bangladesh. Most affected animals recover but leaves them debilitated. Sequelae include decreased milk yield, permanent hoof damage, chronic mastitis, loss of reproduction, reduced draught power, painting etc. Mortality reported high in calves.

As FMDV surveillance and serotyping in Bangladesh is rather casual, animal movement and trade is almost unregulated, even during outbreaks. Proper strategy for prevention and control is almost absent. Sporadic vaccination depends on farmers will. Limited quantity of vaccine is produced at Livestock Research Institute. Investigation of FMD outbreaks in 1999 at Baghabari milk-shed area revealed 63, 51, and 48% incidence in cattle, sheep / goats and buffaloes respectively; mortality in calves was 9.71% (*Howlader et al. 2004*).

The multifaceted economic impacts assessment is very complex; immediate losers are farmers but affect the community also. Economic impact is expected to address the following:

a) Production and productivity
b) Price and market effects
c) Trade
d) Food security and nutrition
e) Health and environment
f) Cost of disease prevention and control.

Islam (2011) estimated hypothetical annual economic loss due to FMD outbreaks in Bangladesh on the basis of BBS statistics*.

Only the direct losses and Veterinary costs are included; indirect economic impacts and the overhead cost of the state veterinary services are not considered.

The calculation based on some assumptions:

a) Every year 5% cattle and 1% sheep and goat get infected with FMD virus.
b) 20% of affected cattle die
c) Affected milch cows produce 25% less milk for 14 days
d) 50% affected working cattle and buffaloes abstain from ploughing or pulling cart.
e) 30% of affected animals are treated with antibiotics and antiseptics.
f) 30% of affected animals are treated with antiseptics only.

Livestock population in Bangladesh (in millions)

Source of Information	Cattle	Buffaloes	Sheep	Goats
BBS;2010	26.82	0.54	1.22	16.24

Table 1.4: Livestock Population in Bangladesh (BBS Statistics, 2010)

Total loss from FMD is BDT 819,021,170 (US$ 10,920,282)* on the basis of BBS statistics; 2010. No information is available on the impact of FMD on market price, trade, food security and nutrition and health and environment in Bangladesh. Difference might be related to calculation procedure (Direct Vs Both). It is fact that FMD is of great concern on economic point of view in Bangladesh.

Sl.No.		Description	Cost
1.	Loss from calves mortality and productivity	Death due to FMD (20%) case fatality in Cattle and buffalo calves (68,158 X 3500/taka)	238,553,000/
2.		Reduction of milk production 25% for 14 days; 1,331,708 X 7 lit X 40/taka)	53,268,320/
3.		Loss of draft power 982,772 X 14days X 200/day)	196,554,400/
4.	Cost of treatment	Antibiotic treatment (30% ; 410,580 X 500/taka)	205,290,000/
5.		Antibiotic treatment (Sheep and Goats) (30%; 52,389 X 200/)	10,477,800/
6.		Antiseptic treatment (Cattle and Buffalo) (30%; 410,580 X 50/)	20,529,000/
7.		Antiseptic treatment (Sheep and Goats) (30%; 52,389 X 50/)	2,619,450/
8.	Veterinarian's fee	At Veterinary hospital 10%; 154,323 X 00	00
9.		Private practitioners (50%;617,292 X 100/)	61,729,200/
10.	Vaccination cost	Cattle vaccination (Local Vaccine) (300000X30/)	9,000,000/
11.		Cattle vaccination (Imported Vaccine) (300,000 X 70/)	21,000,000/
Total			819,021,170/

Table 1.4.1: Partial picture of Economic loss due to FMD (Howlader et al., 2004)

1.5. Aims of the study

The aim of the study was to analyze the antibody response against non-structural polyprotein 3AB3 in different cattle breed blood serum at FMD post-infection serosurveilance. The present study was conducted with the following specific objectives:

1. To identify breed response against 3AB3 non-structural polyprotein.
2. To check vaccination status against 3AB3 non-structural polyprotein.
3. To measure vaccination status in relation with breed predominance.

CHAPTER 2

LITERATURE REVIEW

REVIEW OF LITERATURE

Review of literature is presented with a view to obtaining information related to the present research work. A remarkable number of studies have been carried out at home and abroad on different aspect of Foot and mouth disease virus. The literature related to the research work has been briefly reviewed under the following sub- headings.

2.1 History and Impact of Foot and Mouth disease
2.2 The Agent
2.3 Host affected
2.4 Pathogenesis
2.5 Infection route
2.6 Subclinical and persistent infections
2.7 Epidemiology of the disease
2.8 Non-structural protein as antigen to differentiate infected from vaccinated animals
2.9 ELISA method for Differentiating infected from vaccinated animals

2.1 History and impact of foot-and-mouth disease

The first indication of foot-and-mouth disease (FMD) in the literature can be found in a book from the Italian physician Hieronymi Fracastorii (*Wright, 1930)*, where he describes a cattle disease similar to the signs of FMD. Since then, several indications of FMD can be found in the literature (*Thalmann and Nockler, 2001*). From the second half of the 19th century, on the account of the increase of animal trade due to the development of new transport routes (*O'Rourke and Williamson, 2002*), the disease became more important and started to cause severe economic losses. In Germany, in 1897, a commission had been set up by the Prussian Ministry of Culture, following a request from the "Partei der Landwirte" of the Reichstag to set up measures of FMD control (*Rott and Siddell, 1998*). One year later, Friedrich Loeffler and Paul Frosch wrote in their report that the causative agent of FMD was neither a known bacterium nor a toxin but an "ultravisible, ultrafilterable substance" (*Loeffler and Frosch, 1897*), and described thereby the first animal virus, foot-and-mouth disease virus (FMDV). In 1910, the first FMD research institute was built on the island of Riems in Germany, followed by other research institutes in Europe, e.g. in Pirbright, United Kingdom in 1925 and on Lindholm island, Denmark in 1926. The major milestones in these early years of FMD research were the introduction of guinea pigs as research animals (*Waldmann and Pape, 1920*) and the first steps towards the development of an FMD vaccine in 1937 (*Waldmann et al., 1937).*

After the Second World War, FMD vaccines became available in all European countries and the disease was controlled by systematic vaccination until the end of 1991 when the European Union adopted a non-vaccination policy (Directive 85/511/CEE du conseil du 18 novembre 1985 établissant des mesures communautaires de lutte contre la fièvre aphteuse).

In 1967/1968 the United Kingdom experienced a large FMD outbreak in which more than 430,000 animals were slaughtered (*Gloster et al., 2005*). Since then FMD has occurred only sporadically and only a few minor outbreaks have been detected in Europe, e.g. in Denmark in 1982/1983 (*Christensen et al., 2005*) and in Italy in 1993 (*Nunez et al., 2006*). For the latter it has been shown that the causative virus was closely related to viruses that circulated previously in the Middle East (*Nunez et al., 2006*). In 2001 a large FMD outbreak started in the United Kingdom (*Alexandersen et al., 2003a)* and spread to France, Ireland and The Netherlands. The losses to agriculture in the United Kingdom alone were about £3.1 billion (Thompson et al., 2002). The causative FMDV here showed phylogenetic similarity to the virus from the outbreak in Italy in 1993 and was closely related to viruses that circulated previously in Asia and Middle East (*Knowles et al., 2005*).

2.2 The Agent

Foot-and-mouth disease is an acute, highly contagious viral disease, which has a great potential for causing severe economic losses in susceptible cloven-hoofed animals (*Alexandersen and Mowat, 2005)*.The causative agent of FMD is the foot-and-mouth disease virus (FMDV), a small positive sense ssRNA virus (approx. 8.3 kb) which belongs to the *Aphthovirus* genus of the family *Picornaviridae* (*Belsham, 1993*).

FMDV replicates through negative strand RNA which was recently used in quantitative strand specific real time RT-PCR as a tool to determine strain virulence and replication patterns (*Horsington and Zhang, 2007*). The high genetic and phenotypic variability of FMDV is reflected in the existence of seven serotypes: O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3 and further numerous variants and lineages, described as topotypes (*Knowles and Samuel, 2003*). The antigenic diversity within the serotypes is crucial to consider when selecting vaccine strains, as cross-reactivity may be variable (*Kitching, 2005*).

