

Epidemiological and Clinico-Diagnostic Studies on Foot and Mouth Disease (FMD) in Ruminants

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Epidemiological and Clinico-Diagnostic Studies on Foot and Mouth Disease (FMD) in Ruminants

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Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
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Shuhama Campus Srinagar-190006

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DEDICATED TO
MY BELOVED FAMILY
AND
MAJOR ADVISOR

اللَّهُمَّ اغْفِرْ لِي ذُنُوبِي وَلِوَالِدَيَّ وَارْحَمْ
هُمَا كَمَا رَبَّيَانِي صَغِيرًا

YA ALLAH, FORGIVE MY SINS AND THOSE OF MY PARENTS, AND HAVE MERCY
UPON THEM AS THEY HAD MERCY UPON ME WHEN I WAS SMALL.

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and You make the difficult easy if it be Your Will.

IN THE NAME OF ALLAH, THE MOST GRACIOUS, THE MOST BENEFICIENT, THE MOST COMPASSIONATE AND THE MOST MERCIFUL ALL PRAISES BE TO ALLAH, THE LORD OF THE WORLDS AND PRAYERS AND PEACE BE UPON HIS MESSENGER PROPHET MUHAMMAD ^(PBUH)

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ABSTRACT

A study on the epidemiological pattern of Foot and Mouth disease (FMD) in domestic ruminants (Cattle and Sheep) was conducted from September 2021 to August 2022. The objectives of the study were to investigate the epidemiology, economic losses, clinical manifestations of the disease and identification of FMD virus serotypes prevalent in domestic ruminants of Kashmir valley. A total of ninety-six (96) FMD-infected ruminants (cattle-89 and sheep-7) were included in the study. The cattle of the older age group (>4 years: 68.50%) were more affected with FMD as compared to younger (<1-2 years: 14.60%) and adult (2-4 years: 16.85%) age groups, whereas, young sheep (<1-2 years: 57.14%) were more affected than adult (2-4 year: 42.85%) sheep. Proportionally, the female cattle (68.54%) were more affected by FMD compared to males (31.46%). Out of

the eighty-nine (89) cattle infected with FMD, only a small percentage (3.3%) of FMD-infected cattle were vaccinated in the ongoing year. The economic losses due to FMD were evaluated in terms of reduction in milk yield, treatment cost and mortality losses. In affected animals, the mean post-FMD milk yield (2.01 ± 0.21 L) was significantly ($P \leq 0.05$) decreased than the mean pre-FMD milk yield (10.57 ± 0.29 L). The average treatment cost calculated was about Rs. 1235.83 per FMD-infected animal and the average loss due to mortality was estimated at around Rs. 2014.29 per infected calf. The total revenue losses of about Rs. 10,149.64 per infected cattle were estimated due to FMD. In infected animals, there was a significant ($p < 0.05$) increase in mean rectal temperature, respiration rate (/min) and heart rate (bpm) in comparison to the healthy control group of animals. All (100%) of the FMD-infected cattle and sheep exhibited salivation and prominent clinical lesions in the mouth, foot/h hoof and udder/teats were found in 98.8%, 85.3% and 6.7% of affected animals, respectively. The older age group (>4 years) cattle showed a higher percentage of mouth lesions than young and adult ones. The three major post-FMD complications in affected cattle were heat intolerance/panting (21.34%), hypertrichosis (7.80%) and mastitis (6.74%). A total of 89 samples were subjected to Polymerase Chain Reaction targeting 5'-untranslated region (5'-UTR) gene fragments, out of which 65 samples (73.0%) were detected positive for FMDV. PCR amplification identified serotype O from 65 (73.0%) FMD-positive samples as sequencing results confirmed serotype O from the four representative FMDV-positive samples. The FMDV serotype O identified in the study confirmed its close genetic proximity with FMDV serotype O (PanAsia strain of Pakistan).

Keywords: Foot and Mouth Disease, Cattle, Sheep, Epidemiology, Lesions, Economic losses, PCR, Serotypes, Sequencing.

Signature of Student

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Signature of Major Advisor

Dated: _____

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ABBREVIATIONS

GDP	Gross domestic product
J&K	Jammu and Kashmir
Kgs	Kilograms
IPTG	Isopropyl-beta-D-thiogalactopyranoside
dNTP's	Deoxynucleoside triphosphates
DADH	Department of Animal Husbandry and Dairying
LB	Luria bertani
FMDV	Foot and Mouth Disease Virus
OIE	International des Epizooties
Etc	Et cetera
et al.	et alia
Kb	Kilo Base
VP	Viral Protein
SAT	South African Territories
OD	Optical density
MgCl ₂	Magnesium chloride
⁰ F	Fahrenheit
WGS	whole-genome sequencing
RNA	Ribonucleic acid
Min	Minute(s)
M	Molar
Fig.	Figure
Sec	Second(s)
DNA	Deoxyribonucleic acid
%	Percent
x-gal	5-bromo-4-chloro-3-indoyl-beta-galactopyranoside
V	Volts
Viz.	Namely
nm	nanometers



INTRODUCTION

Chapter-1

INTRODUCTION

Livestock is an important sector of Indian agriculture and contributes significantly to the Indian economy. The livestock sector contributes 4.11% of the country's total GDP and 25.6% of the total Agriculture GDP, while in the Union Territory of Jammu and Kashmir, the livestock sector contributes 5.04% to the state GDP (20th Livestock Census). Cattle, sheep and goats play an important role in the livelihood of a large percentage of the farmer's community in India. Around 192.49 million cattle, 148.88 million goats and 74.26 million sheep form the major livestock population in India. Jammu and Kashmir has around 3 million cattle, 2 million goats and 3.2 million sheep population (20th Livestock Census). In the year 2020-21, India ranked first in milk production with an annual production of 209.96 million tons, while the annual production of meat and wool was 8.8 million tons and 36.93 million Kg, respectively (DAHD, 2022). Despite these promising figures, there are numerous constraints that hamper the overall growth of the livestock sector including the higher prevalence of infectious and non-infectious diseases.

Foot and Mouth Disease (FMD) is one of the highly contagious viral and economically devastating diseases of cloven-hoofed animals throughout the globe (Artz *et al.*, 2011). FMD is on the A list of infectious diseases of animals of the Office International des Epizooties (OIE) (Pawar *et al.*, 2010). The disease affects all cloven-hoofed animals with broad host susceptibility including cattle, buffaloes, swine, sheep, goats, yaks, and many wildlife species (Radostits *et al.*, 2012). Susceptible non-cloven-hoofed species include hedgehogs, kangaroos, rats and mice etc. Exotic and crossbred livestock are more vulnerable to FMD as compared to indigenous breeds (Pawar *et al.*, 2010). The disease is endemic in India and is prevalent throughout the country including Kashmir, where the disease occurs in frequent outbreaks. Since FMD is highly contagious, there are biosecurity concerns regarding the accidental introduction of the virus into non-endemic regions or countries (Pawar *et al.*, 2010).

Because of the contagious nature of the disease, FMD has a great potential for causing severe economic losses, especially in high-producing animals. Direct economic losses due to FMD are attributed to losses in milk and meat production, mortality in young animals and reduced wool production in sheep. Indirect losses occur in the form of decreased draught ability and reproductive disorders including abortions in pregnant animals and infertility in the recovered animals (Howlader *et al.*, 2004).

Foot and Mouth Disease is a viral disease caused by the Picorna virus or FMD virus (FMDV) belonging to the genus *Aphovirus* and the family *Picornaviridae* (Racaniello, 2007). Foot and Mouth disease virus is a small, non-enveloped, positive-sense, single-stranded RNA virus, with a genome of approximately 8.5kb and carries a small polypeptide protein VP (Viral Protein). The four structural polypeptides are VP1, VP2, VP3 and VP4. The viral proteins VP1, VP2 and VP3 are exposed on the surface of the virus while as VP4 is located internally. Seven immunologically distinct serotypes of FMDV have been identified, namely serotypes O, A, C, Asia-1, SAT1, SAT2, and SAT3. The serotypes are named after their place of origin or person, where O depicts the department of Oise in France and A for Allemagne (the French word for Germany). A third serotype named serotype C was identified by Waldmann and Trautwein (Knowles and Samuel, 2003). Southern African Territories (1, 2, 3) are named as SAT (1, 2 and 3) after the place of origin from South Africa. The seventh serotype, Asia-1, was detected in a water buffalo at Okara, Punjab, Pakistan in 1954. Within these seven serotypes, more than 140 immunologically and serologically distinct sub-types have been identified that differ in degree of virulence subtypes (Al-Salihi, 2019). The most prevalent FMD virus serotypes that are reported throughout India are Serotype O, A and Asia-1 (Pattnaik *et al.*, 2012; Subramaniam *et al.*, 2013). Serotype C has not been reported in the country since 1995; so far, the abrupt disappearance of this serotype is not exactly known (Singh *et al.*, 2008b; Verma *et al.*, 2008). Serotypes are further classified into subtypes, topotypes (except for serotype Asia-1 viruses, which comprise a single topotype), genotypes, lineages and sub-lineages (Subramaniam *et al.*, 2013). Antigenic

variability and genetic diversity make FMD virus difficult to eradicate even through vaccinations, as there is no cross-protection between the serotypes (Jangra *et al.*, 2005).

Foot and Mouth Disease virus can spread from one animal to another by inhalation of virus aerosols or by ingestion. In endemic areas, the FMD virus is mainly transmitted by close contact between animals through aerosols and respiratory routes (Brito-Barbara *et al.*, 2016). Indirect transmission is facilitated by the ingestion of infected or contaminated materials, uncooked animal products including milk and also by inanimate objects, especially in non-endemic areas (Pawar *et al.*, 2010; Arzt *et al.*, 2018). There may be 100 percent morbidity due to FMD in a susceptible population of all ages. Mortality in adults can reach up to 2-5 percent and 20 percent in young ones (Radostits *et al.*, 2010). In ruminants, the primary site of virus multiplication is the pharynx. The virus may appear in milk and saliva within 24 hours before the vesicles appear in the mouth. The incubation period of the FMD virus varies between 2-7 days. The severity of clinical signs varies with the strain of the virus, the exposure dose, the age and breed of the animal, the host species and the immune status of the animal. The clinical features of the disease are characterized by anorexia, high fever, excessive frothy-ropy salivation, painful stomatitis, careful chewing behaviour and smacking of lips (Longjam *et al.*, 2011; Knight-Jones *et al.*, 2016). Vesicles contain a thin straw-coloured fluid and are 1-2 cm in diameter that appear on the mouth, especially on the buccal mucosa, dental pad and tongue. These vesicles usually rupture within 24 hours leaving ulcer areas. These vesicles may also appear on teats and on feet especially in clefts and on the coronet area (Mohammed, 2013). Adult animals may suffer weight loss and production losses for a longer period (Brito *et al.*, 2018). In neonates, myocardial necrosis may appear as small greyish foci producing a striped appearance of 'tiger heart' (Pawar *et al.*, 2010). Foot and Mouth disease is suspected on the basis of clinical signs and pathognomic macroscopic lesions in the mouth, udder and feet.

The laboratory diagnosis is obtained by nucleic acid recognition methods, isolation of the FMD virus from clinical samples, immunological methods, or

electron microscopy (Verma *et al.*, 2012). The PCR technique has revolutionized the diagnosis and has provided faster protocols for detection of the highly contagious and fast-spreading viral diseases. Nucleotide sequence analysis has now become the definitive technique for characterizing FMDV strains (Gonzalez *et al.*, 2012). The use of genetic variation techniques enables the FMDV strains to differentiate individually and track their movement across international borders (Knowles and Samuel, 2003). A strong molecular typing technique for outbreak analysis, whole-genome sequencing (WGS) enables quick differentiation between isolates from outbreaks and those from non-outbreaks (Abdelbary *et al.*, 2018).

