LABORATORY TRAINING ON FOOT AND MOUTH DISEASE DIAGNOSIS (2-7 May, 2011)

TRAINING MANUAL

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# CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>An Introduction to Foot and Mouth Disease</td>
<td>1</td>
</tr>
<tr>
<td>II.</td>
<td>Diagnosis of Foot-and-Mouth Disease</td>
<td>5</td>
</tr>
<tr>
<td>III.</td>
<td>Cell culture and Virus isolation</td>
<td>13</td>
</tr>
<tr>
<td>IV.</td>
<td>Identification of FMDV serotype by Sandwich ELISA</td>
<td>16</td>
</tr>
<tr>
<td>V.</td>
<td>Liquid phase blocking ELISA</td>
<td>21</td>
</tr>
<tr>
<td>VI.</td>
<td>Differentiation of Infected from Vaccinated Animals (DIVA) ELISA</td>
<td>26</td>
</tr>
</tbody>
</table>
Chapter I

An Introduction to Foot and Mouth Disease

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease 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 in adult animals it ranks first in the Office International des Epizooties list A diseases (OIE: Manual of standards, 2001) owing to nearly 100% percent morbidity, rapid spread, severe decrease in livestock production and mortality in young animals. 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 India, the disease is most concerned in cattle, buffalo and swine because of negative impact on rural economy principally based on these farm animals. As per an estimate there could be ~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. Furthermore, prolonged convalescence, short term protective immunity with no inter-serotypic cross protection and establishment of carrier status complicates the control and eradication of this devastating disease. Vaccination against FMD is a key element in the control of the disease in addition to slaughter and movement restrictions.

FMD is regarded as one of the most contagious viral disease caused by Foot and mouth disease virus (FMDV), a member of genus *Aphthovirus* in the family *Picornaviridae*. The earliest descriptions of FMD were of an outbreak in Northern Italy in 1514 (Frascatorri, 1545) and in Southern Africa in 1780 (Le Vaillant, 1795). Picornaviruses have played an important role in the history of virology and veterinary medicine as in 1897, Loeffler and Frosch for the first time demonstrated that FMD was caused by an
agent that passed through filters that held back bacteria. A century later the same virus was among the first animal viruses to have its structure resolved at the atomic level by X-ray crystallography. In second half of 19th century and first half of 20th century, repeated rapidly spreading epidemics of FMD resulted in great loses while intensive livestock production systems were developing in industrial countries. In 1884 the United States Congress created the Bureau of Animal Industry (BAI) within Department of Agriculture to deal with FMD and two other diseases, contagious bovine pleuropneumonia and hog cholera.

**Causative agent**

FMDV is a single-stranded positive-sense virus with a RNA genome of ~8.5 Kb. There are seven distinct serotypes, designated as O, A, C, South African Territories (SAT) 1-3 and Asia-1. There is also extensive genetic heterogeneity in individual serotypes, with many distinct virus subtypes/variants occurring within each serotype. Viral genome encodes four structural proteins (VP1-4) and at least 8 non-structural proteins (NSPs) in form of a unique polyprotein from which the different viral polypeptides are cleaved by viral proteases. 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 NSPs of FMDV consist of L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D. The VP1 protein (1D gene) is mostly responsible for virus neutralization and contains a conserved RGD integrin binding motif that binds virus to integrin cellular receptors. Both structural and non-structural antigens induce the production of antibodies in infected animals. But vaccinated animals which have not been exposed to replicating virus will develop antibodies only to the viral antigens (structural proteins) in the inactivated material. This has become basis for differentiation of FMD infected and vaccinated animals (DIVA) assay.

**Pathogenesis**

FMD is a vesicular disease of all cloven hoofed animals including elephants and pigs. The disease is highly contagious, rapidly spreading disease and even some authors refer FMD as Fast Moving Disease. Only cattle and swine exhibit enough characteristic clinical lesions for diagnosis. Sheep and goats, although are susceptible to FMD but exhibits clinical signs similar to other diseases such as PPR and BT. In addition, wild
animals also show variable clinical sings which makes diagnosis difficult. The disease is characterized by fever, depression, excessive salvation, 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°C and is sensitive to both acid and alkaline treatments.

Predominant route of FMDV infection is respiratory, although ingestion of contaminated food and direct inoculation also are both highly effective in transmitting infection although greater doses of virus are required. 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. Compared to cattle, swine secrete thousands of times more aerosolized infectious virus and may be called as FMDV amplifiers. 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. Mastitis may be a sequel. In uncomplicated cases, resolution of the infection is usually complete by 14 days after infection.

**Epidemiology**

FMD was once prevalent all over the world but due to strict control and eradication measures adopted by industrial countries limit the disease prevalence. Seventy countries in the world are already officially recognized by the OIE as free from FMD with or without vaccination while more than 100 countries, including India are still considered as endemically or sporadically infected with the disease. With advent of rapid
transport system and global trade, even the strictest quarantine measures sometimes fails to prevent entry of virus into FMD susceptible population of the country, outbreak in England is a current example. Also the virus keeps on changing genetically resulting in altered phenotype evading immune response, further aggregating to the problem in control and eradication. With the potential of devastation of animal industry, both FMD free and endemic countries are now vigorously monitoring the disease by sero-surveillance and genetic analysis. For effective sero-monitoring, first important thing is to have a dependable test to identify the diseased and carrier animals in a population. FMDV rapidly accumulates mutation to escape immune response in susceptible animals due to error prone replication of its RNA genome. In endemic countries like India where control is mainly sought by vaccination, constant monitoring by antigenic and phylogenetic studies is required for effective control.