The virus capsid is composed of 60 copies of each of the four structural polypeptides designated VP1 to VP4. A region in the G-H loop (around residues 140-160) of the VP1 protein exposed on the surface of the viral capsid was determined to be the main antigenic site recognized by neutralizing antibodies (*Bittle et al., 1982*). The natural FMDV infection is initiated when the RGD motif within the G-H loop binds to certain cellular integrin receptors, which are expressed in the epithelial cells targeted during the acute phase of infection [(*Alexandersen et al., 2003b*) and [*Monaghan et al., 2005*]). In the process of cell culture adaptation, FMDV acquire the ability to use other receptors like glycosaminoglycans (GAGs), which do not require the RGD motif. The host specificity to natural FMDV infection is thought to depend on FMDV binding to specific integrins although there are some other, yet undiscovered, mechanisms which determine FMDV susceptibility.

2.3 Host Affected

Animals that can be affected include cattle, swine, sheep, goats, buffaloes and wild ruminants (*Alexandersen and Mowat, 2005*).

FMD in *Bovinae* is characterized by fever (often above 40 °C), excessive salivation, lameness, depression and decreased milk production. The mucosa of the lips, dorsum of the tongue, and the dental plate are most severely involved. The mortality rate is low and mostly young animals are affected due to myocardial necrosis (*[Alexandersen et al., 2003b], [Gulbahar et al., 2007] and [Kitching, 2002]*).

FMD in pigs primarily affects the feet. It is dominated by rather painful formation of vesicles in the epidermis of the feet (coronary band, interdigital clefts, bulbs), associated with severe lameness. Complications are seen, such as detachment of the hoof and secondary infection of disrupted aphthae (fluid-filled blisters), which may cause purulent arthritis of the pedal joint (*Kitching and Alexandersen, 2002*).

Clinical signs of FMD in sheep and goats are less severe; often only lameness through aphthae and inflammation at the cloves (*Alexandersen and Mowat, 2005)*.

2.4 Pathogenesis

FMD is a very fast moving vesicular disease of all cloven hoofed animals. The disease is characterized by fever, depression, excessive salivation, lameness and formation of vesicles on the mucous membranes of the oral cavity and muzzle, epidermis of the coronary band and inter-digital spaces, udder and teats. Necrosis of the heart muscle occurs in some young animals which is often fatal. The vesicles erode to leave ulcers that result in reduced food consumption, weight loss and emaciation. Secondary bacterial infections occur in the ulcers further complicates the case in some affected animals. While mortality is generally less than 3%, morbidity is very high and economic losses reflect decreased productivity and protracted convalescence of affected animals. FMDV can survive for extended periods in animal secretions and products such as milk and semen. But at the same time FMDV is inactivated by heating above 50 degree Celsius and is sensitive to both acid and alkaline treatments.

The virus can persist in the oral cavity of infected animals for long periods after acute infection. FMD virus may be shed up to 4 days before the onset of clinical signs and movement of infected animals prior to recognition of the disease is the most common means of virus spread. FMDV also survives for days to months in some processed animal and meat products. Clinical disease may develop in 2-14 days after infection depending on virus dose, strain and site of entry. Clinically FMD is characterized by lameness, anorexia, pyrexia, salivation reduced milk production in lactating animals and weight loss.

2.5 Infection route

The mechanism of spread of FMDV is primarily in the form of either aerosolized droplets, saliva, or through indirect contact by personnel or contaminated surfaces and then subsequently back to the respiratory tract (*Alexandersen and Mowat, 2005*). However, infection can also occur through lesion of the skin or mucous membranes, but this is a very inefficient entering route, unless abrasions or cuts are present (*[Alexandersen et al., 2003a] and [Donaldson et al., 1987]*). The site of penetration is influenced by droplet size, since only the smallest inhaled droplets reaching the alveoli of the lungs. Initial infection occurs predominantly at the epithelial surface of the soft palate and adjacent nasopharynx, with subsequent spread of virus through regional lymph nodes (*[Alexandersen et al., 2003a] and [Donaldson et al., 1987]*). The virus can be detected in the oral cavity by real time RT-PCR 1-3 days before the onset of the viraemia (*Alexandersen et al., 2003b*).

2.6 Subclinical and persistent infections

Inapparent infections of FMDV can be divided into two types. First, those animals that become infected and spread the virus without showing clinical signs and second, those animals in which the virus persists after recovering from FMD with clinical signs (*Sutmoller and Casas, 2002*).

FMDV persistently infected animals are defined as those being virus positive for a minimum of 28 days (*Sutmoller et al., 1968*) and usually are referred to as carriers. Persistently infected animals show only low-level excretion of FMDV from the pharynx of ruminants, for periods that are species and virus lineage associated (*Salt, 1993*). The maximum duration of the persistent infection ranges from 4 months in goats to 5 years in African Buffaloes (*Syncerus caffer*) (*Alexandersen et al., 2002b*). FMDV persistence in pigs could not be demonstrated and there is no information about persistence in Asian Buffaloes (*Bubalus bubalis*) (Alexandersen et al., 2002b).

Transmission of FMDV from carrier animals to susceptible hosts, under field conditions, has so far only been shown for African Buffaloes to cattle and impala (*Aepyceros melampus*) (*[Bastos et al., 2000] and [Dawe et al., 1994]*). However, under experimental conditions, saliva obtained from carrier animals injected into cattle and pigs has caused infection, although there is no information as to whether this also can happen under field conditions (*Alexandersen et al., 2002b*), or with closely related species as the Asian Buffaloes.

Unlike persistently infected animals, subclinically infected animals, i.e. those animals not developing obvious clinical signs, may be highly contagious (*[Gibbens et al., 2001] and [Holzhauer et al., 2001]*). Beside the immune status of the host, also the involved FMDV lineage plays a role in the subclinical course of the disease (*Bouma and Dekker, 2002*). If an outbreak is caused by vaccine strains, which shows reduced virulence (*Sa-Carvalho et al., 1997)*, subclinical infections can also be found. For example, during the FMD outbreak in 2007 in

the United Kingdom, which was caused by a vaccine strain (*Enserink, 2007*), only few of the FMDV infected animals showed clinical signs.

Subclinical infection can also be produced within vaccinated ruminant herds, with a high FMDV challenge in those animals with high immunity or a low challenge in animals with low vaccine titer ([*Hutber et al., 1999*] and [*Yadin et al., 2007*]). The possibility of those animals becoming carriers has, to the author's knowledge, not been examined. Nevertheless, it is likely that such animals may play an important role in the maintenance of FMD in endemic countries (*Kitching, 2005).*

2.7 Epidemiology of the disease

FMD is ah highly transmissible disease, and a limited number of infective particles can initiate host infection 66. Contaminated animal products, nonsusceptible animals, agricultural tools, people, vehicles, and airborne transmission (*A.I.Donaldson et al.,1987*) can contribute to the mechanical dissemination of FMDV. The epidemiology of FMD is complex, ant 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 disseminataion of the virus (*H.Nishiura and R.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 [(*N.M.Farguson et al.,2001) (F.Sobrino and E.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. Of the different serotypes prevalent in Bangladesh , type O once were most predominant over other serotypes but recently type A and type Asia 1 has been also found which were not found in the earlier years. The agro climatic and socioeconomic condition , mixed animal husbandry practices, unrestricted movement, and trade among animals and porous international border provide a conductive epidemiology niche for the FMDV to flourish, mutate , and persist over time and to affect the susceptible animal population [(*B.U.Rao,1989)(N. Mishra et al.,1998)].* In Bangladesh FMDV is an endemic disease and every year a staggering amount of economic loss pursued in Bangladesh by this dreadful viral disease. The losses are mainly due to reduction in milk yield, draught power, and breeding capabilities. In Bangladesh, it is a leading cause of loss of livestock economy (direct and indirect losses) due to its endemic nature.