There are higher chances of mutation rates in FMD virus during genome replication and as a result, different strains may emerge with variable antigenicity (Raies *et al.*, 2009; Verma *et al.*, 2010c). Also, there are chances of a change in virulence and antigenicity between the serotypes as each serotype demonstrates distinct characteristics with respect to transmission and exposure. Due to the presence of multiple strains and sub-strains of FMDV, at times, it is difficult to control the disease by therapeutic means and routine vaccination approach.

There is no specific treatment for FMD and only symptomatic treatment is practised to control the disease. FMD is otherwise difficult to control due to variations in the virus serotypes. However, the provision of early diagnosis and vaccination policy is imperative to control FMD outbreaks (Brehm *et al.*, 2008). As the culling policy cannot be practiced in India (due to ethical and socio-economic reasons), routine vaccination is the best way to achieve a protective antibody response against FMD. In India, cattle and buffalo are vaccinated biannually against the FMD viral disease, however, sheep and goats are not usually included in routine FMD control programs (Pattnaik *et al.*, 2012). FMD-infected sheep and goats may transmit the sub-clinical infection to cattle and buffalo as these ruminants are usually grazed together. Therefore, FMD vaccination in small ruminants as practiced in many states could prevent the transmission of FMD to cattle and other species (Parida, 2009).

The unrestricted animal movements, delay in seasonal vaccination, lack of quarantine measures and smuggling of animals are some of the major reasons for

frequent FMD outbreaks in endemic regions like Kashmir. Therefore, periodic investigations based on the epidemiology status and associated risk factors are essential to reduce the disease burden.

In view of the importance of Foot and Mouth disease particularly in Kashmir region, this study was proposed to provide the current cognizance of the disease with respect to the epidemiology, detection and identification of FMDV serotypes prevalent in the ruminant population, with the following objectives.

Objectives:

1. To study the epidemiological pattern and economic losses of Foot and Mouth disease in domestic ruminants.
2. To study the clinical features of Foot and Mouth disease in affected animals.
3. To study the Foot and Mouth disease virus serotypes prevalent in the Kashmir valley.



REVIEW OF LITERATURE

Chapter- 2

REVIEW OF LITERATURE

Foot-and-mouth disease (FMD) is an acute, highly contagious systemic disease of domestic and other cloven-hoofed animals. It is one of the most devastating diseases of livestock due to its impact on the economy (Segundo *et al.*, 2014). Cattle are most susceptible as a small amount of virus can cause severe infection. Although mortality is low, morbidity can reach 100 per cent (Arzt *et al.*, 2011). The first written description of FMD was probably that by Hieronymi Fracastorii (1546). He described the uncommon illness, which struck animals in Northern Italy in 1514. Later in Southern Africa in 1780, a sickness that affected the feet of cattle resulted in inflammation and falling of hooves described by Le Vaillant (1795). In 1897, Loeffler and Frosch described the filterable agent as the cause of FMD and in 1922, Vallee and Carre first showed the existence of two immunological types of FMDV by cross-immunity tests in cattle. Later, it was confirmed that the Aphthovirus of the Picornaviridae family was the causative agent of FMD (Smith, 1992). In the late 19th and early 20th century, many European countries experienced frequent FMD outbreaks which prompted vast and widespread research on the disease. There are seven distinct serotypes of FMD (O, A, C, Asia, SAT-1, SAT-2 and SAT-3), which differ in distribution across the world and comprise more than 65 subtypes (Knowles and Samuel, 2003). More than 70 species of domestic and wild cloven-hoofed animals including cattle, swine, sheep, and goats are affected by FMD (Bachrach, 1968). Although disease signs can vary among species, FMD generally causes fever and vesicular lesions on the tongue, feet and udder of susceptible animals resulting in salivation and lameness. Secondary bacterial infections frequently occur, especially on the feet (Segundo *et al.*, 2014). Various methods and diagnostics have been identified to study the disease pattern and new strategies are continuously being developed and enacted to enhance control and preventive measures. The various research aspects on Foot and Mouth disease investigated by different research workers are reviewed in this chapter.

2.1 Epidemiological study:

Mannan *et al.* (2009) studied the epidemiology of Foot and Mouth Disease and reported a 24.51% prevalence of FMD in cattle. The clinical prevalence of FMD was highest in the month of November (34.69%) and December (36.20%). The males (35.77%) were more susceptible than the females (15.97%). The adult cattle (34.18%) were more susceptible compared to the heifer (23.43%) and young ones (09.72%). Also, 39.18% of Indigenous breeds and 15.38 % of crossbreeds were infected by FMD.

Jamal *et al.* (2010) reported that out of 2484 epithelial samples collected from the outbreak in Pakistan, 1543 samples were found positive for various serotypes of FMDV. Serotype O was found to be the most prevalent followed by serotype Asia-1 and serotype A.

Dupka *et al.* (2011) studied the epidemiology of Foot and Mouth Disease in Bhutan and recorded 230 outbreaks of FMD at the sub-district level located in 19 out of the 20 districts in the country. Cattle were the most affected species, among all other affected ruminant species during the outbreak period. Serotype O constituted 70.6% of the outbreaks the most predominant serotype prevalent in Bhutan followed by A (16.7%), Asia 1 (8.8%) and C (3.9%).

Negussie *et al.* (2011) identified and reported four serotypes (O, A, SAT 2 and SAT 1) among the seven serotypes of the Foot and Mouth Disease virus in Ethiopia.

Sarker *et al.* (2011) in an epidemiological study reported FMD in 347 cattle, among them 154 were males and 193 were females. The overall prevalence of FMD was found to be 25.07%. The clinical prevalence of FMD was highest in the month of November (35.59%) and December (37.14%) and was also higher in old (36.53%) compared to adults (22.22%) and young (8.08%).

Shah. (2011) found that the prevalence of FMD was highest (47.12%) in July followed by September (39.32%) in Kashmir. The rate of infection was 17.13% and 4.16% during the spring and winter seasons, respectively. Viral typing of the 65 samples identified 49 (75.28%) as FMD type O strain, 6(9.23%) as type

A and 3 (4.67%) as Asia-1, whereas, no virus was recovered from 7(10.77%) cases.

Abubakar *et al.* (2012) confirmed that in Pakistan, during a period of five years (2005-2009), out of 590 samples, 180 were found positive, with an overall FMDV of 33.2%. Of the prevalent serotypes, FMDV 'O' serotype caused the most outbreaks (20.7%), followed by serotype A (6.6%) and serotype Asia-1(4.6%).

Depa *et al.* (2012) reported a predominance of serotype O (60.4%) from the FMD outbreak that occurred in Iran. No evidence for the presence of Asia-1 and A serotypes was reported.

Kasanga. (2012) in a study collected 364 samples out of which 167 were FMDV positive. Out of 167 positive samples, 37 (28.4%) were serotype O, 7 (4.1%) serotype A, 45 (21.9%) SAT-1 and 79 (45.6%) SAT-2. Two FMDV serotypes (O and SAT-2) were widely distributed throughout Tanzania, while SAT-1 and serotype A were only found in the Eastern Zone.

Ramanoon *et al.* (2013) carried out a retrospective study of the outbreaks of FMD in Peninsular Malaysia. A total of 270 outbreaks of FMD were recorded. Serotype O (89.95%) and serotype A (7.7%) were the most common serotypes identified.

Subramaniam *et al.* (2013) reported that FMD outbreaks in India were more in number in 2007-2008 than in 2010-2011. Three serotypes of FMD virus O, A and Asia-1 were prevalent. Serotype O was responsible for 80% of the confirmed outbreaks/cases, whereas Asia-1 and A responsible for 12% and 8%, respectively.

Hegde *et al.* (2014) studied the epidemiology of Foot and Mouth disease in Karnataka. Cattle and buffalo were the predominant species affected, being involved in all of the outbreaks reported. The majority of the outbreaks were caused by serotype O (64.04%), followed by Asia-1(19.87%) and A (12.27%). Serotype C was not reported since 1993 in the state.

Hossen *et al.* (2014) reported that out of 151 samples collected from different districts of Bangladesh, 71 (83.52%) were found positive for FMDV of which, 31 (36.47%) were positive for type A, (30.58%) type O and 10 (11.76%) for type Asia-1. Only 4 (4.70%) samples were found as positive for mixed infection having type A and Asia-1.

Ochi *et al.* (2014) in an epidemiological study reported 5 serotypes O, A, C, SAT-1, and SAT-2 in cattle, goats and sheep. Serotypes O and SAT-2 seemed to be the most prevalent in seven states of the country. The overall mean prevalence of FMD in Unity and Lakes states showed the highest and lowest prevalence rate of 56% and 25%, respectively in Sudan.

Tum *et al.* (2015) revealed that the village level prevalence of FMD in the southern provinces of Cambodia was 87% with an overall individual animal prevalence of 30%. Three serotypes: O, A and Asia-1 were detected in this region with a prevalence of 28.5%, 9.5% and 9.3 %, respectively.

Abubakar *et al.* (2015) confirmed that the highest proportion of positives was of serotype O (65.52%), followed by serotype A (24.14%) and serotype Asia-1 (10.35%) from the suspected FMD outbreaks that occurred in Pakistan.

Jemberu *et al.* (2016) reported that in Ethiopia, the most dominant serotype was O, accounting for 70% of all the collected samples that tested positive followed by SAT 2 with 20.8%. During the entire study period, serotype SAT-2 had overtaken the rank of serotype A, which used to be reported as the second most dominant serotype in the country.

Giasuddin *et al.* (2016) reported that out of 134 samples collected from clinically FMD-infected cattle from 15 different outbreak areas of Bangladesh, 73% were positive for FMDV. Three different serotypes (O, A, Asia-1) of FMDV were found to be present in Bangladesh. Among the positive FMDV, serotype O and Asia-1 accounted for about 31% followed by A with 7% incidence.

Soltan. (2016) studied the epidemiology of Foot and mouth disease in Egypt. Serotype O was detected in 73.3% of herds and serotype A was identified in 26.7% of herds.

Abdela. (2017) studied the epidemiology of Foot and Mouth Disease in Ethiopia. The most dominant serotype identified was O, accounting for 72% of the investigated outbreaks, followed by A19.5%, respectively.

Ali *et al.* (2017) reported that prevalence rates for the three FMD serotypes in an outbreak in Pakistan were 70% for serotype O, 25% for serotype Asia-1 and 4.7% for serotype A.

Machira and Kitala. (2017) reported an outbreak in Kenya and found that serotype O was the most isolated serotype out of 109 tested positive for FMDV with 25.15% of the samples being positive. This was followed by SAT-2 (17.5%), Serotype A (11.7%), and SAT-1 (9.4%) positivity, respectively.

Nawaz *et al.* (2018) reported the FMDV outbreak occurred in the Punjab province of Pakistan and collected 109 epithelial tissue samples (42 from buffaloes, 54 from cattle, 10 from goats and 3 from sheep). A total of 77 (70.65%) samples were found positive for the FMD virus. Out of these positive samples, 48 were successfully typed into serotype O (62.33%) followed by 26 (33.77%) into Asia-1 and 3 (3.90%) into serotype A.

Al-Salihi *et al.* (2019) reported that during the FMD outbreak in Iraq, the prevalence rates of FMD were 68.7%, 46.6% and 30.3% in cattle, buffalo and small ruminants, respectively during 2016 and prevalence rate of 18.4%,19.9% and 17.3% respectively, during 2015.

Rafique *et al.* (2020) reported 106 samples as FMD positive, out of the 184 epithelial samples collected in an outbreak in Punjab, Pakistan. Of the three serotypes identified, serotype A was the most prevalent (39.62%), followed by serotype O (33.96%), while the least prevalent was serotype Asia-1 (26.41%).

Tesfaye *et al.* (2020) studied the epidemiology of Foot and Mouth Disease in Ethiopia and analyzed 43 samples by using FMDV screening primers and serotype-specific primers. The contribution of the disease-causing serotype was serotype O (18.60%), serotype A (46.51%) and mixed infection O and A (2.33%).