**Scenario in India**

In India the disease is endemic and occurs in all parts of the country throughout the year. The serotypes prevalent in our country are O, Asia 1, and A. Out of these three serotypes serotype O accounts for more than 85% of the outbreaks followed by serotypes A and Asia 1. Serotype C has not been recorded since 1995. Though the morbidity rate is high, the mortality rate due to FMD is low. The economic losses caused by the disease are mainly due to loss in milk production and reduction in the working ability of work animals. The direct losses due to this disease in the country are estimated to be more than Rs. 4000 crore per year. In addition to this loss, the milk and milk products, meat and hide are not accepted by countries free from the disease causing reduction in the export potential of livestock in our country.

Within each serotypes, there is existence of different lineages/genotypes in causing disease outbreaks. There is no interserotypic protection and even within serotype, protections to different lineages are also not uniform. Hence vaccine strains are carefully chosen which can cover all prevalent lineages and are updated from time to time. Post vaccination, protective titer is consistently monitored in the animals by indigenously developed and produced Liquid Phase Blocking ELISA (LPBE). Virus isolates from field samples are also cross matched with vaccine strain in virus neutralization test (two-dimensional micro-neutralization test) with currently used vaccine strains.
Chapter II: Diagnosis of Foot-and-Mouth Disease

Diagnosis is first important step in control and eradication of Foot and Mouth Disease (FMD). Due to single stranded RNA nature, FMDV 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.

Even FMD free developed countries are scared with this disease due to trade embargo. Vaccination with inactivated vaccine is not practiced by FMD free countries due to its interference in sero-monitoring based on structural proteins. Control is mainly relied upon strict ban on import of animal and animal products from FMD endemic countries and test and slaughter of all FMD positive reactors. The restrictions on trade adversely effects the import and export of animal industry and ultimately to the poor farmers. FMD endemic countries like India are facing problems such as economic barriers and social or religious taboos in implementing test and slaughter policy. Vaccination followed by sero-monitoring is best alternative for effective control in endemic countries. In fact in past many European countries like France have adopted vaccination and after control seized vaccination. But here important issue is to differentiate infected from vaccinated animal population. For the purpose many techniques have been employed but detection based on FMDV non-structural proteins (NSPs) gained high popularity due to high specificity and convenience for mass scale testing.

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 field persons. Also simple test which can be performed on site will greatly help in endemic countries like India. Work in this area is already on progress. Pen-side test, real time PCR based test and DIVA assay have been successfully validated in this series by some laboratories.
1. **Clinical diagnosis:** Disease is characterized by fever, depression, excessive salvation, 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.

   a. Vesicles on the mucous membranes of the oral cavity and muzzle

   ![Vesicles on the mucous membranes of the oral cavity and muzzle](image1)

   b. Lameness and excessive salvation

2. **Laboratory diagnosis:**

   2.1 **Samples to be collected:** Tissue of choice is tongue epithelium, foot lesions, saliva or vesicular fluid. Semen from FMD suspected bulls engaged in semen straw production can also be collected. Where epithelial tissue is not available from ruminant animals, for example in advanced or convalescent cases, or where infection is suspected in the absence of clinical signs, samples of oropharyngeal fluid is collected by means of a probang (sputum) cup (or in pigs by swabbing the throat) for submission to a laboratory for disease diagnosis and virus isolation. Viraemia may also be detected by examining serum samples by means of RT-PCR or virus isolation.

   2.2 **Method of sampling:** Ideally, at least 1 g of epithelial tissue should be collected from an un-ruptured or recently ruptured vesicle, usually from the tongue, buccal mucosa or feet. Epithelial samples is collected in a transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer, pH 7.2–7.6, preferably with added
antibiotics (penicillin, neomycin sulphate, polymyxin B sulphate, mycostatin). FMDV is extremely labile in low pH and buffering of the transport media is critical for successful sample collection. Samples should be kept refrigerated or on ice until received by the laboratory.

2.3 Diagnostic methods

2.3.1 FMD viral antigen detection by Sandwich ELISA: Antigen capture sandwich ELISA for virus serotyping is a highly sensitive method of virus detection and serotyping in clinical materials. In this test plates are coated with serotype specific rabbit polyclonal sera (coating) and viruses present in processed clinical samples are allowed to bind to capturing antibodies. Bound viruses are detected by serotype specific tracing antibody which increases the specificity of the test. Reaction is developed by tracing antibody specific conjugated antibody and substrate solution.