2.8 Non-structural protein as antigen to differentiate infected from vaccinated animals

Non structural proteins are continually found as an effective mean of differentiating infected from vaccinated cattle to effectively monitoring post-vaccination sero-serveillance and help to identify career cattle from the vaccinated animals. Recombinant non structural protein has a major advantage of use directly on living cattle in the field where NSPs were evaluated in Delayed Type Hypersensitivity skin tests which measures Th1 type cellular immune responses, where the most T cell immunogenic non-structural proteins were 2B, 2C and 3D and that could be a very useful tool to differentiate infected from vaccinated cattle (*Foster, M., et al 1998*). Constructed gene encoding non-structural protein of O/SKR/2000 FMDV to express under polyhedron promoter of Baculovirus and the expression was confirmed by immunoflourescence assay and western blotting. In this case, expressed NSP used in indirect trapping ELISA where monoclonal antibody against expressed NSP as trapping antibody was developed to differentiate infected from vaccinated cattle and proved suitable for a differential diagnosis method in Cattle (*Kweon et al 2003*). A radioimmunoprecipitation method in mice reported the presence of antibodies against non structural protein inoculated with FMD virus and by thus it was possible to differentiate between cattle exposed or non-exposed as well as protected and possible virus carriers in the field (*Berger, H.G. et al 1990*).

2.9 NSP ELISA for differentiating Infected from Vaccinated animals (DIVA)

Control of FMD mainly encompasses vaccination and slaughter policy, particularly for the FMD-free countries, and slaughter policy is considered a fundamental measure. However, some restrictions from economic situation, social culture, geographical and natural environment limit this policy to be applied in a large-scale range in the endemic areas. Consequently, vaccination or the combination policy of vaccination and slaughter remain the most effective counter measure against FMDV. Though different type of vaccine comprising subunit vaccine, peptide vaccine, DNA vaccine are developed, FMD inactivated vaccine still play a key role in control campaigns and eradication of FMD *(Doel TR et al.,2003)* in the majority FMD epidemic countries and territories because of perfect protection potency. However, another new problem arises from inactivated vaccine of FMD is that it is difficult to distinguish vaccinated from infected animals. And then, A range of ELISA techniques are currently being evaluated with the intention of producing ELISAs for routine diagnostic use which are capable of detecting antibody to FMD virus NSP's. These methods were described as following:

3D Protein 3D, also known as the virus infection associated antigen (VIAA) since antibodies to this antigen would be detected in serum from recovery animals *(Cowan KM et al.,1966)*, is the

core subunit of the virus-encoded RNA-dependent RNA polymerase *(Flanegan JB et al., 1977)* and responsible for proteolytic cleavage and viral replication *(Clavijo et al., 2004)*.

The antigenicity of 3D protein is highly conserved among all serotypes *(O'Donnel VK et al.,* 1996) holding out the possibility of a single serological test capable of detecting infection with any of the seven serotypes of the virus. 3D polymerase is the first NSP to be used to distinguish FMDV infected from vaccinated animals. The traditional VIAA is a semi-purified antigen prepared from the virus grown in tissue culture. When it is used in ELISA, there are problems of inadequate reproducibility appearing to be an inherent characteristic of the semi-purified nature of the VIAA preparations (World Reference Laboratory, unpublished findings). In order to address these problems, recombinant VIAA antigen has considerable attractions. When used in a simple indirect ELISA, recombinant 3D can differentiate infected from naive cattle *(Villinger F et al., 1989)*. The sensitivity of the test is only slightly lower than the conventional liquid phase blocking ELISA of Hamblin et al. *(Villinger F et al., 1989)* and the specificity is approximately 95%. Moreover, the 3D antibody tests can be used to monitor viral activity in large cattle populations and for certification of FMDV free animals for import and/or export testing *(O'Donnel VK et al.,* 1996) Whereas the following research showed that repeatedly vaccinated animals can develop antibodies to 3D, which demonstrate 3D is insufficient to differentiate infection from vaccination.

3B Protein 3B, also known as VPg, is the viral genome-bound protein. It exists in three nonidentical copies and covalently bound to the 5' end of the genome and serves as a primer for virus genome replication *(Hamblin C et al., 1986)*. Besides, the VPg copy number also has a substantial association with the host range and virulence *(Paul AV et al., 1998)*. An epitope-blocking ELISA (EB-ELISA) exploring combination of MAb to 3B core repeat motif (QKPLK) and purified recombinant 3AB protein was developed by Oem et al. *(Oem JK et al., 2007)* to evaluate FMD-free herds and vaccinated cattle, pigs*(Filgueira MP et al., 2000)*

Non structural protein 3AB efficiently expressed in Baculovirus system (Sorensen KJ et al). In DIVA technique to identify infected from vaccinated animals, non-structural polyprotein 3AB detect antibodies from 10 to as late as 900 days post-infection with higher sensitivity (96%) than commercial 3ABC ELISA kit particularly with samples collected from the late stages of infection(*Mohapatra JK et al 2011*). Experiment drawn to distinguish FMDV infected farm from immunized farms with commercial vaccine strategy, epitope blocking ELISA using monoclonal antibodies to target 3AB and 3B core repeat motif with 99.8-100% specificity (*Oem JK et al 2007)*. Non-structural polyprotein 3AB were giving positive reactions when serial studies were performed to test FMD virus against sera isolated from pigs and sows of 16 weeks and 3.5 years post outbreak period. No positive reaction was recorded in sows with at least 10 vaccinations. Maternally derived 3AB antigen to piglets lasts for 13 weeks indicating a good substitute for sow serum to monitor infection status and differentiate infected from vaccinated pigs (Chung WB et al 2002). Non structural polyprotein 3ABI is also used as an alternative to imported kits for FMD internal screening and transboundary sero –surveillence *(Jaworski,JP et al 2011*). Recombinant

non structural polyprotein 3AB was expressed as a formation of inclusion bodies in E.Coli and then purified with Ni-NTA HisBind Resins. Indirect ELISA based on 3AB as a coating antigen that could specifically react with FMDV infection antibodies but no reaction with immune antibodies induced by vaccine and speculated as a promising moleculer marker for DIVA ELISA *(Shao J. et al 2011).*

3ABC The polypeptide 3ABC, playing an important role during the different stages of viral replication [*(Ryan MD et al.*, 1989)*(Xiang W et al., 1997)(O'Donnell VK et al., 2001)*] is perceived by the most researchers as the most appropriate antigen to distinguish infection from vaccination because of its high immunogenicity and relatively low concentration in FMDV-infected cell lysates. Numerous different mode of 3ABC-ELISAs based on the E.coli expression system were developed to discriminate naïve, infected, vaccinated pig/cattle/sheep and detect silent infections/subclinical cases, or survey field samples in FMD-vaccinated or infected populations [*(Rodriguez A et al.,1994)(Diego DM et al., 1997)(Brocchi E et al., 1998)(Blanco E. et al., 2002) (Kweon YH et al., 2003)]*. In order to imitate native protein as far as possible, the NSPs expressed in baculovirus expression systems [*(Mezencio JM et al.,1998)(Sorensen KJ et al., 1998)*] was searched. In 2003, Kweon et al established Mab linked indirect-trapping ELISA using the baculovirus expressed 3ABC (mainly amino acid 1417-1835) and MAb against 3A. The equal sensitivity and specificity were acquired in comparing with commercial kits (baculovirus expressed 3ABC I-ELISA from USDA and Mab (3A) linked E. coli expressed 3ABC I-ELISA from IZSLE) during retrospective sero-surveillance. Then, Sørensen et al. *(Sorensen KJ et al., 2005)* modified the method of Kweon et al. and removed non-specific reactions in sera of cattle and sheep by filtration and inactivation. Due to the antigen capture strategy can sharply simplify the purification step of recombinant protein, Clavijo et al. [76] *(Clavijo A et al., 2004)*established a biotinylated 3ABC competitive ELISA(cELISA), which demonstrated no differences between species (cattle, sheep, pigs) and virus serotypes. Other indirect and competitive ELISAs detecting antibodies to 3ABC have been shown to have equivalent diagnostic performance characteristics [*(Brocchi E et al., 2006)(Lu Z et al., 2007)]*. However, the NSPs expressed in E. coli and baculovirus expression systems may sometimes create problems in the interpretation of the results on account of non-specific reactions. Furthermore, the number of epitopes found on such a long recombinant protein may interfere with antibodies to other picornaviruses.Moreover, chemically synthesized synthetic peptides of NSPs were used for DIVA [*(Meyer RF et al., 1997)(Oem JK et al., 2005)(Shen F et al.,1999)]*. Subsequently, Foord et al. *(Foord AJ et al., 2007)* provided a C-ELISA format on the basis of complete bacterial expression systems in 2007. The combination of recombinant antibody single chain variable fragments (scFv) from phage display libraries with E.coli-derived recombinant 3ABC reflected the best performance in detecting sera from cattle, sheep and pigs representing naïve, FMDV-vaccinated or FMDV-infected animals. The biggest advantage of this test is safe, economical and without the need for infectious virus, the use of laboratory animals or the costly maintenance of viable hybridoma cell lines. The results indicated that scFv displayed the potential to replace polyclonal or monoclonal antibodies in such assays.