Mpoloka *et al.* (2021) reported 176 FMD-positive samples from a total of 453 bovine epithelial tissue samples from 33 FMD outbreaks that occurred in southern African countries. Serotype SAT-2 was found in 105 samples, serotype SAT-1 in 32 samples and serotype SAT-3 in 21 samples.

Ali *et al.* (2022) carried out an epidemiological study of Foot and Mouth Disease in Punjab and collected 64 samples. The FMD outbreaks started during the month of January, culminated in February (56.25%), and stopped during the month of April 2019. The most common serotype identified was O (45.83%), followed by Asia-1 (29.17%) and A (13.89%). In some farms, mixed infection with serotype A and O (9.72%), serotype O and Asia-1 (1.39%) were identified.

2.2 Clinical Study:

Sanson. (1994) studied the clinical characterization due to the FMD outbreak in animals in New Zealand, which was manifested by the formation of vesicles or erosions in the mucosa of the mouth, including the tongue, lips, gums, pharynx, and palate; and is associated with a sharp rise in body temperature of 3-4°C. The typical vesicle had a blanched covering, with the release of clear, sometimes turbid, colourless or straw-coloured fluid. Vesicles were found on the coronary band and in the interdigital cleft. Vesicles were also present on the teats of lactating animals.

Davies. (2002) reported vesicular lesions on the coronary band of the hooves and in the mucosa of the mouth including the tongue palates prominent clinical findings in FMD-affected animals. The vesicles typically contained clear or straw-coloured fluid before they burst and healed.

Kitching. (2002) reported pyrexia (40°C), a variable number of vesicles on the tongue, hard plate, dental pad, lips, gums, muzzle, coronary band and interdigital space in the affected animals. Vesicles were also seen on the teats, particularly of lactating cows. Acutely infected cattle salivated profusely and developed a nasal discharge, first mucoid and then mucopurulent which covered the muzzle.

Alexandersen *et al.* (2003) reported lameness, manifested by foot flicking, a tucked-up stance and reluctance to stand or walk and inappetence in FMD-affected animals. Vesicles were seen on the muzzle, teats, mammary gland, prepuce, vulva and other sites of skin.

Musser. (2004) recorded the clinical signs of FMD included excessive salivation, fever, vesicles and erosions of the oral and nasal mucosa, coronary band, interdigital areas and teats, lameness, sloughing of claws, reluctance to move, anorexia, mastitis, decreased milk production and abortion or weak newborn.

Alexandersen *et al.* (2005) in a study reported that major clinical signs of FMD were fever, drooling of saliva, formation of vesicles in and around the mouth and on the feet (interdigital space, bulb of the heel and the coronary band), muzzle, teats, mammary gland, prepuce, vulva and other sites of skin and mucosa including lameness and inappetence.

Rhyan *et al.* (2008) observed that clinical signs of FMD in North America as fever, lameness, inappetence and ptyalism. Physical examinations on bison revealed numerous small vesicles and erosions affecting tongue, gingiva, muzzle, hard and soft palates, coronary bands and interdigital skin.

Klein. (2009) studied clinical signs of FMD characterized by fever (often above 40°C), excessive salivation, lameness, depression and decreased milk production. The mucosa of the lips, the dorsum of the tongue and the dental plate were severely involved. In sheep and goats, only lameness through aphthae and inflammation at the cloves was reported.

Ghanem and Hamid. (2010) observed the clinical signs of FMD in Holstein cows in Egypt which had, profuse salivation, and lameness followed by heat intolerance (panting) syndrome, hirsutism and reduced milk production.

Pawar *et al.* (2010) studied the clinical signs of FMD in cattle and found the characteristic signs of high fever, salivation, and vesicle formation in the mouth and feet followed by lameness. The animals further showed debilitating effects,

including weight loss, decrease in milk production and loss of draught power, resulting in loss of productivity for a considerable time.

Sarker *et al.* (2011) recorded the prominent clinical signs of FMD including fever, vesicular eruption in the mouth, nares, muzzle, foot, teats and other hairless soft areas of the body. The disease had a debilitating effect, including weight loss and a decrease in milk production.

Ayelet *et al.* (2012) reported fever, salivation and vesicular eruptions on the feet and mouth in clinically FMD-affected animals. Morbidity estimated was 100% in susceptible animal populations but mortality was low, particularly in adults.

Rout *et al.* (2012) recorded initial clinical signs in cattle as fever of 103-105°F, dullness, anorexia and a drop in milk production. These signs were followed by excessive and drooling salivation, serous nasal discharge, shaking/kicking of the feet, or lameness followed by vesicle (blister) formation. Sites of predilection for vesicles were the tongue, dental pad, gums, soft palate, nostrils, muzzle, interdigital space, coronary band and teats.

Yoon *et al.* (2012) studied the clinical signs of FMD incidences in the Republic of Korea and the most commonly observed clinical signs were profuse salivation, vesiculation, lameness, ataxia and ulceration, irrespective of the species. About 54% of the infected dairy farms reported vesicles on the teats as primary clinical signs.

Jamal *et al.* (2013) reported high temperatures, excessive salivation and formation of vesicles on the oral mucosa, interdigital spaces and coronary bands of feet in FMD-affected cattle.

Chakraborty *et al.* (2014) recorded a high rise in temperature, vesicles (blisters) on the mouth, muzzle, tongue, nose, teats, inter-digital space of feet and other hairless parts of skin that resulted in off-feeding and lameness in cattle.

Delgado *et al.* (2014) found the clinical signs of FMD in affected cattle as drooling, lameness, fever, loss of appetite and the formation of blisters in the

mouth or at the top of the hooves. Also, affected cattle in the herd appeared depressed and reluctant to move.

El-Bayoumy *et al.* (2014) observed clinical signs in FMD-affected cattle started with a high fever (42-43%), which persisted for 3-4 days, lameness, anorexia and loss of general health condition. About 20% of infected sheep showed vesicles and/or ulcers on the dental pad and dorsum of the tongue. Foot lesions along the coronary band and interdigital space were obvious for 2-3 weeks. Some animals had only high fever (42-43°C) without developing any mouth or foot lesions.

Admassu *et al.* (2015) recorded the clinical signs of FMD in cattle in Ethiopia which included fever, depression, excessive salivation, lameness and formation of vesicular-type lesions on the mucous membrane of the mouth (tongue, dental pad and gums) and the skin of the muzzle, interdigital spaces, udder, teats and coronary band.

Islam *et al.* (2016) recorded the clinical signs of FMD in cattle which included high fever (104-106°F) (in 100% of cases), frothy salivation (81.8%), sore foot (72.7%), sore tongue (63.6%), involvement of foot region (94.6%), lack of appetite, anorexia, loss of milk production, hoof deformities and lameness.

Rout *et al.* (2016) in a study summarized the results of a clinico-molecular investigation of FMD in sheep and goats across several states in India during 2008–2014, where a total of 51 clinical epithelial tissue samples (vesicle/tongue/gum/foot epithelium) from sheep and 78 from goats were found positive for FMD virus (FMDV) serotype O in serotyping ELISA and multiplex reverse transcription-PCR.

Soltan. (2016) reported that all affected animals in a single farm had either severe or mild clinical form due to FMD in Egypt. The severe form was characterized by fever (40.5-41°C) and extensive lesions in the mouth and interdigital spaces of the feet. In some cases, the entire oral mucosa got sloughed during the oral examination. The mild infection was characterized by fever (40-40.5°C) and small vesicular lesions on the oral mucosa.

Hegazy *et al.* (2018) reported clinical signs of FMD in Egypt, which included fever which ranged from (39°C-42°C) with severe salivation, vesicular lesions of the mouth, feet, tongue and teats. Mouth lesions varied from an elevated area of hydropic degeneration on the upper surface of the tongue to a red area and severe ulceration that resulted from ruptured vesicles found in the upper lips, tips of the tongue, dental pad and upper third of the tongue. Lameness due to the foot lesions which were represented by severe vesicular lesions in the digits and coronary bands ended by severe ulceration in interdigital space.

Sobhy. (2018) performed a clinical examination of FMD-affected animals in Egypt and found anorexia, fever, recumbency, hurried respiration, tachycardia with a rhythmic pulse, excessive salivation and vesicular lesions on the tongue and feet resulted in lameness.

Tesfaye. (2019) studied the clinical signs of FMD in bovines in Ethiopia, characterized by fever (above 40°C), excessive salivation, lameness, depression and decreased milk production. The mucosa of the lips, the dorsum of the tongue and the dental plate were most severely involved. Lameness was reported as the first indication of FMD in sheep and goats. The animal separated itself from the rest of the flock.

Wong *et al.* (2020) found FMD-infected animals developing lesions on the tongue, muzzle, oral cavity, coronary bands and teats. Other signs frequently observed include fever, loss of appetite, weight loss, hypersalivation, depression, growth retardation and severe decrease in milk production.

2.3 Economics of FMD:

Kazimi *et al.* (1980) analyzed the data from an FMD outbreak in Sahiwal cattle and found that 70% of the herd was affected, even though it had been vaccinated previously. Of the cows affected, 69% had reduced milk yields (74 liters) and 74% had lost weight (an average 18 kg).

Saxena. (1994 a,b) estimated the losses in milk production due to FMD was 3508 million kg. Losses due to loss of draught power, animal deaths and cost of

treatment were about Rs 18,130 million/year. That 80 % of the total direct loss caused by FMD was due to a drop in milk production.

Mulei *et al.* (2001) calculated the economic loss due to FMD for a period of 95 days, with the greatest direct financial impact due to milk losses (42.0%), followed by the purchase of additional feeds (13.6%) and culling of milk cows that developed chronic mastitis (12.5%). The other direct costs were extra labor inputs (8.9%), veterinary fees (3.3%), transport (3.0%), deaths (3.0%), treatment (2.9%), abortions (1.4%) and chemicals (0.5%).

James and Rushton. (2002) estimated economic losses on a dairy farm with 280 cattle that experienced FMD in Colombia. Seventy-four percent of the animals were affected leading to a 26% drop in milk production, an increase in mortality (0.7% died) and an average loss in weight per animal.

Howlader *et al.* (2004) estimated that in Bangladesh, the incidence of FMD varied significantly ($p < 0.01$) in different host species with a higher incidence in cattle (63.41%) followed by sheep/goats (50.96%) and buffaloes (48.02%). A total of 125 (9.71%) calves died of FMD and the financial loss incurred from this mortality was estimated to be US\$ 6250 at the rate of US\$ 50/calf. The economic losses due to calf mortality, reduced milk yield and draft power would stand at US\$ 163329 for cattle alone.

Thirunavukkarasu and Kathiravan. (2005) estimated the economic losses in livestock farms due to FMD outbreaks in four districts of Tamil Nadu. The loss due to the treatment of affected animals has been estimated to be Rs 89.16 lakh. An amount of Rs 300.88 lakh has been found to be lost due to reduced milk yield, while the loss in earnings because of the reduced draught power has been observed as Rs 9.26 lakh. A total of Rs 31.71 lakh has been estimated to be lost due to the mortality and culling of FMD-affected stock. The total economic loss due to abortions in FMD-affected bovines has been estimated to be Rs 6.50 lakhs. The total economic losses accrued as a result of the FMD outbreak have been found as Rs 448.48 lakh.

Mathew and Menon. (2008) calculated the economic loss due to FMD in India by taking into consideration milk loss, losses due to abortion and treatment charges. The total economic loss was calculated as Rs. 313900, out of which loss in milk production accounted for 80.68 %.

Thirunavukkarasu and Kathiravan. (2010) estimated the economic losses due to milk production losses and the reduction of draught power in FMD-affected bovines in 4 districts of Tamil Nadu. The prevalence of FMD was higher in cattle than in buffaloes. The total loss due to treatment was Rs.88.89 lakhs, a greater proportion of which was for the treatment of cattle (93.27%). The losses due to reduced milk output and draught power were Rs. 300.88 and 9.26 lakhs, respectively. The total economic loss due to FMD outbreak in the studied area was Rs. 1133.53 lakhs.