2.3.2 PCR based diagnosis: Newer methods of typing have been widely utilized now a day based on serotype specific multiplex polymerase chain reaction (mPCR) for simultaneously serotype identification. Multiplex PCR has been developed for FMDV serotyping (Vangrysperre and De Clercq, 1996; Callensand De Clercq, 1997). In order to improve the serotyping percentage, an mPCR was developed for differentiation of FMDV serotypes in India (Giridharan et al., 2005). The assay was found to be sensitive
and specific superior to the currently used diagnostic test, ELISA. Real-time assays have been developed using FMDV sequence-specific primers and TaqMan probes to conserved regions of the internal ribosomal entry site within the 5' untranslated region in a 2-step PCR assay (Reid et al., 2001) or a one-step reverse-transcription (RT) PCR method to conserved regions of the RNA polymerase (3D) gene using TaqMan probes (Callahan et al., 2002) or linear hybridization probes (Moonen et al., 2003). Real-time assays have been developed using FMDV serotype sequence-specific primers and TaqMan probes to differentiate three serotypes prevalent in the country.

2.3.3 Cell culture isolation: Virus isolation in mammalian cell culture (e.g. BHK-21 or IBRS cell lines) is routinely done for confirmatory diagnosis and also for vaccine matching of outbreak strains. For this, full-grown monolayer BHK-21/IBRS-2 cells in tubes are infected with 10 % PBS tongue epithelium suspension in the presence of antibiotics and after an hour adsorption the supernatant is discarded. The medium is filled up and the tubes are incubated for 24-48 hrs for cytopathic effect (CPE). Like this, every material is given at least 3-5 blind passages for presence of cytopathic effect. Presence of FMDV is suspected by appearance of cytopathic effects (CPE) of rounding and cell detachment of monolayer cells after 18-24 hours of inoculation. Confirmation of positive results is made by virus typing in sandwich ELISA and multiplex PCR.

2.4 Serological diagnosis: Serologic diagnosis of FMD virus infection is done by ELISA, agar gel immunodiffusion test (AGID) and VNT. In humoral immune response against FMDV, antibodies against both structural and non-structural are produced. The problem associated with diagnosis by serology is use of vaccination in control programme and time required for generation of antibodies against FMDV. Although ELISA against structural protein is much sensitive method then virus isolation but if vaccination by inactivated vaccine is done than antibody against structural protein of vaccine interfere in correct diagnosis.

3. Genetic and antigenic characterization

3.1 Micro-neutralization test: In order to estimate the antigenic relationship of the field isolates, 2D-MNT is performed as per standard method (Rweyemamu et al., 1978. BHK-21 cells are used as indicator system in neutralization test. Each test is repeated thrice on three different days and the averages are considered to avoid manual errors. On
every testing day, along with the field isolates, the homologous vaccine virus is also tested to avoid any bias. The end point titre of the serum is calculated as the reciprocal of the last dilution of serum that neutralizes 100TCID$_{50}$ in 50% of the wells. The one-way antigenic relationship (r-value) is calculated as the ratio between heterologous and homologous serum titre. As an ‘r’-value of more than 0.3 indicates close antigenic relatedness.

3.2 Molecular epidemiology: The molecular epidemiology of FMD is based on the comparison of genetic differences between viruses. VP1 based phylogenetic comparison is performed in order to analyze genetic relatedness. Comparison of whole genome sequences can provide further discrimination between closely related viruses and help to recreate the transmission pathways between farms within outbreaks. RT-PCR amplification of FMDV RNA, followed by nucleotide sequencing, is the current preferred option for generating the sequence data to perform these comparisons. Phylogenetic analysis of Indian FMDV serotypes A, O and Asia1 are carried out regularly which revealed extreme genetic heterogeneity among the viruses circulating in the country. Dendogram showing phylogenetic relationships of Indian isolates is presented in Fig1-3
Fig1. Phylogenetic analysis of FMDV type A virus. UPGMA tree showing complete VP1 sequence based global phylogeny and topotype/genotype distribution.
Fig. 2 Mid point rooted Neighbour Joining Phylogenetic tree at 1D genomic region of Ind2001 lineage of FMD virus serotype O during 2010-2011.
Fig3. Phylogenetic analysis of FMDV type Asia1 virus. Neighbour-joining tree depicting phylogenetic relationship of type Asia1 field isolates.
Chapter III

**Cell culture and Virus isolation**

Cell culture is the complex process by which cells are grown under controlled conditions at an appropriate temperature and gas mixture (typically, 37°C, 5% CO₂ for mammalian cells) in a cell incubator. Passaging of cells involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence associated with prolonged high cell density. For adherent cultures, cells first need to be detached; this is commonly done with a mixture of trypsin-EDTA. A small number of detached cells can then be used to seed a new culture. BHK-21/IBRS-2 cell lines are maintained and used for FMD virus isolation and neutralization test. The cell lines amplify the amount of virus present, express the viral antigens and in many cases die as a consequence of the viral infection producing characteristic cytopathic effects in the cell monolayer. The amplified viruses are then available for further identification by molecular techniques to determine whether they are common or new strains of the virus.