Other NSPs or NPs ELISAs based on other NSPs and NPs or assays using infection-specific epitopes of NSPs to remove cross reactivity are being explored [*(Höhlich BJ et al., 2003),(Silberstein E et al., 1997),(Meyer RF et al., 1997)]*. Moreover, chimeric proteins that infection related B-cell epitopes of FMDV NSPs, which self-assembled into chimeric tymovirus-like particles(TVLPs), was selected as a candidate antigen for use in I-ELISA for DIVA of different species from the field *(Foord AJ et al., 2007)*. The function of the nonstructural proteins 2B is not very clear, although it has hydrophobic domains [*(Hema M. et al., 2007)(Robertson BH et al.,1985)]*.

However, a 2B peptide ELISA, described by Inoue et al using a chemically synthesized 2B peptide (RSTPEDLERAEKQ) as antigen showed more competitive strength than other NSP tests in the detection of the early period of infection because of the characteristic of earlier induction (as early as 1-2 weeks after infection) and longer persistence of the antibody to 2B. Then, Ko et al. reported rP13C ELISA in 2009, which explored the recombinant protein (rP13C) expressed in insect cells as a diagnostic antigen. The higher endpoint titers than LPB-ELISA and virus neutralization test (VNT) was represented in the measure of sera from goats challenged with FMDV post-vaccination.

CHAPTER 3

MATERIALS & METHODS

3.1. Study Location

This study was carried out at Bangladesh Livestock Research Institute, Savar and Central Disease Investigation Laboratory, Dhaka, Bangladesh.

3.2. Study Area and subjects

The study area of this investigation was different government dairy farm, private owned dairy farm. Samples were collected from Bangladesh Livestock Research Institute, Savar Millitary Dairy Farm, Central Cattle Breeding Station, Jessore Military Dairy Farm, private owned dairy farm from Sinduria and Mothertek village, Jessore, Hazaribag Abattoir and Kaptanbazar Abattoir. Total 218 numbers of blood sera were collected during sampling time. All samples were collected as to check post-infection serosurveilance with or without clinical signs.

3.3. Study Design

The study subjects were devided into the following categories based on their vaccination status.

Animal Details	No vaccination	Vaccinated Stock	Vaccine history not found	Vaccination strategy not properly taken	Total
Cross breed Cattle	**31**	**55**	**14**	**13**	113
Native Cattle (Non descriptive)	**73**	**0**	**0**	**7**	80
Pabna Cattle	**0**	**6**	**0**	**0**	6
Red Chittagong cattle	**0**	**19**	**0**	**0**	19
Total	**104**	**80**	**14**	**20**	218

Table 3.3: Study Design

All the blood sera were collected for post-infection serosurveillance after FMD outbreak has occurred during November 2011 to May 2012. All sera were tested against 3AB3 non-structural protein as antigen in DIVA ELISA method.

The study groups were compared in the following parameters:

1. Antibody response against crossbreed cattle and native cattle.
2. Vaccination status versus antibody response against NSP 3AB3.
3. Vaccination status versus breed variation.

3.4. Laboratory Diagnosis for DIVA ELISA

3.4.1. Sample Collection

Cattle blood was collected into 10ml fresh syringe with needle and put it into 45^0angle for effective serum separation. After collection, serum was isolated into ependorf tube. To separate serum from other blood impurities, Hemocentrifuger was used immediately to isolate serum from blood impurities.

Fig 3.4.1: Blood collection

3.4.2. Serum isolation

12µl of blood serum is needed for each sample. Fresh, refrigerated or previously frozen serum may be tested.

Fig 3.4.2: Serum sample

3.4.3. Serum Preservation

All serum was preserved at -20^0c after haemocentrifugation upto test.

Fig 3.4.3: -50^0C,-20^0 C and 4^0 C freezer

3.4.4. NSP antibody production in FMDV exposure

In an animal exposed to FMD virus, antibodies against NSPs are detectable by about 7-10 days post infection and will reach a peak by one month. The peak level will be maintained for about more than a year and then gradually wanes. Hence the most ideal time for sample collection is one month post infection.

3.4.5. 3AB3 NSP Based ELISA Kit

A 3AB3 NSP based indirect ELISA kit with about 90% sensitivity and 98% specificity for DIVA has been designed, developed and evaluated completely using in house produced and standardized reagents at central laboratory, Project Directorate on FMD, Muktesswar very much in toe with the OIE approved test for assessing the FMD prevalence in the country. This kit is useful for bovines only.

3.4.6. Test principle

Seroconversion against NSPs (3AB3) is observed since 10-14 days after FMD virus infection. Whereas if the animal is not exposed to FMD virus infection but vaccinated with inactivated purified polyvalent FMD vaccine no anti-NSP immune response is elicited in host's body . This differential induction of anti-NSP antibody is exploited in DIVA ELISA to discriminate between infected and vaccinated animals. In this DIVA test reactivity of anti-3AB3 antibodies present in the serum of an infected animal (bovine species only) is assessed against purified recombinant 3AB3 ($\sim 38kd$) NSP in an indirect ELISA format. A sample producing OD value more than the fixed cut-off ratio (i.e., percent positivity value or PP value ≥ 40%) is qualitatively diagnosed as positive for FMD infection.

$$\text{Fixed cut off ratio} = \left\{ \left[\frac{\text{Test serum sample mean OD}}{\text{Positive control serum means OD}} \right] \text{X } 100 \right\}$$

The currently used FMD vaccines consist of purified preparations of inactivated virions and therefore induce antibodies almost exclusively to the structural capsid proteins of the virus in the vaccinated host, provided the vaccine batch is significantly free from non structural proteins . But replication of virus during infection results in the production of a number of NSPs , of which some are immunogenic and these NSPs can be exploited as markers for detection of FMD infection. Hence while antibodies to the capsid proteins are induced by both vaccination and infection, antibodies towards NSPs are elicited only during FMD virus infection. Serological tests like liquid phase blocking ELISA detect antibodies to the structural capsid proteins of the virus. It is not possible, therefore, to differentiate animals that have been infected from those that have been vaccinated based on the detection of antibody to structural protein in these tests. Hence differentiation of infection from vaccination based on the detection of the antibodies to NSPs, in particular 3ABC or 3AB has been established to be a reliable indicator.

Differentiation of these two categories of animals (DIVA) is important during serological surveys to detect evidence of infection and circulation of virus, as a follow up to intensive vaccination campaign in FMD endemic countries and for import/export serology. Besides, this NSP based antibody screening can be used for detection of subclinical carrier status, where the virus is actively replicating but without conspicuous signs. Anti-3AB3 antibodies have been detected in animals where no virus or occasional virus isolation from oropharyngeal fluid could be done. The

usefulness of this diagnostic test is more appropriate in context of mass scale herd based surveillance rather than for judging individual animal infection status.

The present DIVA kit as described here is a recombinant 3AB3 NSP based indirect ELISA kit designed , developed and evaluated using in-house produced and standardized reagents at the central FMD laboratory, project directorate on FMD, Muktesswar , This DIVA kit is very much in toe with the OIE approved test as a companion test for inactivated vaccines.