Gorsi *et al.* (2011) estimated the economic losses due to FMD in district Sahiwal, Punjab as around Rs.27448000 during the study period (6 months) in infected /outbreak villages. In a total population of 2571 cattle, morbidity, mortality and case fatality rates were 53.20%, 15.25% and 28.65%, respectively.

Depa *et al.* (2012) studied the economic loss due to FMD in dairy animals. The total cost of that outbreak was estimated at 9-13 billion dollars, where there were at least 4.5 billion dollars in direct costs and 9 billion dollars in agricultural and tourism sectors.

EI-Hussein and Daboura. (2012) recorded the costs of the outbreak in Khartoum state, Sudan, assessed in terms of losses in milk production, costs of drugs and mortality. The overall loss to the dairy farmers in the state was estimated at USD 1771924 with the loss of milk.

Knight-Jones *et al.* (2013) estimated that the annual impact of FMD in terms of visible production losses and vaccination in endemic regions alone amounted to between USD 6.5 and 21 billion. In addition, outbreaks in FMD-free countries and zones caused losses of >\$USD 1.5 billion a year.

Singh *et al.* (2013) calculated the economic loss due to FMD in animals. Among different components of losses, the maximum loss of 49.83% was

observed due to milk loss (direct and indirect), followed by opportunity cost (16.15%) and reduction in growth (12.20%). The total losses per infected animal due to FMD in cattle, buffalo, sheep, goat and pigs were Rs. 12,532, 21,682, 2,023, 3,046, and 2,830, respectively.

Young. (2013) calculated the average post-FMD losses in animals that occurred in Southern Cambodia, which varied from USD 216.32, a 54% reduction from the pre-FMD value, because of weight loss and treatment costs, to USD 370.54, a 92% reduction from pre-FMD value, of animals.

Jemberu *et al.* (2014) calculated the economic losses of the FMD outbreak in Ethiopia due to milk loss, draft power loss and mortality were on average USD 76 per affected herd and USD 9.8 per head of cattle.

Ali *et al.* (2015) studied the economic impact due to FMD and analyzed it by recording milk loss. Their average milk yield was observed at 9.2 litres before the onset of the disease which decreased dramatically after the disease. Milk loss of 225 and 195 litres was recorded for buffalo and cattle, respectively during 70 days of the study period.

Mdetele *et al.* (2015) reported the economic impacts of FMD and reported major losses were associated with treatment costs (87.5%), milk productivity (85.0%), draught power (80.0%), livestock market loss (67.5%), lower weight gain (60.0%), lower fertility (37.5%), abortion (35.0%), death of animals (25.0%) and vaccine supply cost (2.5%).

Baluka. (2016) estimated that large herds experienced the highest milk losses due to FMD. Total income earned per month at the processing level was reduced by 23%. Animals were sold at 83% and 88% less market value, respectively due to FMD impact.

Farooq *et al.* (2017) studied economics due to FMD in cattle and buffaloes in Pakistan. The estimated milk loss was 307.8 liters per animal in 45 days. The total damage due to FMD in sampled buffaloes was estimated as much as Rs.6.7 million.

Govindaraj *et al.* (2017) reported an outbreak of FMD in Karnataka state, India and estimated that in indigenous cattle, the highest loss due to FMD was because of distress sale (208 USD) followed by other losses, whereas, in crossbred cattle, the highest loss was mortality loss (515 USD) followed by distress sale (490 USD), milk yield loss (327 USD), treatment cost (38 USD) and extra labor engagement expenses for nursing of FMD-affected bovines (30 USD).

Sinha *et al.* (2018) estimated a production loss of Rs 3184.00 in crossbred cows and Rs 3062.50 in buffaloes. There was a reduction in milk yield, accounted Rs.894.60 and Rs.510.00, representing 20.88% and 13.60% of the total loss in crossbred cows and buffaloes, respectively.

Govindaraj *et al.* (2019) evaluated the economic losses of FMD in India and reported that among the indigenous cattle, the milk reduction loss per animal ranged from INR 427 to INR 2949 and in crossbred cattle INR 1,145 to INR 7829. In buffaloes, the milk reduction loss ranged from INR 1689 to INR 8886. The mortality loss ranged from INR 2,027 to INR 50,000 per animal in indigenous cattle and INR 1912 to INR 35,920 in crossbred cattle. In crossbred animals, the distress sale loss ranged from INR 4,000 to 32,286. In indigenous cattle, the treatment cost per animal ranges from INR 246 to INR 2,700 whereas, in crossbred cattle, the treatment cost ranged from INR 250 to INR 2,068.

Alhaji *et al.* (2020) estimated the economic impact of FMD occurred in Northern Nigeria due to production losses and treatment costs which were estimated to be USD 16,055,368.00. The value of visible losses to the herders was estimated at USD 15,591,694, 30 and the cost of control by treatment of secondary infection was USD 463,673.70.

Govindaraj *et al.* (2020) studied the economic loss due to FMD in cattle and buffaloes in India. The total estimated loss due to FMD in cattle and buffalo was INR 20,897 crore during 2013–14. The total losses estimated comprise milk yield reduction, unavailability of draught power, distress sale, treatment cost, mortality and opportunity cost of labour.

Tadesse *et al.* (2020) reported the herd level morbidity of FMD in the outbreak-affected part of Northwest Ethiopia and estimated 57.2% for cattle and 8% for sheep and goats. The economic losses due to milk loss, draught power loss, mortality and treatment cost were on average USD 34 per affected herd.

Govindaraj *et al.* (2021) estimated that in India the milk loss per animal ranged from USD 6.87-47.44, 18.42-125.88, 16.33-91.43 and 27.17-123.62 in indigenous cattle, crossbred cattle, local and improved buffalo, respectively. Mortality loss per animal ranged from USD 32.61-804.27, 30.76-577.7, 65.36-502.2 and 188.04-413.7. Distress sale loss per animal ranged from USD 3.22-188.63, 64.34-519.3, 214.47-341.8 and 209.11-450.3 and cost of labour per animal from USD 5.49-54.29, 5.49-67.78, 7.95-31.37 and 9.83-72.38, respectively.

2.4 Molecular Study:

Reid *et al.* (1998) collected 166 clinically affected FMD samples from the field out of which 78 were positive by RT-PCR. The RT-PCR detected FMD viral RNA in 11 of the 86 samples assessed as negative by virus isolation/ELISA.

Waheed *et al.* (2007) found 36 (23%) samples collected from FMD-suspected animals from the Punjab province of Pakistan as positive following RT-PCR analysis. A total of 156 samples of vesicles from the mouth, hooves and tongue tissues of dead animals were collected and subjected to RT-PCR analysis.

Balinda *et al.* (2010) detected FMD virus RNA by diagnostic RT-PCR in 9 of the 12 tissue samples collected (oropharyngeal tissue samples) from 12 cattle (Uganda). All samples identified belonged to the SAT-2 serotype.

Saeed *et al.* (2011) detected FMD in 182 out of 250 (72.8%) tissue samples and 92 out of 175 (52.6%) secretion samples. Samples collected were 250 FMD clinically positive tissue samples (mouth and hoof epithelium and vesicle swabs, tongue tissue) and 175 secretion samples (milk, saliva, serum, plasma) that were evaluated by RT-PCR for the diagnosis of FMD.

Waheed *et al.* (2011) detected positive 32 samples from 58 clinical samples. Out of the 32 samples that were positive for FMDV with serotype O in 31 and serotype A in 1 animal.

Lee *et al.* (2012) collected 55 samples from FMD-affected animals and by use of reverse transcriptase PCR (RT-PCR), serotype O was found in (n=34), serotype A was found in (n=17) and serotype Asia-1 was found in (n=4).

Rashtibaf *et al.* (2012) collected soft palate samples immediately after slaughter for the detection of FMDV by RT-PCR and the results showed that 37.7 % of cattle (96 of 255) were carriers of the virus. Among positive samples (96), 58 (60.4%) belonged to serotype O. No evidence was detected for the presence of Asia-1 and A serotypes were found.

Al-Rodhan and Salem. (2014) reported that out of 83 samples collected, only 68 (81.9%) were successfully amplified by RT-PCR and only 19 (22.9%) of samples were found serotype-A positive and 26 (31.3%) were found serotype-O positive. The samples were collected from clinically FMD-suspected cattle, which included samples from mouth vesicles, serum and saliva.

Al-Rodhan. (2014) collected blood, saliva and vesicle fluid from 73 FMD-affected cattle and tested by RT-PCR. RT-PCR based detection of FMDV for primary and serotype-specific diagnosis was done with universal primer sets 1F/1R, A-1C612 and O-ARS4 with the expected bands of 328, 865 and 1301bp, respectively.

Hossen *et al.* (2014) estimated 71 (83.52%) FMD-positive cases by RT-PCR from 151 samples collected from different districts of Bangladesh, out of which 31 (36.47%) were positive for type A, 26 (30.58%) for type O and 10 (11.76%) for type Asia-1. Only 4 (4.70%) samples were found as positive for mixed infection having type A and Asia-1.

Kadir and Ahmed. (2014) reported 90% of positive FMD samples collected from cattle of two different outbreak areas of Bangladesh, out of which 70% were identified as serotype O and 20% were identified as Asia-1. One set of universal primers was used in RT-PCR for the detection of FMDV.

Alam *et al.* (2015) collected 12 samples comprising tongue epithelium (n=8) and inter-digital tissue (n=4) from FMDV-suspected cattle in Bangladesh. RT-PCR was performed using three sets of primers corresponding to the serotype O, Asia-1 and A respectively. Out of the 12 samples, 10 (83.33%), were found to be positive for FMDV, with all of them sequencing to serotype O.

Ali *et al.* (2015) confirmed FMD, from different outbreak areas of Faisalabad. On the basis of clinical signs and then RT-PCR out of a total 88 samples, 73 were found positive which were then serotyped into type O (n=44), Asia-1 (n=18) and A (n=6).

Giasuddin *et al.* (2016) collected 134 samples from clinically FMD-infected cattle from outbreak areas in Bangladesh. All samples were subjected to RNA extraction and RT-PCR for detection and serotyping of FMD. Out of 134 samples, 73 % were positive for FMDV. Among the positive FMDV, serotype-O and Asia-1 accounted for about 31% followed by A (7%) serotype.

Soltan *et al.* (2016) reported an outbreak of FMD in Egypt and collected vesicular epithelia from 41 animals which were tested by RT-PCR. Consequently, 92% of examined samples were positive.

Ali *et al.* (2017) reported that out of a total of 72 samples collected from different FMD outbreaks in Faisalabad, 65 were found positive, for FMD which were then serotyped into type O (n=30), Asia-1 (n=19) and A (n=5). Some samples (n=5) were found positive for more than one serotype. RT-PCR was employed for the confirmation of the FMDV genome.

Algayed *et al.* (2018) confirmed that the clinically FMD-infected animals were positive with FMDV serotype A (16.3%), O (79.3%) and SAT-2 (4.4%) respectively in samples. A total of 10 collected samples tested positive by using conventional RT-PCR.

Mansour *et al.* (2018) conducted a study on cattle suspected of foot-and-mouth disease (FMD) in Iraq. They reported FMD in 55 of 73 cases (75.3%); of these, 37 (67.3%) were females and 18 (32.7%) were males, with high significance ($p < 0.01$) between males and females in the PCR positivity ratio. The products of

RT-PCR were sent for RNA sequencing and the results were 100% positive for serotype A, which meant that it is the predominant type in Iraq.

Ali *et al.* (2019) confirmed FMDV from a total of 30 (15 calves and 15 adults) tongue epithelial tissue samples from clinically FMD-affected cattle in Bangladesh. Serotyping (A, O and Asia-1) was performed by RT-PCR. All 30 samples were found FMDV positive and mixed infection of all samples with serotype Asia-1 and serotype O were observed.

Diab *et al.* (2019) reported that out of 51 oral tissue samples collected from cattle and buffaloes in 13 farms samples suspected to be FMD (in Egypt), 44 (86.27%) were positive by RT-PCR using universal primers. Serotype O was predominant being detected in 31 samples (70.45%), serotype A was detected in 9 samples (20.45%) and then serotype SAT-2 was identified in 4 samples (9.10%).