**Cell culture media and reagents**

**Amino Acid Stock Solution (For 2 liters)**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>1.680 g</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.960 g</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.768 g</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.096 g</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.096 g</td>
</tr>
<tr>
<td>Lysine (monohydrochloride salt)</td>
<td>2.924 g</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.320 g</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.904 g</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.320 g</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.448 g</td>
</tr>
<tr>
<td>Valine</td>
<td>1.872 g</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.600 g</td>
</tr>
<tr>
<td>Inositol</td>
<td>0.140 g</td>
</tr>
<tr>
<td>Phenol red (0.5%)</td>
<td>0.160 ml</td>
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(L-cystine is dissolved separately in 5 ml in NaOH solution)
Store at -20°C

**Vitamin Stock Solution (For 500 ml)**

- Choline chloride \(250 \text{ mg}\)
- Folic acid \(250 \text{ mg}\)
- Nicotinamide \(250 \text{ mg}\)
- Pantothenic acid \(250 \text{ mg}\)
- Pyridoxine HCl \(250 \text{ mg}\)
- Thiamine HCl \(250 \text{ mg}\)
- Riboflavine \(25 \text{ mg}\)

(Folic acid is dissolved separately in 5 ml of IN NaOH solution)

Stored at -20°C.

**BHK-21 maintenance medium (Glasgow modification)**

(Composition for making 2 ltr of medium)

- NaCl \(12.8 \text{ g}\)
- KCl \(0.800 \text{ g}\)
- CaCl\(_2\).2H\(_2\)O \(0.530 \text{ g}\)
- MgSO\(_4\).7H\(_2\)O \(0.400 \text{ g}\)
- NaH\(_2\)PO\(_4\).2H\(_2\)O \(0.280 \text{ g}\)
- Glucose \(9.0 \text{ g}\)
- L-glutamine \(1.170 \text{ g}\)

(Part of distilled water is added to dissolve these salts)

- Phenol red (Sodium salt) \(0.034 \text{ g}\)
- NaHCO\(_3\) \(5.5 \text{ g}\)
- Penicillin \(2 \text{ Lakh I.U.}\)
- Streptomycin \(0.2 \text{ g}\)
- Amino acid stock \(100 \text{ ml}\)
- Vitamin stock \(8 \text{ ml}\)
- Tryptose phosphate broth \(6.0 \text{ g}\)
- Distilled water \(\text{Upto 2000 ml}\)

pH adjusted to 7.4
Sterilize by positive pressure seitz filtration. Incubate for overnight at 37°C before use. Store at 4°C.

**BHK-21 growth medium (Glasgow modification)**

- BHK-21 maintenance medium - 900 ml (pH 7.4)
- Healthy calf serum - 100 ml

Sterilized by positive pressure seitz filtration. Incubated for overnight at 37°C before use. Stored at 4°C.

**Trypsin-versene solution**

- NaCl - 5.0 g
- KCl - 0.125 g
- Na$_2$HPO$_4$.2H$_2$O - 0.950 g
- KH$_2$PO$_4$ - 0.125 g
- Trypsin - 0.850 g
- Versene (EDTA) - 0.700 g
- Phenol red (0.5%) - 0.5 ml
- Distilled water - To make 500 ml

pH adjusted to 7.4

Mixed by stirring on a magnetic stirrer and sterilized by positive pressure seitz filtration. Incubated overnight at 37°C before use. Stored at +4°C.
Chapter IV

Identification of FDMV serotype by Sandwich ELISA

**Principle:** Antigen capture sandwich ELISA for virus serotyping is highly sensitive method of virus detection and sero-typing in clinical materials. In this test plates are coated with serotype specific rabbit polyclonal sera and viruses present in processed clinical samples are allowed to bind to capturing antibodies. Bound viruses are detected by serotype specific tracing antibody which increases the specificity of the test. Reaction is developed by tracing antibody specific conjugated antibody and substrate solution. The steps in s-ELISA are described step by step:

1. Coating with trapping antibodies
2. Capture of target antigen
3. Tracing of captured antigens
4. Detection of bound antibodies with species specific antibodies conjugated with HRPO
5. Development of color reaction after addition of chromogen and substrate

**Kit Components:**
1. 96-well Nunc Maxisorp ELISA plates (cat. No. 442404)
2. Freeze dried anti-FMDV coating serum: Type specific anti-146S FMDV serum raised in rabbits.
3. Freeze dried anti-FMDV tracing serum: type specific anti-146S FMDV serum raised in guinea pig.
4. Anti-guinea pig conjugate: Rabbit/goat anti-guinea pig immunoglobulin HRPO conjugate (DAKO)
5. FMDV inactivated antigens: sero-type specific inactivated freeze dried antigens
6. Chromogen: OPD (Orthophenylenediamine dihydrochloride, Sigma)
7. Hydrogen peroxide ($H_2O_2$)
8. Phosphate buffer saline vials (Sigma)
9. Substrate buffer capsules (Sigma)
10. Coating buffer tablets (Sigma)
11. Tween-20

**Materials needed but not provided:**

1. Precision pipettes: multi channel pipettes variable range from 50 to 200µl & Single channel pipettes variable range from 1 to 20µl, 20 to 100µl, 50 to 200µl and 200 to 1000µl along with disposable tips.
2. Distilled water
3. H₂SO₄
4. Wash bottle fitted with Immunowasher
5. 1 container: 1 to 2 liters
6. ELISA plate reader, 492 nm filter.
8. Refrigerator: range of +2°C to +6°C
9. Freezer: range of -15 to -20°C
10. Incubator: warm wall incubator maintained at +35°C to 37°C.
11. pH meter: with accuracy of 0.01pH units or good quality pH strips with varying range from 2 to 10 can be used. Glassware/ plastic ware: flasks (50-5000ml), graduated cylinders (10-2000ml), graduated pipettes (1-100ml).
12. Weighing balance

**Preparation of solutions/ reagents**

1. **Coating buffer**: dissolve one capsule of coating buffer in 100ml of distilled water. Check pH if necessary bring pH to 9.2.