3.4.7. Materials

1. 96- Well Nunc Maxisorp ELISA plates (Cat. No. 442404)
2. Freeze dried recombinant 3AB3 protein (Store at -20^{o} C)
3. Freeze dried positive and negative control sera (Store at -20^{o} C)
4. *E.Coli* lysate (Store at -20^{o} C)
5. Lapin Anti Bovine – HRP conjugate (DAKO; Cat. No. P0159) (Store at 4^{o} C)
6. Skimmed milk powder (Merck; Cat. No. 1.15363.0500)
7. Chicken serum Sera laboratories International Ltd.; Cat. No. S-606-HSL (Store at -20^{o} C)
8. Salts for Phosphate buffer solution, pH 7.4
9. Tween – 20
10. Carbonate – Bicarbonate coating buffer (Sigma; Cat No. C3041)
11. Phosphate – Citrate substrate buffer (Sigma; Cat. No. P4809)
12. OPD (Sigma; Cat No. P1526-100G) (Store at -20^{o} C)
13. 30% H_2O_2 (Merck; Cat. No. 1.07210.0250)
14. 1M H_2SO_4

3.5. Laboratory Methods

3.5.1. Preparation of Freeze dried recombinant 3AB3 protein

Each vial is to be dissolved in 1ml of Carbonate-Bicarbonate coating buffer, pH 9.5 and then added to 10 ml of additional coating buffer. This reconstituted vial is sufficient for coating two 96-well immunoplate. After reconstitution the vial is to be used immediately without storing further.

3.5.2. Preparation of Freeze –dried positive and negative control sera

Each vial is to be dissolved in 160 µl of distilled water and then distributed into single use aliquots for storage at – 20º C, if to be consumed in more than a month's time or else store the entire reconstituted vial at 4ºC. Working dilution of 1: 20 in diluent buffer for control serum is recommended.

3.5.3. Preparation of *E. Coli* lysate

Each vial of E. coli lysate is to be reconstituted with 70 µl of PBS and stored at – 20º C.

3.5.4. Composition of ELISA BUFFERS

3.5.4.1 Washing Buffer (1X): 1Lit

- NaH_2PO_4. $2H_2O$ 0.39g (0.0025M)
- $Na_2HPO_4.2H_2O$ 1.336 g (O.0075M)
- NaCl 29.325 gm (2.93% w/v)
- Tween-20 500µl (0.05% v/v)
- Distilled water to make 1 liter

(Check pH. If required adjust pH with IN NaOH as 7.2- 7.4)

3.5.4.2 Coating (Carbonate-Bicarbonate a) Buffer

One Carbonate-Bicarbonate capsule dissolved in 100ml distilled water. Check the pH as 9.6. Store at 4º C.

3.5.4.3 Diluent Buffer: 20 ml

- Skimmed milk powder 0.6gm (3%w/v)
- Chicken serum 2 ml (10% v/v)
- *E.Coli* lysate 2.5 µl (0.01% v/v)
- Washing buffer Up to 20 ml

3.5.4.4 Substrate (Phosphate-Citrate)Buffer : 100 ml

One phosphate-citrate tablet dissolved in 100 ml distilled water. Check pH as 5.0. Store at 4ºC.

3.5.4.5 Substrate solution : 7.5 ml (Prepare immediately before use)

- Substrate (Phosphate - Citrate) Buffer 7.5 ml
- OPD 5 mg
- H_2O_2 (30%) 4 µl

3.5.4.6 Conjugate solution

- Diluent buffer 6 ml
- Anti-bovine HRP conjugate 3 µl

3.5.4.7 Stopper solution (1M H_2SO_4): 100 ml

- H_2SO_4: 95-97% 5.55 ml
- Distilled water 94.45 ml

3.6. Laboratory Procedure

3.6.1 Recombinant protein preparation for ELISA plate Coating

A. Dissolve freeze dried recombinant protein in the vial with 1ml coating buffer first.
B. Then add it into another 4.5ml of coating buffer to another bottle. The reconstituted protein vial should be used for coating immediately (96 well standard DIVA ELISA plate) to all well at 50µl per well.
C. Store the plate at 4^oc incubation overnight after gentle tapping from all side.
D. Remove the plates from the refrigerator and thaw them at 37^0C for 15 minutes.

3.6.2 Serum Dilution

E. In a low protein binding Perspex plate dilute the test and the supplied negative and positive control sera @ 1: 20 in diluent buffer.
    a. Take 228 µl of diluent buffer in each well of Perspex plate; dispense 12 µl of serum in each well.
    b. So, total amount will be 240 µl. Here dilution ratio is 1: 20.
    c. Dilution act is performed by frequent dispensing.
    d. Each time of dilution pipette tip has to be changed.

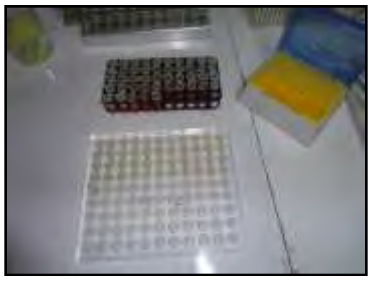


Fig 3.6.2 : Persplex plate for sample dilution with coating

F. Only serum from bovine host is compatible with this ELISA plates in duplicate. On a coated ELISA plate a total of 45 test sera samples, one positive and one negative control

serum and two background controls can be accommodated. For background controls only 100 µl of diluent buffer is dispensed without any serum.

G. Incubate for 1 hour at 37^{O}C and tap the plate gently from all sides at every 15 minute intervals.

Fig 3.6.3: 37^{0}C incubator

H. Give three continuous wash (no hold time) with wash buffer.
I. Transfer 100 µl of the serum and diluent buffer mixture from Perspex plate to the ELISA plate in duplicate wells.
J. Incubate for 1 hour at 37^{O}C and tap the plate gently from all sides at every 15 minutes intervals or incubate for 30 minutes in a plate shaker at 37^{O}C with20-30 rpm.
K. Give three washes of 3 minutes soak period each.

3.6.3 Addition of anti-bovine – HRP conjugate

L. Dispense anti-bovine – HRP conjugate diluted in the diluent buffer (1:2000) @ 50 µl per well.
M. Incubate for 1 hour at 37^{O}C and tap the plate gently from all sides at every 15 minute intervals.
N. Give three washes of 5 minute soak period each.

3.6.4 Addition of Substrate solution

O. Add freshly prepared substrate solution @ 50 µl per well and incubate for 15 minutes at 37^{O}C without shaking.

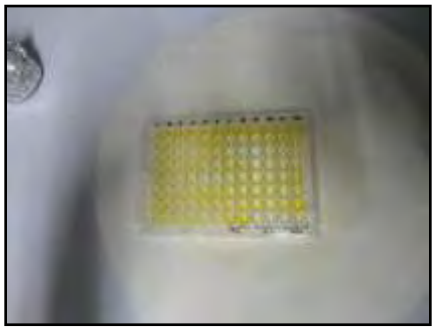


Fig 3.6.4: ELISA plate after substrate addition

3.6.5 Addition of Stopper solution

P. Then stop color reaction by adding stopper solution 1M H2SO4 at 50 µl per well.

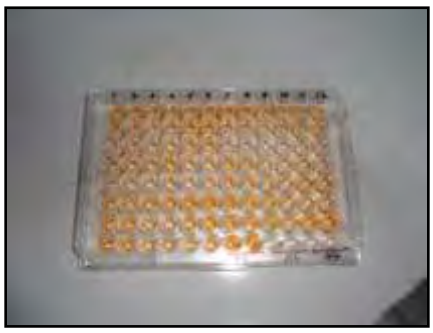


Fig 3.6.5: DIVA ELISA plate after addition of stopper solution

Q. Measure the optical density values at 492nm using Gene 5 software and get the result.

Fig 3.6.6: ELISA plate reader and washer

3.7. Performance and Interpretation

The test is to be considered valid provided the mean absorbance of the positive control wells is not less than 0.8. Likewise the plate has to be rejected if the mean absorbance of the supplied negative control serum is > 0.3. The OD in back ground control wells should be less than 0.1.