Singh *et al.* (2019) reported that a total of 25 tongue epithelial samples were collected from suspected cases of FMD and subjected to RT-PCR assay for diagnosis. Out of 25 samples, only 20 were found positive for the presence of the FMD virus, among which 12 were serotype-O and 8 were serotype-A.

Teya *et al.* (2019) reported that serotypes O and A were detected from clinical samples by RT-PCR. Samples collected were epithelium and oral swabs (n=20) from cattle from four districts in Southern Ghana that experienced FMD outbreaks.

Dubie and Amare. (2020) confirmed that out of 27 samples detected by conventional RT-PCR, 12 FMDV samples were found to be FMDV positive by universal primers in Ethiopia.

El-Mayet *et al.* (2020) reported that 32 out of 45 samples (tongue epithelium, vesicular fluid) from FMDV clinically suspected cases showed a positive result in RT-PCR. The isolated viruses were identified and serotyped using RT-PCR that confirmed the 3 different serotypes A, O and SAT-2 with different ratios for their prevalence in Egypt.

Julieth. (2020) revealed that 72% of samples (n=26) were positive for the FMDV genome from 36 epithelial samples collected from cattle in Tanzania and laboratory analysis of the samples was performed by RT-PCR. Molecular typing of the FMDV genome positive samples was achieved by using serotype-specific primers, of which serotypes A were 85% (n=22) and O were 15% (n=4).

El-Damaty *et al.* (2021) collected 54 samples from water buffaloes with acute signs of FMD. Oral desquamated epithelial and oropharyngeal fluid samples have been tested for FMDV by reverse transcriptase PCR (RT-PCR). The results showed that all the examined samples were confirmed positive for FMDV serotype SAT-2.

Islam *et al.* (2021) carried out a study in FMD-suspected crossbred cattle (2-3 years old) and collected 16 tongue epithelial samples, in which 14 samples were positive by RT-PCR. The detection rate of Foot and Mouth Disease by RT-PCR was 87.50%. The samples were further sero-grouped and serotyped for FMDV. It was detected in 42.86%, 100%, 21.43% and 21.43% of serotype O, Asia-1, A, and mixed infection with Asia-1 and A, respectively.

Khan *et al.* (2021) detected viral genome in 51 (85%) of the samples using pan-FMDV primers set, 1F/1R from a total of 60 epithelial samples collected from suspected cases of FMD using RT-PCR assays.

Salem *et al.* (2021) in a study, collected 30 oral epithelial samples from vaccinated animals (14 native cattle and 16 water buffaloes), which showed clinical signs of FMD. All the collected samples were tested by using conventional RT-PCR. Fifty per cent of the examined samples were positive for FMDV and serotyped as serotype O (40%), serotype SAT-2 (33%) and serotype A (27%), respectively.

Rahman *et al.* (2022) detected FMDV in 55.56 per cent (5/9) of the suspected samples collected from 1-2-year-old black Bengal goats suspected to have FMD, using RT-PCR. However, FMDV serotype A was reported in 100 per cent (5) positive samples.



MATERIAL AND METHODS

Chapter-3

MATERIALS AND METHODS

3.1 Epidemiological study

The epidemiology of foot and mouth disease in ruminants (cattle and sheep) was investigated in outbreak sites from September 2021 to August 2022. A total of 96 FMD-infected animals (cattle and sheep) were included in the study from four different districts of Jammu and Kashmir *viz*: Baramulla, Bandipora, Ganderbal and Srinagar. The detailed qualitative parameters were taken into consideration while investigating the epidemiology of foot and mouth disease (Table 1).

Table 1: Proforma for epidemiological investigation

A: Animal affected

Species	Age	Gender
Reduction in milk yield	No. of animals died/case-fatality	Disease duration (days)

B: History

Feeding	Vaccination status	Disease pattern

C: Clinical signs

Salivation pattern	Temperature (°F)	Heart (Rate/Min)	Respiration (Rate/Min)	Complications

D: Site of lesions

Oral mucosa/tongue/soft Palate	Udder/teat	Hoof/Feet	Any other site

3.2 Estimation of economic losses occurred due to FMDV

The estimated losses due to FMD were evaluated based on the response of the FMD-affected farm families on various qualitative and quantitative parameters (Govindaraj *et al.*, 2015).

Economic losses caused by the FMD disease were evaluated in terms of:

1. Productivity loss (Reduction in milk yield)
2. Treatment cost
3. Mortality/Death

3.2.1 Average short-term loss due to milk yield reduction per animal was calculated as per the formula

$$S_Y = 1/n \sum (E-A) \times D \times P$$

Where S_Y = average short-term loss due to milk yield reduction per animal;

E = expected milk yield (litres/day);

A = actual milk yield till recovery from FMD (litres/day);

D = duration of infection in lactating animals (days);

P = litre of milk;

n = number of lactating animals recovered from FMD.

3.2.2 Average treatment costs per animal were calculated as per the given formula

$$L_T = 1/n \sum (F+M+I)$$

Where L_T = average treatment costs per animal;

F = fees for veterinarians/animal/farm family;

M = cost of medicines/animal/farm family;

I = cost of drugs/treatment during the infected period;

n = total number of animals infected by FMD.

3.2.3 Average loss due to mortality per animal was calculated as per the given formula

$$L_M = 1/n \sum A_j \times V_j$$

Where L_M =average loss due to mortality per animal in cattle/sheep/goat;

j = category of animals, viz. in milk, dry, bull, immature males, heifer, male calf and female calf;

A_j =number of animals in different categories;

V_j =average value/cost of animals;

n = total number of animals infected by FMD/died.

3.2.4 Total losses

Total losses were estimated by the sum of average short-term loss due to milk yield reduction (S_Y), average treatment costs per animal (L_T) and average loss due to mortality per animal (L_M).

$$\text{Total loss per animal} = S_Y + L_T + L_M$$

3.3 Therapeutic trial study

Eighteen FMD-affected cattle were selected for a clinical trial and were divided into 3 groups of 6 animals each. It was ensured that the groups were uniform in terms of age and the severity of the lesions. Group 1st and 2nd animals were given antibiotics along with supportive treatment (for 5 days) and an immunomodulator (7 days), while as, in group 3rd affected animals, only antibiotic and supportive treatment (for 5 days) were given (without any immunomodulator). The average number of days required for the remission of clinical signs was evaluated to compare the effectiveness of different therapeutic regimens. The different therapeutic regimens evaluated are given in table 2.

Table 2: Therapeutic trial study in FMD-affected animals

<p style="text-align: center;">Group I (N=6)</p>	<p style="text-align: center;">Group II (N=6)</p>	<p style="text-align: center;">Group III (N=6)</p>
<ul style="list-style-type: none"> • Antibiotic (Enrofloxacin @ 2.5mg/Kg b.wt.) x I/M for 5 days. • Anti-inflammatory (Meloxicam & Paracetamol @ 10mL) x I/M for 5 days. • Anti-allergic (Pheniramine maleate @10 mL) x I/M for 5 days. • Emollient and antiseptics (Boro-glycerine Paste, Potassium Permanganate 1% mouthwash) • Immunomodulator (Restobal @ 50mL for large animals OD for 7 days) 	<ul style="list-style-type: none"> • Antibiotic (Gentamicin @ 2.5 mg/Kg b.wt.) x I/M for 5 days. • Anti-inflammatory (Meloxicam & Paracetamol @ 10mL) x I/M for 5 days. • Anti-allergic (Pheniramine maleate @10 mL) x I/M for 5 days. • Emollient and antiseptics (Boro-glycerine Paste, Potassium Permanganate 1% mouthwash) • Immunomodulator (Tissue aid 2 boli/day for 7 days) 	<ul style="list-style-type: none"> • Antibiotic (Enrofloxacin @ 2.5mg/Kg b.w) x I/M for 5 days. • Anti-inflammatory (Meloxicam & Paracetamol @ 10mL) x I/M for 5 days. • Anti-allergic (Pheniramine maleate @10 mL) x I/M for 5 days. • Emollient and antiseptics (Boro-glycerine Paste, Potassium Permanganate 1% mouthwash) • No immunomodulator

3.4 Molecular epidemiology

3.4.1 Sampling

Samples were collected from FMD-infected animals (cattle and sheep) during the study period. Samples included vesicular tissues from the lesions present on mouth, tongue, hard palate, udder, teats and feet/hooves (Plate 1). A total of ninety-six (96) tissue samples were collected from the suspected animals exhibiting pathognomic FMD signs. The tissue samples obtained were directly

preserved in TRIzol reagent (Sigma, USA) and kept at -80°C until further processing.

3.4.2 Extraction of total RNA from tissue samples

RNA was extracted from tissue samples using TRIzol reagent as per the protocol described by (Chomeynski and Sacchi., 1987).

3.4.3 Homogenization

Tissue samples (50 to 100 mg) were homogenized in 1 ml of TRIzol reagent using a power homogenizer (Polytron, Tekmar's TISSUEMIZER). Samples were subsequently incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Samples were centrifuged to remove cell debris and the supernatant was transferred to a new tube.

3.4.4 Phase Separation

Two hundred (200) microliter of chloroform was added per ml of TRIzol sample. Samples were vortexed vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes. Centrifugation was done at 12,000 x g for 15 minutes at 2-8°C. Following centrifugation, the mixture separated into lower red, phenol-chloroform phase, interphase and a colourless upper aqueous phase. RNA remained exclusively in the aqueous phase. The upper aqueous phase was transferred carefully without disturbing the interphase into a fresh tube.

3.4.5 RNA Precipitation

RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol was used per ml of TRIzol reagent sample for the initial homogenization. Samples were incubated at 15 to 30°C for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 2 to 4°C. The RNA precipitated which was invisible before centrifugation formed a gel-like pellet on the side and bottom of the tube.



Plate 1: Collection of sample (tongue epithelium, foot and dental pad)

3.4.6 RNA Wash

RNA pellet was washed twice with 75% ethanol. Samples were mixed by vortexing and centrifuged at 7500 x g for 5 minutes at 2 to 8°C. All the leftover ethanol was removed.

3.4.7 Redissolving RNA

RNA pellet was air-dried for 5-10 minutes and was not allowed to dry completely as this greatly decrease its solubility. RNA was dissolved in (DEPC)-treated water by passing solution a few times through a pipette tip.

3.4.8 Spectrophotometric analysis

One microliter of RNA was diluted with 39 µl of DEPC-treated water (1:40 dilution). Two microliters of it were used. The purity of extracted RNA samples was assessed by measuring the absorbance at 260/280nm in a spectrophotometer (Biophotometer plus, Eppendorf, Germany). The absorbance range and RNA concentration of samples obtained were between 1.8-2.0 and 5-20 ng/µL respectively.

3.5 First-strand complementary DNA (cDNA) synthesis

All the components of first strand cDNA synthesis kit (K1622, Thermo Scientific) were thawed on ice. The following reagents of kit were added in 0.2 ml sterile nuclease-free PCR tubes on ice as depicted in table 3.

Table 3: Thermal cyclers mixture

S. No	Components	Concentration (volume)
1.	Template (total RNA)	0.1ng-5µg (2-3 µL)
2.	Random Hexamer Primer	1µL
3.	Nuclease free water	8-9µL
	Total volume	12µL

The contents were mixed gently, centrifuged briefly and incubated at 65°C for 5 minutes. The samples were subsequently chilled on ice. The following components were then added sequentially as depicted in table 4.

Table 4: PCR reaction mixture

S. No	Components	Volume
1	5 x Reaction buffer	4 µL
2	Ribolock RNase inhibitor 20U/µL	1µL
3	10 m MdNTP Mix	2µL
4	Revert Aid M-MuLVRT (200U/µL)	1µL
Total volume		20µL

Contents were mixed gently and centrifuged briefly, and incubated for 5 minutes at 25°C followed by 60 minutes at 42°C. The reaction was terminated by heating at 70°C for 5 minutes. The reverse transcription reaction product was subsequently used in polymerase chain reaction (PCR).