2. **Washing buffer**: dissolve one PBS capsule in 1000ml of distilled water check pH and if necessary adjust to pH 7.4. Add tween-20 to final concentration of 0.1% and mix it well.

3. **Blocking Buffer for 1 plate**
   - Lactalbumin hydrolysate (LAH) 450mg
   - Healthy rabbit serum 750µl
   - Healthy calf serum 750µl
   - Washing buffer 13.5ml
4. **Substrate Buffer**: dissolve one capsule in 100ml of distilled water. Check pH if necessary adjust pH to 5.2.

5. **Substrate solution for one plate : (prepare fresh)**

   - Phosphate-Citrate buffer (pH 5.0) - 7.5 ml
   - Orthophenylene-diamine dihydrochloride (OPD) - 5 mg
   - \( \text{H}_2\text{O}_2 \) (30% w/v) - 4µl

**Procedure**

1. Dilute all the 4 coating sera, as suggested, with coating buffer sufficient for the number of plates to be used (2ml of working dilution per serum per plate). Dispense the diluted coating serum in 50µl volumes per well as indicated later in the plate layout. Gently tap the plates to ensure that the liquid has covered the whole well area and incubate at 37°C for 1 hour.

2. Wash the plate by adding washing buffer using immune-wash and discard the contents of wells by abrupt downward hand motion. Repeat the washing 3 times with 5 minutes of hold period between each wash. Slap the inverted microplate 3-4 times onto a dust free absorbent pad to remove all residual contents in the wells.

3. Dispense 50µl of test sample/ antigen per well according to plate layout. In background wells dispense 50µl of blocking buffer in place of antigen and in positive controls dispense 50µl of respective controls provided. Cover the plate with lid and incubate at 37°C for 1 hour with intermittent gentle shaking followed by washing as described in step 2.

4. Dilute all the 4 tracing serum, as suggested, with blocking buffer sufficient for the number of plates required. Dispense 50µl volumes per well as indicated in plate layout.

5. Incubate the plates at 37°C for 1 hour with intermittent gentle shaking followed by washing as described in step 2.

6. Prepare working dilution of conjugate, as suggested, in blocking buffer in sufficient volume. Dispense 50µl of diluted conjugate to all the wells of ELISA plate and incubate the plates for 1 hour followed by washing as described in step 2.

7. Prepare substrate solution, as described, and dispense 50µl of substrate solution to each well. Cover the plate with lid and incubate at 37°C for 15 min in dark. Stop the color reaction by adding 50µl of stopping solution to each well.
8. Measure the optical density of each well at wavelength of 492nm and reference wavelength of 620nm in ELISA reader.

**Interpretation of test sample result**

Performance of a test can be determined from positive reaction of the known antigens employed in the test and a clear background reaction of all the serum. The interpretation of the result should be done on the basis of the corrected OD value (OD of test well - OD of background well).

When a test is conducted properly, the background reaction of all the serum provided will lie between 0.00 to 0.02 OD with substrate. If the reactivity (OD value) of the test antigen with a particular FMDV serotype serum is $\geq 0.10$ (with no heterologous reactivity), then antigen in question can be identified as belonging to that serotype (OD limit 0-3.0).

**Precautions:**

1. All the glass wares to be used should be clean and sterile.
2. Slight drop in pH (less than 7.2) in washing buffer can adversely affect antigen antibody reaction. Ensure correct pH every day before use.
3. Microbial contamination/ precipitation formation of any degree in any of the reagents/ buffer will very much reduce the specificity of the test.
4. Mark the plate orientation properly with water proof marker before starting the test to avoid any confusion which may arise later.
5. Do not stack plates one over other in incubator.
6. Prepare fresh substrate solution every time.
7. Do not store prepared buffers more than 1 month.
8. Check pH of every buffer every day before starting the test.

**Trouble shooting:**

**1. No OD in any wells including positive controls:**

- Improper reconstitution/ preparation of reagents/ buffers
- Cross check the composition of buffers and reagents
- Use of conjugate other than anti-guinea pig conjugate.
  - Cross check the conjugate used
- Lower pH of washing buffer
  - Cross check the pH of washing buffer

2. **Even OD in all the wells**
- Cross contamination of tips used for conjugate and substrate solution.
- Coating of wells with tracing antibodies (in spite of coating serum).