To reduce inter-run variation due to differences in absolute absorbance between runs, final results for each test serum needs to be expressed as the PP value calculated by deviding the reaction of the test serum by that of the positive control serum and then multiplying with 100. The results should be interpreted based on the following cut-off zones :

- 3AB3 NSP reactivity positive: If PP value is more than 40%
- 3AB3 NSP reactivity negative: If PP value is less than 40%

3.8. Statistical analysis of the data

Data analyses were carried out using the Microsoft Excel and Profession Strata 9.0 version software. Following data input into the excel sheet, the acquired result were analyzed by using Microsoft Excel Equation and professional Strata software. Various relationships among variables have been explored to match the possible outcome of the test. Microsoft Excel Software was used for making various graphs, tables. Strata 9 was used for graph preparation and measurement of significance level as comparison between the values of different variables were than analyzed by , Pearson's Chi-square test to evaluate statistical differences or significance between and/or within variables, where appropriate. A chi-square value of $\leq .05$ was the criterion for a statistically significant result between variables.

CHAPTER 4

RESULTS & DISCUSSIONS

4.1 Results

The DIVA ELISA reported here uses a non-structural 3AB3 recombinant protein and two polyclonal antisera, one as capture (Bovine) and the other as detector (Guinea pig). It is based on the ability of the antibodies anti-3AB3 contained in the test serum to block the trapping of 3AB3 antigen by capture antibody.

A sample producing OD value more than the fixed cut-off ratio (i.e., percent positivity value or PP value ≥ 40%) is qualitatively diagnosed as positive for FMD infection assured good differentiation between infected and vaccinated cattle.

4.1.1. Screening for antibodies against NSP

A total of 218 cattle sera were collected from Bangladesh livestock research institute, Savar Military Dairy Farm, Central Cattle Breeding Station dairy farm, Jessore Military Dairy Farm, Lalteer Livestock dairy Farm, Hazaribag Abattoir and Kaptan bazaar abattoir, Jessore and two village, namely Sinduria and Mothertek near BLRI.

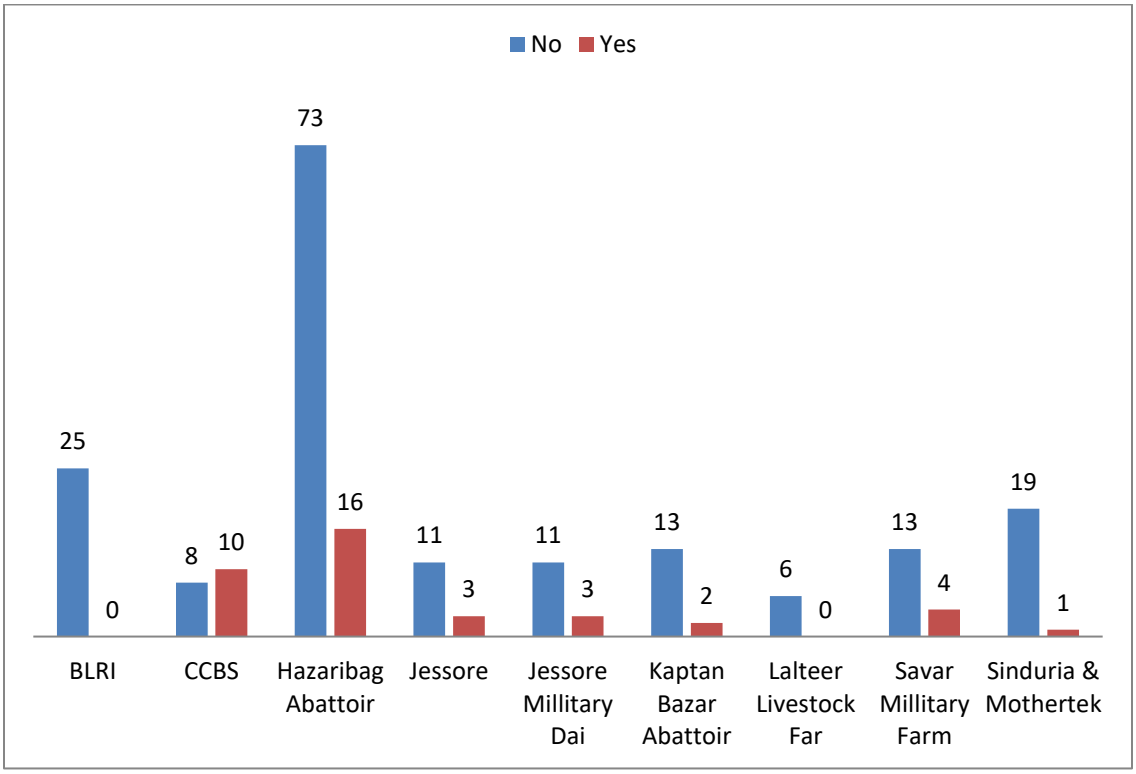


Fig 4.1.1: Sample collection status from different farms with DIVA positive result.

Native cattle (included Pabna cattle and Red Chittagong cattle) and crossbreed cattle sera were grouped into several orders in respect of vaccination strategy taken by the authorities or owners. A total of 104 cattle sera irrespective to breed were fallen into no vaccination category, 80 cattle sera irrespective to breed were fallen into vaccination group, 14 cattle sera irrespective to breed were fallen into the group where vaccination history was not found and 20 other cattle sera were

fallen into group where vaccination history was not properly maintained (Table).Body condition of the animal at the particular time of sample collection includes salivation, blister in tongue epithelium, emaciated , previously infected with no obvious sign and symptoms and apparently healthy stock.

Animal Details	No vaccination	Vaccinated Stock	Vaccine history not found	Vaccination strategy not properly taken	Total
Cross breed Cattle	**31**	**55**	**14**	**13**	113
Native Cattle	**73**	**0**	**0**	**7**	80
Pabna Cattle	**0**	**6**	**0**	**0**	6
Red Chittagong cattle	**0**	**19**	**0**	**0**	19
Total	**104**	**80**	**14**	**20**	218

Table 4.1: Vaccination status of the collected serum

The prevalence of antibodies against NSP r3AB3 within herds with visible clinical signs of FMD ranged between 13% and 56%. Among the farms, CCBS dairy farm had high prevalence of antibodies elicited against NSP , Jessore and Jessore Military Dairy Farm had 21.42% prevalence rate respectively, Hazaribag Abattoir had 17.97% prevalence rate during that time frame while Kaptanbazar abattoir and Savar Military farm were experiencing 13.33% and 13.52% prevalence rate. A 5% prevalence rate of antibodies elicited against NSP in 20 cattle detected in sera collected from two nearby villages at BLRI (Table 4.2)

Antibody against 3AB3

Place	No	Yes	NSP Positive (%)	Total
BLRI	**25**	**0**	**0**	25
CCBS	**8**	**10**	**55.55**	18
Hazaribag Abattoir	**73**	**16**	**17.97**	89
Jessore	**11**	**3**	**21.42**	14
Jessore Military Dairy Farm	**11**	**3**	**21.42**	14
Kaptan Bazar Abattoir	**13**	**2**	**13.33**	15
Lalteer Livestock Farm	**6**	**0**	**0**	6
Saver Military Dairy Farm	**13**	**4**	**13.52**	17
Sinduria & Mothertek	**19**	**1**	**5**	20
Total	**179**	**39**		218

Table 4.2: Antibody status against NSP 3AB3 in collected serum samples.

All sera collected from BLRI dairy farm and Lalteer Livestock dairy farm were negative in NSP-3AB3 ELISA. Altogether cattle sera collected from two villages with a history of recent FMD outbreak had significant lower prevalence of antibodies against NSP-3AB3 then herds recovering from FMD outbreaks. As authorities in these farms are known to receive and/or exchange animals, this may have lead to acquisition of these positive animals on the farms.

All blood samples were collected from November 2011 to May 2012 after a major outbreak of foot and mouth disease in Bangladesh.

4.2. Discussion

FMD is one of the most contagious epidemic diseases of livestock and can spread very rapidly. Serological tests that allow discrimination between antibodies resulting from infection and vaccination are now becoming available and should permit more accurate monitoring of control and eradication programme based on mass vaccination. FMD vaccination is , unfortunately , still carried out in a haphazard manner in many countries , resulting in the disease remaining endemic for long periods (Maddur et al., 2008).