3.6 Detection of Foot and Mouth Disease Virus (FMDV) by Polymerase Chain Reaction (PCR)

The PCR assays in this study were performed in 25µl reaction volume containing cDNA template, Go-green Mastermix (premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, MgCl₂ and reaction buffers), specific primers and Nuclease free water. Sterile distilled water served as negative control template. Amplification was carried out in a Master cycler nexus GX2 (Eppendorf, Germany). All the samples were subjected to PCR, targeting untranslated region (5-UTR) of FMDV; as described by (Madi *et al.*, 2015) depicted in table-5. PCR condition consisted of initial denaturation for 94°C for 5 min, denaturation for 94°C for 1 min, annealing for 55°C for 1 minute, extension for 72°C for 2 min and final extension for 72°C for 10 min. Number of cycles were 30.

Table 5: The primer set used in the detection

S.No	Primer Name	Primer sequence (5'-3')	Target gene region	Size	Reference
1	1F	GCCTGGTCTTTCCAGGTCT	5-UTR	328 bp	Madi <i>et al.</i> , 2015
2	1R	CCAGTCCCCTTCTCAGATC			

3.6.1 Electrophoresis and documentation

Agarose gel (1% w/v) was made by heating the appropriate amount of agarose (Sigma Aldrich, St. Louis, USA) with 50 ml 1X tris acetate EDTA (TAE) buffer in a 500 ml Erlenmeyer flask. The flask was cooled to 60 °C and ethidium bromide added to the final concentration of 0.5 µg/ml. The warm agarose solution was poured into a plastic holder with a suitable comb (with 0.5-mm or 1-mm wells) and allowed to completely set at room temperature for 30 min. The comb was removed and the gel was mounted on electrophoresis tank (Amersham Pharmacia Biotech, UK) and the tank filled with 1X TAE buffer. PCR samples were loaded in separate wells on the submerged gel. Standard molecular weight (100 bp) DNA Ladder (Promega) was also loaded in one well, also sterile distilled water served as negative control template. The voltage 1-5V/cm was applied across the gel until the yellow dye of master mix is migrated to an appropriate distance. The gel was removed and visualized under ultraviolet illumination and photographed with Gel Documentation System (Ultra-lum Inc. Imaging System, UVP, UK).

3.7 Serogrouping of Foot and Mouth Disease Virus (FMDV)

The cDNA of samples that were detected positive in detection were subjected to serogrouping.

3.7.1 Polymerase chain reaction (PCR)

The PCR assays in this study were performed in 25µl reaction volume containing cDNA template, Go-green Mastermix (premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, MgCl₂ and reaction buffers), specific primers (table 5) and Nuclease free water. Sterile distilled water served as a negative control template. Amplification was carried out in a Mastercycler nexus GX2 (Eppendorf, Germany).

Table 6: List of Primers used for Serogrouping

S. No	Primer Name	Sequence	Product size	Location on genome	Reference
1.	O (ARS4) _____ NK61	ACCAACCTCCTTGATGTGGCT / GACATGTCCTCCTGCATCTG	1301 bp	<u>1C</u> 2B	Knowles <i>et al.</i> , 2005
2.	A (A-1C562) _____ NK61	TACCAAATTACACACGGGAA / GACATGTCCTCCTGCATCTG	863-866 bp	<u>1C</u> 2B	Knowles <i>et al.</i> , 2005
3.	Asia-1 (AS1-1C505F) _____ NK61	TACTACTGCTTCTGACGTGGC / GACATGTCCTCCTGCATCTG	911 bp	<u>1C</u> 2B	Knowles & Samuel, 1995.

The primers used for serogrouping are depicted in table-6 and PCR conditions with O Primer set consisted of initial denaturation for 94°C for 5 min, denaturation for 94°C for 1 min, annealing for 60°C for 45 seconds, extension for 72°C for 2 min and final extension for 72°C for 10 min. The number of cycles were 30.

3.7.2 Agarose gel electrophoresis of PCR product

PCR products were analyzed by agarose gel electrophoresis using 1% agarose gel. An appropriate mass of agarose was weighed on digital balance and was put in a conical flask. Then electrophoresis buffer (1X TAE buffer) was added to the flask. The agarose/buffer mixture as mentioned above was mixed and was

allowed to boil in a microwave. The contents were swirled every 30 sec to mix well. Agarose was allowed to cool either on the benchtop to 65° C and Ethidium Bromide was added to a concentration of 0.5 µg/ml. Comb was inserted into the gel tray to create the wells. The molten agarose was poured into the gel tray and was allowed to set at room temperature. After the gel was set, the comb was removed and the gel was placed in the buffer tank.

3.7.3 Sample loading and Electrophoresis

The buffer tank was filled with the fresh TAE buffer till the gel was fully immersed in it. Samples were loaded into the wells. Also, an appropriate volume of 5 microliters of the 100bp DNA ladder are mixed with 1 microlitre loading dye was loaded in one well. Sterile distilled water served as negative control. The lid was put on to the buffer tank and the voltage 1-5V/cm was applied across the gel until the yellow dye of master mix is migrated to an appropriate distance.

3.7.4 Visualization

When electrophoresis was completed, power supply was turned off and lid of the buffer tank was removed. The gel was removed from the buffer tank and excess buffer was removed from the surface of the gel. The gel was removed from the tray and was exposed to UV light in UV trans-illuminator picture of the gel was taken in gel UV Gel Documentation system. At the end, the gel was properly disposed of.

3.8 Cloning of representative FMDV positive samples

3.8.1 Gel extraction

Representative FMDV-positive samples from 4 districts of Kashmir valley with unique banding pattern in gel were purified using MinElute gel extraction Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Briefly, a bulk PCR of representative FMDV positive samples were performed and gel excision was done with a clean, sharp scalpel. The excised gel fragment was weighed and 3 volumes of buffer QG was added to 1 volume of the gel. Incubated at 50°C for 10min in a thermomixer, Eppendorf for dissolving the gel completely. Then 1

volume of isopropanol was added. The sample was added to the QIAquick column to bind DNA and centrifuged for 1 min at $\geq 10,000\times g$. Flow-through was discarded and QIA quick column was put back in the same collection tube. Then 750 μ l of buffer PE was added to wash the DNA and centrifuged for 1 min. Supernatant was discarded and additional 1 min. centrifugation was done to remove the residual ethanol. QIAquick column was placed into 1.5ml microcentrifuge tube, eluted with 30 μ l buffer EB or water in the centre of QIAquick column membrane, allowed to stand for 1 min and centrifuged for 1 min at 13000 rpm. 2 μ l of gel extracted DNA was analyzed on a gel and kept at -20°C for further use.

3.8.2 Ligation

After gel extraction of representative FMDV positive samples, they were ligated to plasmid pGEM-T Easy vector (50ng) using DNA cloning kit (Promega Corporation, Madison, USA). Rapid ligation buffer (2x), pGEM-T Easy Vector (50ng), T4 DNA ligase (3 weiss units/ μ l) and distilled water were thawed and placed on ice. The ligation reaction mixture was prepared by adding 5 μ l of Rapid ligation buffer (2x), pGEM-T Easy Vector (50 ng/ μ l), 1 to 4 μ l of gel extracted product (35 to 65 ng) and 1 μ l of T4 DNA ligase (3 weiss units/ μ l) in a 0.2ml PCR tube. The volume was made upto 10 μ l with distilled water. The ligation-reaction mixture was mixed by pipetting and incubated overnight at 4°C to get the maximum number of transformants.

3.8.3 Preparation of competent DH5 α cells

Escherichia coli strain DH5 α was used for the preparation of competent cells. One ml of overnight culture of *Escherichia coli* DH5 α was inoculated into 50 ml of Luria Bertani (LB) broth (Difco) in one-liter conical flask and incubated at 37°C in a shaker (N-BIOTEK) until the OD A600 reached 0.5-0.6. This took about 2-3 hours. Culture flask was chilled on the ice for 30 minutes. The culture was transferred aseptically into pre-chilled sterile centrifuge tubes and spun down at 6000 rpm for 8 minutes in a refrigerated centrifuge (Eppendorf, centrifuge 5804R, Hamburg Germany) at 4°C. The supernatant was discarded and the cell pellet was

re-suspended in 15 ml of ice cold 0.1M calcium chloride solution and incubated on ice for 30 minutes. The culture suspension was again centrifuged at 6000 rpm for 8 minutes at 4°C and supernatant was discarded. To pellet 600 µl of chilled 0.1M Calcium chloride was added to it and resuspended. The cells were kept cold on ice during the entire procedure.

In prechilled 1.5ml microcentrifuge tubes, 100 µl of competent cells were aliquoted aseptically taking care not to remove the tubes from ice. Competent cells were now ready and were used immediately for the transformation.

3.8.4 Transformation by Heat shock method

To an aliquot of 100 µl of competent cells in each 1.5 ml centrifuge tubes, 3µl of the ligation mixture was added, mixed and incubated on ice for 30 minutes. Water bath was set at a temperature of 42°C and heat shock was given to cells at 42°C for 50 seconds and incubated back on ice for 5 minutes. 800 µl of LB broth was added to the tube and incubated at 37°C in shaking incubator for 45 minutes. This was to allow bacteria to recover and express the antibiotic resistance. 200 µl of this culture was pipetted out to properly labeled LB agar plates containing Ampicillin (100 µg/ml, X-gal (80 µg/ml) and isopropylthio-β-D-galactoside (IPTG, 20mM) and spreaded thoroughly using L- shaped spreader. Plates were incubated overnight at 37°C and White screening was done the very next morning to confirm the transformed and non-transformed cells. The white colonies from the plates were screened for the presence of insert through colony touch PCR and simultaneously streaked on the LB-agar plate containing ampicillin.

3.8.5 Screening of recombinant colonies

The white colonies from the plates were screened for the presence of insert through colony touch PCR.

3.9 Sequencing

3.9.1 Plasmid extraction

The plasmids from the clones with desired insert were extracted using QIAprep Miniprep Kit (Qiagen, Hilden, Germany). Single colonies were picked up

from freshly streaked LB-Ampicillin plates and inoculated into 15ml centrifuge tubes containing 5 ml LB broth containing ampicillin (100µg/ml). The tubes were incubated overnight at 37°C with vigorous shaking in shaking incubator. Bacterial cells were harvested by centrifugation at 10,000 rpm in a table-top microcentrifuge (Centrifuge 5418 R, Eppendorf, Germany) for 3 min at room temperature (15-25°C). Pelleted cells were resuspended in 250 µl Buffer P1 and transferred to a microcentrifuge tubes. 250 µl Buffer P2 was added and mixed thoroughly by inverting the tubes 4-6 times. Finally, 350 µl Buffer N3 was added and mixed immediately and thoroughly by inverting the tubes 4-6 times. The tubes were centrifuged for 10 min at 13,000 rpm (~17,900×g) in table-top microcentrifuge and the supernatant applied to the QIAprep spin columns by pipetting. The columns were centrifuged for 30–60 s and the flow-through was discarded. To the QIAprep spin column was added 0.5 ml Buffer PB, for the plasmid DNA to bind the silica membrane and centrifuging for 30–60 s. The flow-through was discarded and the spin column was washed by adding 0.75 ml Buffer PE and centrifuged for 30–60 s. The flow-through was discarded, and the column centrifuged for an additional 1 min to remove residual wash buffer. To elute DNA, QIAprep column was placed in a clean 1.5 ml microcentrifuge tube and 50 µl Buffer EB was added to the centre of each QIAprep spin column, allowed to stand for 1 min, and centrifuge for 1 min.

3.9.2 Restriction digestion of recombinant plasmid

The isolated plasmids were digested with restriction enzymes EcoRI for the confirmation of insert (Plate 2). Each of the digestion reaction consisted of ≈5µg of plasmid (2µl), 1µl of fast-digest green buffer, 10 units of EcoRI enzyme and remaining nuclease-free water to make up a total volume of 10 µl. the reaction mixtures were incubated in water bath at 37°C for 20 minutes and then 2µl of this plasmid was run in 1% agarose gel and visualized under UV gel documentation system for the presence of insert. The concentration of the extracted plasmid was checked by absorption at λ260 (BioPhotometer Plus, Eppendorf, Germany) and commercially outsourced for sequencing.