3. **Low OD**
- Improper dilution of reagents
- Cross check the dilutions recommended for each batch
- Expiry of reagents of kits
- Cross check the expiry of reagents
- High percentage of tween-20 in washing buffer
- Cross check the tween-20 concentration in washing buffer
- Lower/ higher temperature in incubator
- Cross check the temperature of incubator
Chapter V

**Liquid Phase Blocking ELISA**

**Principle:** The test is aimed at the quantification of protective antibody level in the animals following vaccination. A serial two fold dilutions of test serum are mixed with equal volume of a constant dose of viral inactivated antigens in a liquid medium and allowed to react overnight at 4°C. The antigen-antibody reaction is carried out in a suspension (or liquid medium), and the antigen is blocked by the homologous antibodies, if present, in the test serum for subsequent detection by guinea pig serum. Next day, the antigen which are not completely blocked by the antibodies in the test serum are trapped to the wells of the ELISA plates by the pre-coated type-specific rabbit antibodies. Antigen and background controls are added containing antigen without serum and blocking buffer respectively as described in plate layout. Subsequently, the presence of antigen is traced by adding pre-titrated guinea pig (type-specific) serum and anti-guinea pig-HRPO conjugate and substrate reaction is followed in a standard ELISA procedure. Color reaction is stopped and measured in terms of optical density (OD) at 492 nm wavelength with reference to 620nm. OD of reference antigen controls are used for the calculation of 50% inhibition in OD for calculation of the titers.

**Kit Components:**

1. 96-well Nunc Maxisorp ELISA plates (cat. No. 442404)
2. Freeze dried Anti-FMDV coating serum: Type specific anti-146S FMDV serum, raised in rabbits.
3. Freeze dried Anti-FMDV tracing serum: type specific anti-146S FMV serum, raised in guinea pig.
4. Freeze dried inactivated Type O, A, and Asia1 antigens.
5. Anti-guinea pig conjugate: Rabbit/ goat anti-guinea pig immunoglobulin HRPO conjugate (DAKO)
6. Chromogen: OPD (Orthophenylenediamine dihydrochloride, Sigma)
7. Hydrogen peroxide (H$_2$O$_2$)
8. Phosphate buffer saline vials (Sigma)
9. Substrate buffer capsules
10. Coating buffer tablets
11. Tween-20

**Materials needed but not provided:**

1. Precision pipettes: multi channel pipettes variable range from 50 to 200µl & Single channel pipettes variable range from 1 to 20µl, 20 to 100µl, 50 to 200µl and 200 to 1000µl along with disposable tips.
2. Distilled water
3. \( \text{H}_2\text{SO}_4 \)
4. Wash bottle fitted with Immunowasher
5. 1 container: 1 to 2 liters
6. ELISA plate reader, 492 nm filter.
8. Refrigerator: range of +2°C to +6°C
9. Freezer: range of -15°C to -20°C.
10. Incubator: warm wall incubator maintained at 37°C.
11. pH meter: with accuracy of 0.01pH units or good quality pH strips with varying range from 2 to 10 can be used.
13. Weighing balance

**Preparation of solutions/ reagents**

1. **Coating buffer:** dissolve one capsule in 100ml of distilled water. Check pH if necessary bring pH to 9.2.
2. **Washing buffer:** dissolve one PBS capsule in 1000ml of distilled water check pH and if necessary adjust to pH 7.4. Add tween-20 to final concentration of 0.1% and mix it well.
3. PBS 0.05% tween: add tween-20 in PBS to final concentration of 0.05%.
4. **Blocking Buffer for 1 plate**
   
   Lactalbumin hydrolysate (LAH) 450mg
Healthy rabbit serum       750µl
Healthy calf serum        750µl
Washing buffer            13.5ml

5. **Substrate Buffer:** dissolve one capsule in 100ml of distilled water. Check pH if necessary adjust pH to 5.2.

6. **Stopping solution:** add 9

7. **Substrate solution for one plate : (prepare fresh)**
   - Phosphate-Citrate buffer (pH 5.0)    - 7.5 ml
   - Orthophenylene-diamine dihydrochloride (OPD)  - 5 mg
   - H$_2$O$_2$ (30% w/v)      - 4µl

**Procedure:**

1. **Coating:** Coat the individual plates with type-specific (O, A, Asia1) rabbit (dilute the serum as specified in the serum vial) serum in coating buffer (Carbonate-Bicarbonate buffer, pH 9.6). Dispense 50µl of diluted serum to each wells of ELISA plate. Tap gently for even distribution of coating solution to wells and cover with lid. Incubate at 37°C for 1 hr followed by incubation at 4°C overnight.

2. **Preparation of Ag-Ab mixture:** Prepare two-fold dilutions (4 dilutions starting from 1:16) of serum samples in a low binding perspex plate with PBS containing 0.05% Tween-20. Distribute 75 µl each dilutions of the serum sample to four separate (labeled O, A, and Asia1) perspex plates. Dilute the antigen as specified in the antigen batch provided. Add equal volume of diluted antigen (75µl) to all the distributed serum samples. For antigen control, add equal volume of PBS-Tween 20 (0.05%) to the already diluted antigen. The dilution of each antigen and antibody in this step will be strictly made with PBS containing tween-20 (not the PBS containing 0.1% tween-20 as used for washing of the plate). Incubate the diluted antigen and antibody mixture at 4°C overnight without disturbance.