Vaccination plays an important role in the control of FMD in Asia, Middle East, Africa, and South America. Using conventional diagnostic techniques, up to now it was not possible to distinguish FMD infected animals from purely vaccinated animals. In vaccinated areas disease control authorities had limited possibilities to monitor virus presence or circulation. An ELISA using

baculo virus expressed 3AB as the antigens has been demonstrated to successfully differentiate vaccinated from infected cattle (Sorensen et al., 1998).

The results of NSP-3AB3 DIVA ELISA test were proved the presence of antibodies against NSP of FMDV in cattle population in various governmental and non-governmental dairy farms which may be attributed to natural infection of FMDV or lack of effective vaccination strategies. As shown in Table the number of total positive samples is 39 samples out of 218 samples (17.88%). The highest percent of positive were found in Central Cattle Breeding Station (CCBS) dairy farm (55.55%). It is also worth to note that not a single case was found positive at Bangladesh livestock research institute and Lalteer livestock dairy farm.

Among organized farms, Bangladesh Livestock Research Institute Farm and Lalteer Livestock Farm (though infected) serum sample collected for DIVA testing had revealed 100% negative result.

In case of BLRI Farm vaccination is strictly practiced and vaccination strategy is well maintained and hence serum sample did not produce any antibody response against non-structural protein (r3AB3).

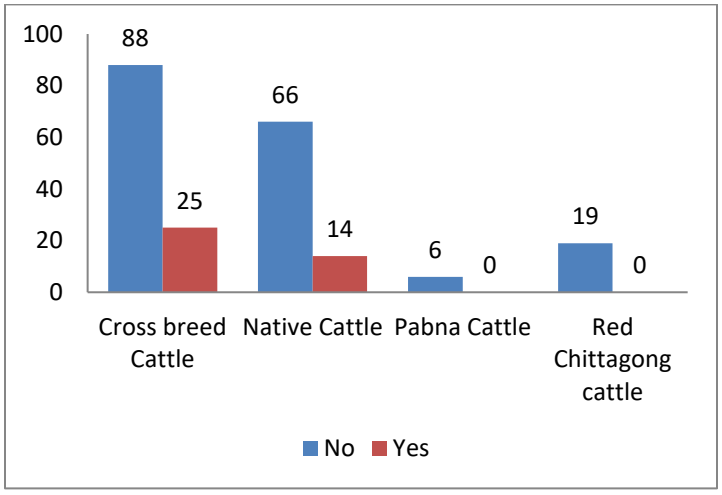


Fig 4.2: Antibody response against NSP-3AB3 in different cattle breeds.

Antibody against 3AB3

Animal Details	No	Yes	NSP positive (%)	Total
Cross breed Cattle	**88**	**25**	**22.12**	113
Native Cattle	**66**	**14**	**17.5**	80
Pabna Cattle	**6**	**0**	**0**	6
Red Chittagong cattle	**19**	**0**	**0**	19
Total	**179**	**39**	**17.88**	218

Table 4.3: NSP positivity versus breed status

In case of Lalteer Livestock there was no antibody response observed against non-structural protein because samples were collected within 10 days of FMDV infection and that's why seroconversion against NSP's didn't take place. Seroconversion against NSPs is observed since 10-14 days after FMD virus infection. But, in this study cattle blood was collected 4 days after post infection. Hence no antibody response is observed against NSP-3AB3.

In Jessore and Jessore Military Dairy Farm, 21.42% serum sample was identified as positive against NSP-3AB3 recombinant protein indicates vaccine that was either complicated with non-structural protein part or proper cool chain was not duely maintained. Another cause might be natural infection by one of the serotype where previous infection by another serotype couldn't confer any immunity. In Hazaribag and Kaptan bajar abattoir, cattle were mostly transferred from local cattle selling point and most of the cattle were either trespassed or imported from India.

This study revealed a staggering 55.55% of infection among the cattle sera collected from Hazaribag Abattoir during the period of March 2012 to May 2012. Most of the cattle sera collected from that abattoir were from native cattle, without any visible lesion and disease history. They were carrying silent infection among them. At Kaptanbazar abattoir, 13.33% silent infection was found in March 2012. At Savar Millitary dairy farm it was found 13.52% positive case against NSP-3AB3 protein in spite of rigorous vaccination strategy. This incidence was supported by the fact that locally produced vaccine was not able to confer immunity against NSP-3AB3. In this case, it is suggested that vaccine batch either contaminated with non structural protein or proper chain of standard during vaccination transportation was not ideally maintained.

As shown in Table the number of positive samples among indigenous cattle is 14 samples out of 105 samples (13.33%) while number of positive samples of crossbreed cattle is 25 samples out of 113 samples (22.12%) which is not significant (Table).

Breed Variation	Antibody against 3AB3		
	Yes	No	Total

Indigenous breed	14	91	105
Cross breed	25	88	113
Total	39	179	218
Pearson chi2=2.8631			Pr = 0.091

Table 4.4: Chi-square test result between different breeds in respect of antibody production against NSP 3AB3.

In table it is found that 17 cattle sera gave positive reation among 80 vaccinated cattle irrespective of breed preference (21.25%) whereas 21 out of 118 non-vaccinated cattle irrespective of breed preference (17.79%) gave positive reaction and only 5% cattle gave positive reaction among 20 cattle in which vaccination strategy was irregularly maintained at two nearby village at Savar district. It was also found insignificant in terms of antibody production against vaccination status. The backdrop behind it might be the cause of not maintaining appropriate vaccination strategy and contaminated vaccination is applied.

Antibody against A3B3			
	Yes	No	Total
Vaccinated	17	63	80
Non vaccinated	21	97	118
Irregularly vaccinated	1	19	20
Total	39	179	218
Pearson chi^2 =2.8777			Pr= 0.237

Table 4.5: Chi-square result between vaccination status and Antibody production status against NSP 3AB3

On the other hand in Bangladesh, people don't pay that much attention to native cattle than cross-breed cattle. According to Table it has shown that 55 cattle are regularly vaccinated and 13 cattle were irregularly vaccinated where 45 animals were not vaccinated among 113 cross breed cattle. On the other hand 25 and 7 native cattle were either vaccinated or irregularly vaccinated out of 105 native cattle. It clearly signifies that people are more considerate to their crossbreed animals than native animals. An interesting event was notified in case of the cattle of BLRI. Not a single case was recorded positive (Antibody response) against NSP-3AB3 (Table).

Breed Variation	**Vaccinated**	**Non vaccinated**	**Irregularly vaccinated**	**Total**

Indigenous breed	25	73	7	105
Cross breed	55	45	13	113
Total	80	118	20	218
Pearson chi2=19.4267			Pr= 0.000	