3.9.3 Sequencing

The extracted plasmids were screened for correct size insert as well as quality of the plasmid, both by running 2 µl of extracted plasmid DNA on the agarose gel (1%) as well as by restriction digestion with EcoRI (Fermentas Life Sciences) to release the insert and then analyzed by agarose gel electrophoresis. The concentration of the extracted plasmid was checked by absorption at λ_{260} (BioPhotometer Plus, Eppendorf, Germany) and then sent in 1.5 ml microcentrifuge tube on an ice pack for sequencing. The plasmids were got sequenced commercially from Bionivid Technology Pvt. Ltd Kasturi Nagar, Bengaluru.

3.10 Phylogenetic analysis of FMDV Serotype “O” Gene fragment

A comparison was made among nucleotide sequences of serotype “O” gene fragments FMDV with the reported serotype “O” sequences Genbank using the Clustal-W (CLUSTAL 2.1 multiple sequence alignment) to generate sequence alignment reports. The evolutionary history was inferred by using the Maximum Likelihood method based on (Jones *et al.*, 1992). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein *et al.*, 1985). A phylogenetic tree was constructed using Molecular Evolutionary Genetic Analysis-7 (MEGA-7) software (Kumar *et al.*, 2016) to ascertain the genetic relationship among FMDV serotypes involved in an outbreak that occurred in Kashmir Valley.

3.11 Statistical Analysis:

The qualitative data were presented in terms of percentage and the quantitative data were analyzed by One-way ANOVA. Post-hoc analysis was performed by Duncan’s multiple range test (DMRT) using SPSS-20. The statistical differences were determined at the 5% level of significance (Snedecor and Cochran, 1994).

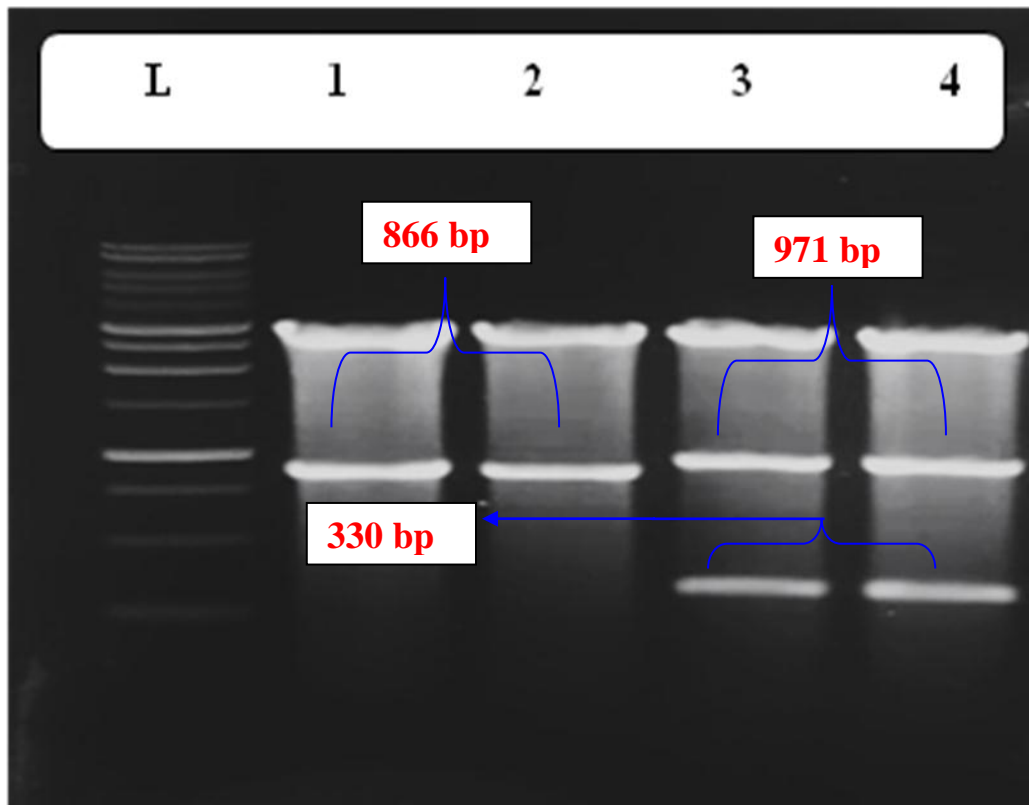


Plate 2: Restriction digestion of recombinant plasmid of Serogroup O and A

L= 100bp ladder

Lane1= Sample 5A (Restriction digested (cut) plasmid

Lane2= Sample 11A Restriction digested (cut) plasmid

Lane3= Sample 5 O Restriction digested (cut) plasmid

Lane4= Sample 11O Restriction digested (cut) plasmid



EXPERIMENTAL FINDINGS

CHAPTER – 4

EXPERIMENTAL FINDINGS

4.1 Epidemiological study

A study on the epidemiology of Foot and Mouth Disease (FMD) in affected ruminants (cattle and sheep) was carried out during the FMD outbreak period (September 2021 to August 2022). A total of 96 ruminants (cattle-89 and sheep-7) with characteristic FMD clinical signs *viz*; fever, altered vital parameters, pathognomic lesions in the mouth, muzzle, hoof and udder/teats were selected for the study from Baramulla, Bandipora, Ganderbal and Srinagar districts of Kashmir valley.

4.2 Age-wise FMD-affected ruminants

4.2.1 Age-wise FMD-affected Cattle:

The percentage of FMD-infected cattle in different age groups was recorded (Table 7, Fig 1). The percentage of FMD-infected cattle in age groups *viz.*, <1-2years, 2-4 years and > 4 years was 14.6, 16.85 and 68.53 percent, respectively. FMD infection rate in older age group cattle (>4 years) was found to be higher compared to the other two age groups (young and adults).

Table 7: Age-wise FMD-affected Cattle

Age category (years)	No. of animal (n)	Percentage (%)
<1-2 (Young)	13	14.61
2-4 (Adults)	15	16.85
>4 (Older)	61	68.54
Total	89	100.00

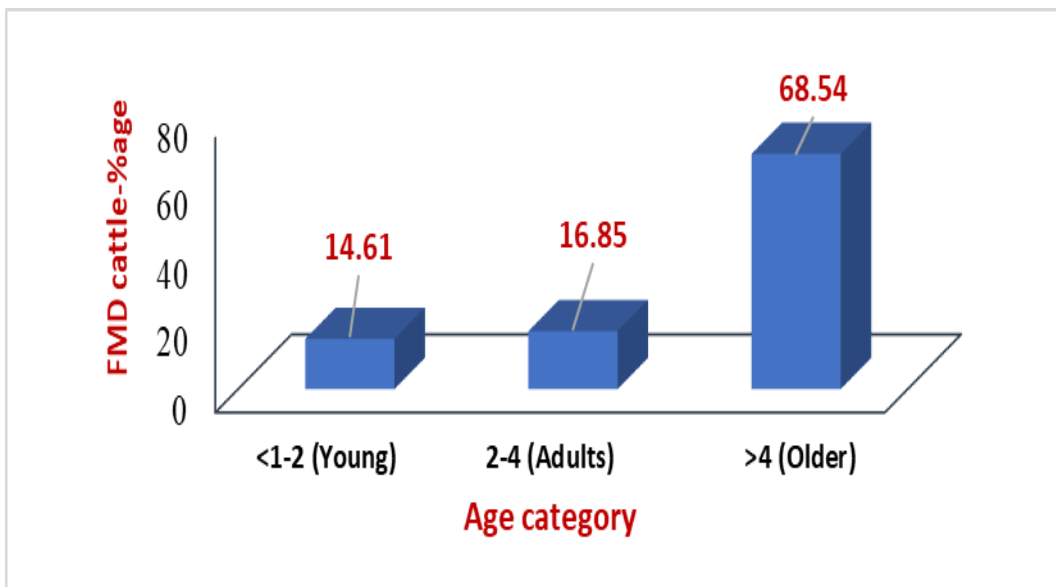


Fig. 1: Age wise FMD-affected Cattle

4.2.2 Age wise FMD-affected Sheep:

The percentage of FMD infected sheep was 57.14 percent and 42.86 percent in the age group of <1-2 years and 2-4 years, respectively (Table 8, Fig 2). The frequency of FMD-infected sheep was found higher in the young age group (<1-2 years) as compared to the adult age group (2-4 years).

Table 8: Age wise FMD-affected Sheep:

Age category (years)	No. of animals (n)	Percentage (%)
<1-2 (Young)	4	57.14
2-4 (Adults)	3	42.86
Total	7	100.00

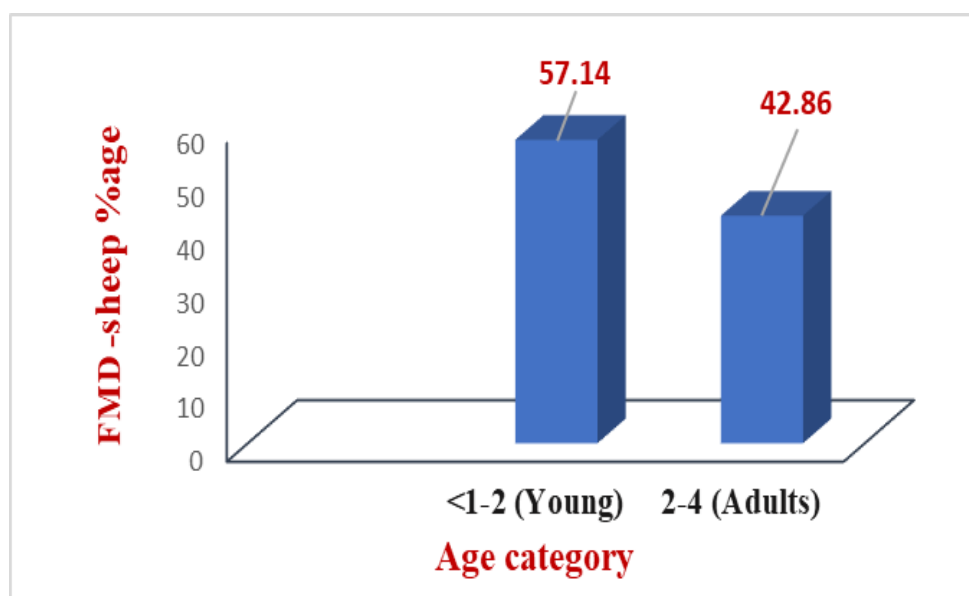


Fig. 2: Age wise FMD-affected Sheep

4.3 Gender-wise FMD affected cattle

Out of the total number of FMD affected animals the percentage of males and females infected with FMD in cattle was 31.46 and 68.54 percent, respectively. Proportionally the percentages of FMD-affected females were more than FMD-affected males (Table 9, Fig. 3).