3. **Washing:** Wash the plate by adding washing buffer using immune-wash and discard the contents of wells by abrupt downward hand motion. Repeat the washing 3 times with
5 minutes of hold period between each wash. Slap the inverted microplate 3-4 times onto a dust free absorbent pad to remove all residual contents in the wells.

4. **Dispensing of antigen-antibody (Ag-Ab) mixture:** Dispense 50µl of the overnight incubated Ag-Ab mixture in duplicate to the ELISA plates as instructed in the plate layout. Incubate the plates at 37°C for 1 hr.

5. Wash the plate as in the step 3.

6. **Addition of tracing antibodies:** Dilute the guinea pig serum, as specified in the serum vials, in blocking buffer. Add the 50µl of diluted tracing serum to each type-specific plate. Incubate at 37°C for 1 hr. Wash the plate as in the step 2.

7. **Conjugate:** Add the anti-guinea pig-HRPO conjugate diluted in blocking buffer to all the wells. Incubate at 37°C for 1 hr. Wash the plate as in step 2.

8. **Substrate reaction:** Add freshly prepared substrate solution to the plate and keep in the incubator at 37°C for 15 minutes.

9. Stop the color reaction by adding 50µl of stopping solution. Read the plate at 492 nm and reference wavelength of 620nm in ELISA reader.

**Interpretation of test sample result**

Performance of a test can be determined from positive reaction of the known antigens (approximately 1.0) employed in the test and a clear background reaction. The interpretation of the result should be done on the basis of the corrected OD value (OD of test well OD of background well).

Percent reactivity against each serum dilution is calculated as follows:

\[
\text{% Reactivity} = \left( \frac{\text{Mean OD of test wells}}{\text{Mean OD of antigen control wells}} \right) \times 100
\]

Titer of serum sample is expressed as the reciprocal of the serum dilution giving 50% optical density (OD) as compared to the antigen control or in other words, reciprocal of the serum dilution which inhibits 50% of the guinea pig serum binding to the homologous virus.
Precautions:
1. All the glass wares to be used should be clean and sterile.
2. Slight drop in pH (less than 7.2) in washing buffer can adversely affect antigen antibody reaction. Ensure correct pH every day before use.
3. Microbial contamination/ precipitation formation of any degree in any of the reagents/ buffer will very much reduce the specificity of the test.
4. Mark the plate orientation properly with water proof marker before starting the test to avoid any confusion which may arise later.
5. Do not stack plates one over other in incubator.
6. Prepare fresh substrate solution every time.
7. Do not store prepared buffers more than 1 month.
8. Check pH of every buffer every day before starting the test.

Troubleshooting:

1. No OD in any wells including positive controls:
   - Improper reconstitution/ preparation of reagents/ buffers
     - Cross check the composition of buffers and reagents
   - Use of conjugate other than anti-guinea pig conjugate.
     - Cross check the conjugate used
   - Lower pH of washing buffer
     - Cross check the pH of washing buffer

2. Even OD in all the wells
   - Cross contamination of tips used for conjugate and substrate solution.
   - Coating of wells with tracing antibodies (in spite of coating serum).

3. Low OD
   - Improper dilution of reagents
   - Cross check the dilutions recommended for each batch
   - Expiry of reagents of kits
   - Cross check the expiry of reagents
   - High percentage of tween-20 in washing buffer
   - Cross check the tween-20 concentration in washing buffer
   - Lower/ higher temperature in incubator
   - Cross check the temperature of incubator
Chapter VI

Differentiation of Infected from Vaccinated Animals (DIVA) ELISA

(r3AB3 NSP based)

**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 (~38 kD) NSP in an indirect ELISA format. A sample producing OD value more than the fixed cut-off ratio \((\text{test serum sample mean OD}/\text{positive control serum mean OD}) \times 100\) i.e., percent positivity value or PP value ≥ 40% is qualitatively diagnosed as positive for FMD infection.

**Kit Components:**

For testing 450 serum samples in duplicate:

1. 96-well Nunc Maxisorp ELISA plates (Cat. No.442404): 10 plates
2. Freeze dried recombinant 3AB3 protein (~38 kD): Ten vials (Store at -20°C)
3. Each vial is to be dissolved in 1ml of Carbonate-Bicarbonate coating buffer, pH 9.5 and then added to 4.5 ml of additional coating buffer. This reconstituted vial is sufficient for coating one 96-well immunoplates. After reconstitution the vial is to be used immediately without storing further.
4. Freeze-dried positive and negative control sera: One vial each (Store at -20°C)
5. Each vial is to be dissolved in 160 µl of distilled water and then distributed into single use aliquots for storage at -20°C, if to be consumed in more than a month’s time or else store the entire reconstituted vial at 4°C. Working dilution of 1:20 in diluent buffer (as appears in the protocol) for control serum is recommended.
6. *E. coli* lysate: One vial (Store at -20°C)
7. Each vial of *E.coli* lysate is to be reconstituted with 70 µl of PBS and stored at -20°C.
8. Lapin Anti Bovine-HRP conjugate (DAKO; Cat. No.P0159): 35 µl (Store at 4°C)
9. Skimmed milk powder (Merck; Cat. No.1.15363.0500): 8 gram
10. Chicken serum {Sera Laboratories International Ltd. (SLI); Cat. No. S-606-HSL}: 25 mL (Store at -20°C)
11. Salts for PBS, pH 7.4
12. Tween-20: 5mL
13. Carbonate-Bicarbonate coating buffer (Sigma; Cat.No.C3041): 1 capsule (for preparing 100 mL of buffer)
14. Phosphate-Citrate substrate buffer (Sigma; Cat.No.P4809): 1 tablet (for preparing 100 mL of buffer)
15. OPD (Sigma; Cat.No.P1526-100G): 50 mg (Store at -20°C)
16. 30 % H₂O₂ (Merck; Cat.No.1.07210.0250): 50 µl
17. 1M H₂SO₄: 70mL

*All the reagents are only to be procured from the concerned suppliers as mentioned above for best result.