Table 4.6: Chi-square result between breed variation and vaccination status

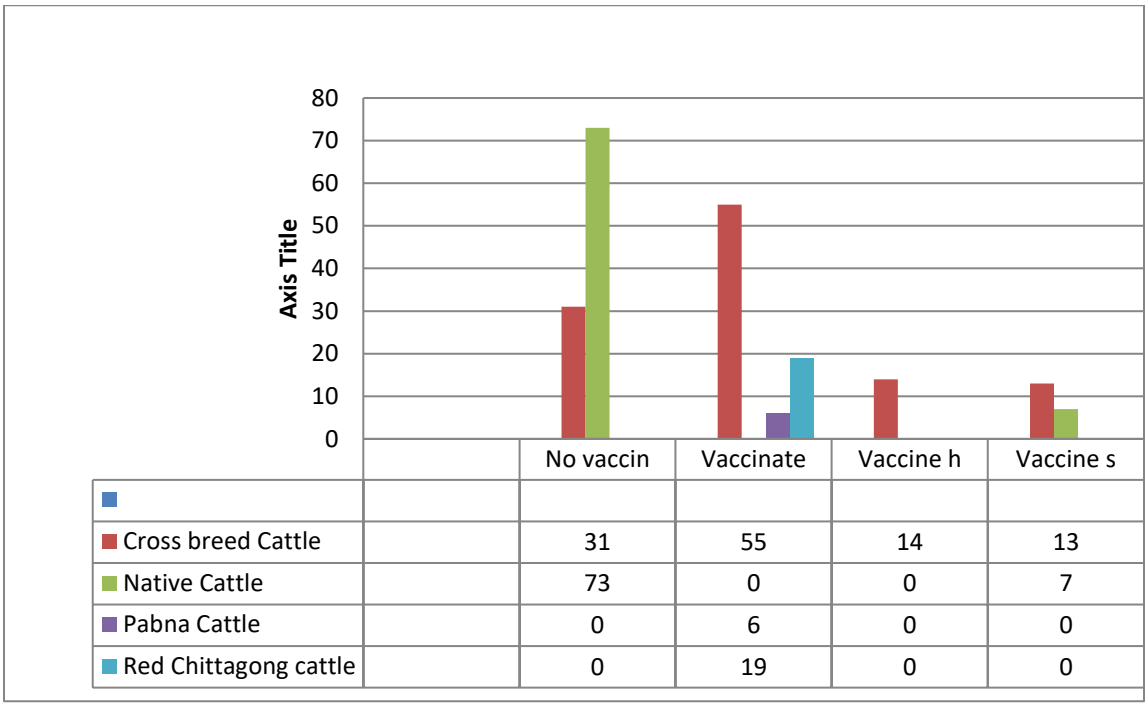


Fig 4.3: Vaccination status of native and crossbreed cattle identified from collected serum sample.

In case of Lalteer Livestock dairy farm, seroconversion was not completed in the cattle blood after 4 days of post infection. So no case was recorded as NSP-3AB3 positive in this case.

These results indicate that precise evaluation of FMD status in regions with a vaccination program is necessary to monitor the progress of control program of a control campaign. Serologic assays that detect antibody to nonstructural proteins have shown specificity in assessing the post infection state and offer various advantages for use in epidemiologic surveys and in serology for certification of FMD free animals. These results indicate that the ELISA-3AB3 method could be used as a complementary method for sero-epidemiological studies as an indirect indicator of viral activity among native and crossbreed cattle in this country. Such studies need to be undertaken to remediate baseline obstacles for more profitable livestock growth and boost up country economy every year.

Chapter 5

Conclusion

Conclusion

In Bangladesh livestock is the major controller of village economy till days. In village based economy Foot and Mouth disease single handedly cause a great damage mostly remained unanswered. It decrease milk and meat production, working ability has been seriously hamplered what turned into lower economic return and death of calves become an obvious reason for farm loss. People are not very much careful about vaccinating their livestock sincerely in both village and organized farm setup. Post infection sero surveillance study is not duely taken into consideration till now. In that particular reason, FMD carrier status among cloven hoofed livestock animals has remained as it was and livestock constantly become affected. The proper vaccination policy cannot be ensured in village level at all. This leads to spread of the virus. The virus is very much rapidly occupying the place surrounding 300km around by water course and merrily 60 km spread by air from the initiating point of FMDV infection.

In the above mentioned impact, the 3AB3 NSP based ELISA bears promising sensitivity and specificity to distinguish post infection FMDV serosurveillance by differentiating infected animals from vaccinated animals. This kit has also been demonstrated to be useful for monitoring the progress of the FMD eradication program in Bangladesh.

The baseline result helps in our understanding that FMD is endemically present in different government and military dairy farm of Bangladesh and random import of cattle from India without proper seromonitoring gives rise to FMD endemicity in this area due to very clinical state of this FMD disease what remains in disguise in imported animals' blood. The result also indicated that repeated vaccination also helps to induce NSP-antibodies as well as viral infection in clinical nature.

The present study was conducted to observe post infection serosurveillance status of various crossbreed and native animals in different farm setup which may in future be helpful for analyses of mechanisms why local vaccine could not give proper immunity to our domestic cattle and what implications are really necessary to take effective measure against Foot and mouth disease spread. This study also helps to draw post infection seromonitoring at herd level more quickly and efficiently that would definitely be helpful to induce proper vaccination strategy in our country.

CHAPTER 6

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Training Manual

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Book

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CHAPTER 7

APPENDICES

7.1. Materials needed but not provided with the test kit

1. Haemocentrifuger machine	2. Precision pipettes

3. Microcentrifuge tube	4. Disposable pipette tips
5. Distilled Water	6. Wash bottle
7. I Container : 1 to 2 litres of PBS-Tween	8. ELISA plate reader, 492 nm Filter
9. ELISA plate filter paper 2 bundle	10. 5mm Syringe for serum collection
11. Falcon tube for sample preparation	12. Weight balance
13. Sample rack	14. Measuring Cylinder: 1lit., 500ml.
15. Persplex plate : 2 plate	16. Permanent Marker pen
17. Stopwatch	

7.2 DIVA ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS1	1	2	2	3	3	4	4	5	5	6	6
B	7	7	8	8	9	9	10	10	11	11	12	12
C	13	13	14	14	15	15	16	16	17	17	18	18
D	19	19	20	20	21	21	22	22	23	23	24	24
E	25	25	26	26	27	27	28	28	29	29	30	30
F	31	31	32	32	33	33	34	34	35	35	36	36
G	37	37	38	38	39	39	40	40	41	41	42	42
H	43	43	44	44	45	45	PC	PC	NC	NC	BG	BG

@PC-Positive Control @NC- Negative Control @BG-Background

7.3. Preconsidering points during NSP-3AB3 DIVA ELISA

I. Carefully read and follow all instructions.

II. Store the kit and all reagents as mentioned.
III. Handle all materials according to the Good Laboratory Practice.
IV. Care should be taken to prevent contamination of kit components.
V. Do not use test kit beyond date of expiry.
VI. Use a separate pipette tip for each sample.
VII. Do not pipette by mouth.
VIII. Include a positive, negative serum and background control on each plate series.
IX. Use only distilled water for preparation of reagents.
X. All unused biological materials should be disposed according to the local, regional and national regulations.

7.4. Troubleshooting

1. No OD in any wells including positive controls:
    a. Improper reconstitution/preparation of reagents/buffers.
        i. Cross check the composition of buffers and reagents.
    b. Use of conjugate other than anti-cow conjugate.
        i. Cross check the conjugate used.
    c. Lower pH of washing buffer
        i. Cross check the pH of washing buffer.
2. Even OD in all the wells
    a. Cross contamination of tips used for conjugate and substrate solution.
3. Low OD
    a. Improper dilution of reagents.
        i. Cross check the dilutions recommended for each batch.
    b. Expiry of reagents of kits.
        i. Cross check the expiry of reagents.
    c. High percentage of tween-20 in washing buffer.
        i. Cross check the tween-20 concentration in washing buffer.
    d. Lower/higher temperature in incubator.

Cross check the temperature of incubator.

7.5. Advantages of NSP ELISA Technique as a New Tool for FMD Diagnosis

I. Detection of infected animals among a vaccinated herd.
II. Most reliable herd based surveillance test for assessing the prevalence rate in a region.
III. For retrospective FMD diagnosis in outbreaks where no antigen could be detected/ no virus isolated in suspected clinical sample due to low antigenic content, delay in sample collection, mild nature of the infection, deterioration of transport medium etc.
IV. The test can also be used for detection of subclinical carrier status, where the virus is actively replicating but without conspicuous signs.
V. Faster, more sensitive and can be carried out at a lower level of biocontainment.

7.6. Limitations of the Study

1. It is only a serotype independent diagnostic tool (The test can only identify an FMD infected animal but not the serotype involved).
2. It cannot differentiate recent infection and past infection as the NSP antibodies persist for a quite long time (about one to one and half years post infection).
3. The test cannot rule out the possibility of infection before one year.
4. If the vaccine is not completely pure (free from NSPs), vaccination can also induce some NSP antibody response (All modern FMD vaccines are based upon highly purified antigens, which are considered free from NSPs of the FMD virus).
5. Usefulness of this diagnostic test is more in context of herd based surveillance rather than for judging individual status. The DIVA system/approach makes possible the mass vaccination of a susceptible animal population without compromising the serological identification of convalescent individuals.