NB: All the reagents are only to be procured from the concerned suppliers as mentioned above for best result

**Materials needed but not provided:**
1. Precision pipettes
2. Disposable pipette tips
3. Distilled water
4. Wash bottle
5. 1 container: 1 to 2 litres for PBS-Tween
6. ELISA plate reader, 492 nm filter

**Specimen information**

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

**Procedure:**
1. Dissolve the freeze dried recombinant protein in the vial with 1 ml of coating buffer and add another 4.5 ml of coating buffer to it (One vial is sufficient for coating one 96-well ELISA plates). The reconstituted protein vial should be used for coating immediately without storing. Reconstitute the freeze dried vials of positive (one
month post-infection serum) and negative (one month post vaccination serum) control serum with distilled water as appears on the sticker and store at – 20°C after reconstitution in single use aliquots. Dissolve the content of freeze dried vial carrying E. coli lysate in 70 µl of PBS and store at –20°C after use.

2. Coat 96-well polystyrene (Nunc Maxisorp) Immuno plates with the diluted recombinant protein @ 50 µl per well (~50 ng of purified recombinant protein per well). Tap the plate gently from all sides and incubate (refrigerate) the plate at 4°C for overnight.

3. Remove the plates from the refrigerator and thaw them at 37°C for 15 minutes.

4. In a low protein binding Perspex plate dilute the test and the supplied negative and positive control sera @ 1:20 in diluent buffer. Only serum from bovine host is compatible with this test. Prepare a total volume of 220 µl of diluted serum so that 100 µl of the mixture can be transferred to the coated 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.

5. Give three continuous wash (no hold time) with wash buffer.

6. Transfer 100 µl of the serum and diluent buffer mixture from Perspex plate to the ELISA plate in duplicate wells. Incubate for 1 hour at 37°C and tap the plate gently from all sides at every 15 minute intervals or incubate for 30 minutes in a plate shaker at 37°C with 20-30 rpm.

7. Give three washes of 3 minute soak period each.

8. Dispense anti-bovine-HRP conjugate diluted in the diluent buffer (1:2000) @ 50 µl per well. Incubate for 1 hour at 37°C and tap the plate gently from all sides at every 15 minute intervals or incubate for 30 minutes in a plate shaker at 37°C with 20-30 rpm. 6ml of diluted conjugate solution is sufficient for an ELISA plate.

9. Give three washes of 5 minute soak period each.

10. Add freshly prepared substrate solution @ 50 µl per well and incubate for 15 minutes at 37°C without shaking. Then stop the colour reaction by adding 1M H₂SO₄ @ 50 µl per well.

11. Measure the optical density values at wave length of 492 nm (Reference 620 nm).
F. 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 O.D. in background 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 \( \left( \frac{\text{test serum sample mean } \text{OD}}{\text{positive control serum mean } \text{OD}} \right) \times 100 \) i.e., percent positivity value or PP value \( \geq 40\% \), calculated by dividing 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:

1. 3AB3 NSP reactivity positive: If PP value is more than 40%
2. 3AB3 NSP reactivity negative: If PP value is less than 40%

Brownish yellow colour indicates positive reactivity in DIVA ELISA

Precautions:

1. Carefully read and follow all instructions.
2. Store the kit and all reagents as mentioned.
3. Handle all materials according to the Good Laboratory Practice.
4. Care should be taken to prevent contamination of kit components.
5. Do not use test kit beyond date of expiry.
6. Use a separate pipette tip for each sample.
7. Do not pipette by mouth.
8. Include a positive, negative serum and back ground control on each plate series.

9. Use only distilled water for preparation of reagents.

10. All unused biological materials should be disposed according to the local, regional and national regulations.

**Trouble shooting:**

1. **No OD in any wells including positive controls:**
   - Improper reconstitution/ preparation of reagents/ buffers
     - Cross check the composition of buffers and reagents
   - Use of conjugate other than anti-cow conjugate.
     - Cross check the conjugate used
   - Lower pH of washing buffer
     - Cross check the pH of washing buffer

2. **Even OD in all the wells**
   - Cross contamination of tips used for conjugate and substrate solution.

3. **Low OD**
   - Improper dilution of reagents
     - Cross check the dilutions recommended for each batch
   - Expiry of reagents of kits
     - Cross check the expiry of reagents
   - High percentage of tween-20 in washing buffer
     - Cross check the tween-20 concentration in washing buffer
   - Lower/ higher temperature in incubator
     - Cross check the temperature of incubator
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