Table 9: Gender wise FMD affected cattle

Gender	No. of animals (n)	Percentage (%)
Males	28	31.46
Females	61	68.54
Total	89	100.00

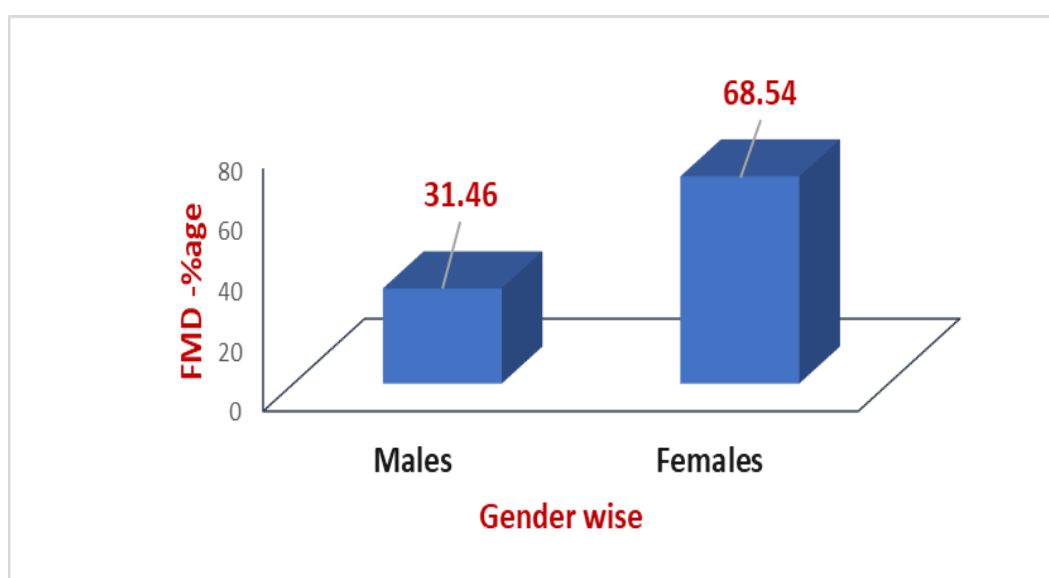


Fig. 3: Gender wise FMD affected cattle

4.4 Vaccination status

4.4.1 Vaccination status of FMD affected cattle

Out of the eighty-nine cattle affected with FMD, a small percentage (3.37%) of cattle was vaccinated whereas, the majority of cattle (96.63%) were not vaccinated for foot and mouth disease in the ongoing year (Table 10, Fig. 4).

Table 10: Vaccination status of FMD affected cattle

Status	Unvaccinated	Vaccinated
No. of animals (n)	86	3
Percentage (%)	96.63	3.37

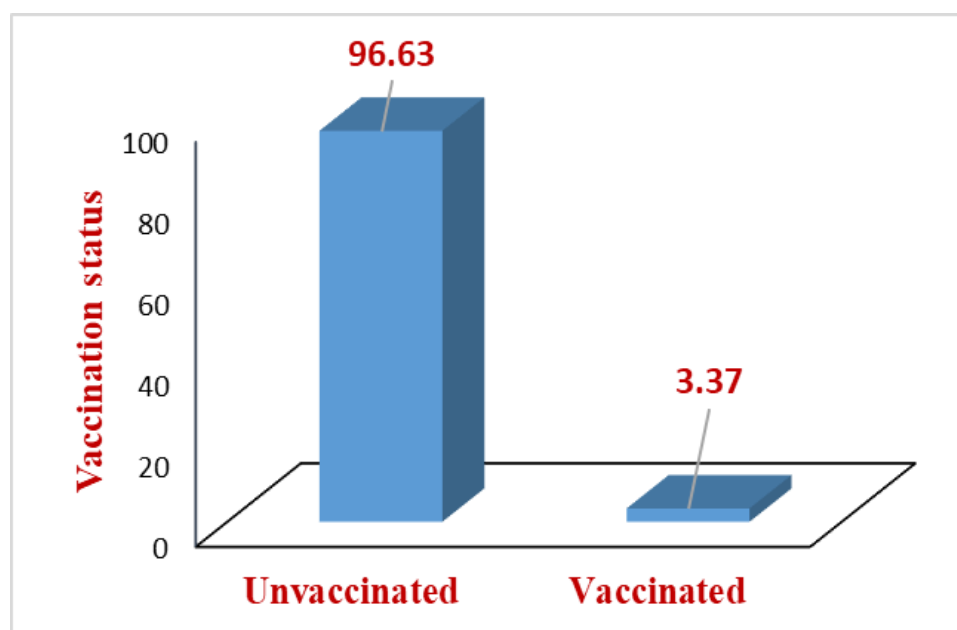


Fig. 4: Vaccination status of FMD- affected cattle

4.4.2 Age-wise vaccination status of the affected cattle

The 3.37 percent of vaccinated animals belonged to the young age group (<1-2 years) and 96.62 percent of unvaccinated animals were all adult (2-4 years) and older (>4 years) age group animals, respectively (Table 11).

Table 11: Age-wise vaccination status of the affected cattle

Age category (Years)	No. of animals (n)	No. of animals Vaccinated (n)	Percentage (%)
<1-2 (Young)	13	3	3.37
2-4 (Adults)	15	0	-
>4 (Older)	61	0	-
Total	89		3.37

4.4.3 Vaccination status of the affected sheep

Out of the total of seven sheep affected with FMD, none of the animals was vaccinated in the ongoing year.

4.5 Economic losses associated with Foot and Mouth Disease

The economic losses due to FMD were evaluated in terms of reduction in milk yield, treatment cost and mortality losses. In dairy cattle, milk production (L) was gauged both before and after FMD infection. In affected animals, the mean pre-FMD milk yield (10.57 ± 0.290 L) was found significantly ($P \leq 0.05$) higher than the post-FMD milk yield (2.01 ± 0.21 L). Similar to this, mean pre-FMD milk yields in four representative districts, Baramulla, Bandipora, Ganderbal and Srinagar were (10.57 ± 0.32), (10.50 ± 1.50), (11.00 ± 0.930) and (9.33 ± 1.20) litres, respectively. It was Significantly ($P \leq 0.05$) higher than the post-FMD milk

yield (2.01 ± 0.24), (1.00 ± 0.00), (2.50 ± 0.65) and (1.00 ± 0.50) in liters, respectively (Table 12, Fig. 5).

Table 12: Pre-FMD and Post-FMD milk losses in different districts (Mean \pm S.E.)

Districts	Pre-FMD milk yield (L)	Post-FMD milk yield (L)
Baramulla	10.57 ± 0.32^a	2.01 ± 0.24^b
Bandipora	10.50 ± 1.50^a	1.00 ± 0.00^b
Ganderbal	11.00 ± 0.930^a	2.50 ± 0.65^b
Srinagar	9.33 ± 1.20^a	1.00 ± 0.50^b
Total	10.57 ± 0.290^a	2.01 ± 0.21^b

The different superscripts (row-wise) indicate a significant difference ($P \leq 0.05$)

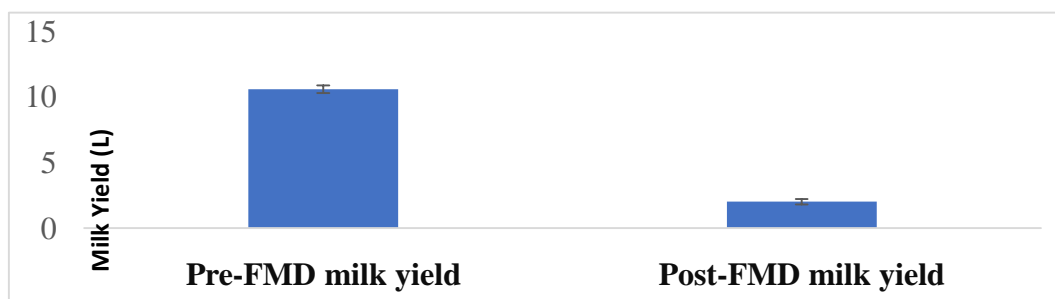


Fig. 5: Pre-FMD and Post-FMD milk losses

4.5.1 Average short-term milk loss (SY) per animal

The average short-term losses resulting from milk yield reduction in FMD-affected cattle were calculated according to the formula $SY = 1/n (E-A) \times D \times P$ and details are given in table 13. The average short-term milk loss per FMD-infected animal was found about 6899.52 Indian rupees.

Table 13: Average short-term milk loss (SY) per animal

Parameters	Notations	Average Estimates
Expected milk yield (litres/day)	E	6
Actual milk yield (litres/day)	A	0.5
Duration of infection (days)	D	30
Price of milk in litres	P	40

4.5.2 Average treatment costs per animal

The average treatment cost was calculated as per the formula $LT = 1/n \sum (F+M+1)$ and details are given in table 14. The average treatment cost was estimated at around Rs.1235.83 per FMD- infected animal.

Table 14: Average treatment costs per animal

Parameters	Notations	Average Estimates
Fees for veterinarians	F	200
Cost of medicines	M	447.5
Cost of drugs indigenous treatment	I	100

4.5.3 Average loss due to mortality per animal

Average mortality losses due to FMD was calculated as per the formula $(LM = \sum A_j \times V_j)$.

This was formulated as the number of animals that died due to the disease multiplied by the average price of the animal. In this study, 7 animals (calves below two months of age) died due to FMD. The average mortality cost was estimated at about Rs. 2014.29 per infected animal (Table 15).

Table 15: Average loss due to mortality per animal (LM)

Parameters	Notations	Average Estimates
Number of animals	A _j	7
Average value/cost of animals	V _j	2000

The total losses per infected animal were calculated as the sum total of average short-term milk loss (SY = 6899.52), average treatment costs (LT = 1235.83) and average loss due to mortality (LM = 2014.29).

Total loss per FMD affected animal = Rs.6899.52 + Rs.1235.83 + Rs.2014.29 = Rs.10,149.64.

Therefore, the total losses per FMD-infected cattle were estimated at around Rs.10,149.64.

4.6 Clinical evaluation (Vital Parameters)

The mean rectal temperature (°F) recorded in FMD affected and the healthy control group of cattle are represented in table 16. In FMD affected group, the mean rectal temperature (°F) recorded on the 2nd and 3rd day (of clinical disease) was 102.26 ± 0.18 in males and 102.52 ± 0.16 in females. There was a significant increase (p ≤ 0.05) in overall mean temperature in infected cattle (102.44 ± 0.13 °F) than in the healthy control group of animals (100.97 ± 0.05 °F).

Table 16: Rectal Temperature (°F) in FMD-affected cattle (Mean ± SE)

Gender	Healthy control (n=89)	FMD infected cattle (n=89)
Males (n=28)	100.38 ± 0.06 ^a	102.26 ± 0.18 ^b
Females (n=61)	101.24 ± 0.02 ^a	102.52 ± 0.16 ^b
Total	100.97 ± 0.05 ^a	102.44 ± 0.13 ^b

Values with dissimilar superscripts differ significantly ($P \leq 0.05$)

The mean respiration (rate/min) recorded in FMD-infected cattle was 40.43 ± 2.18/min in males and 43.00 ± 2.46/min in females. There was a significantly ($p \leq 0.05$) higher rate of respiration (42.19 ± 1.82)/min in affected cattle as compared to the healthy control cattle (21.99 ± 0.15)/min (Table 17).

Table 17: Respiration (rate/min) in FMD-affected cattle (Mean ± SE)

Gender	Healthy Control (n=89)	FMD infected cattle (n=89)
Male (n=28)	21.36 ± 0.26 ^a	40.43 ± 2.18 ^b
Female (n=61)	22.28 ± 0.18 ^a	43.00 ± 2.46 ^b
Total	21.99 ± 0.15 ^a	42.19 ± 1.82 ^b

Values with dissimilar superscripts differ significantly ($P \leq 0.05$)

The mean heart rate (bpm) in infected cattle was 75.04 ± 2.01 in males and 79.00 ± 2.02 in females (table 18). There was a significant ($p \leq 0.05$) increase in

heart rate (bpm) recorded in affected cattle (77.75 ± 1.53) compared to the healthy control group (61.42 ± 0.14).

Table 18: Heart Rate (bpm) in FMD-affected cattle (Mean \pm SE)

Gender	Healthy Control (n=89)	FMD infected cattle (n=89)
Male (n=28)	61.21 ± 0.23^a	75.04 ± 2.01^b
Female (n=61)	61.51 ± 0.18^a	79.00 ± 2.02^b
Total	61.42 ± 0.14^a	77.75 ± 1.53^b

Values with dissimilar superscripts differ significantly ($P \leq 0.05$)

4.7 Clinical Lesions in FMD-affected Animals

4.7.1 Clinical lesions in FMD-affected cattle

All affected cattle and sheep (100%) were found salivating consistently and few animals (1.12%) had a blood-stained mucopurulent nasal discharge (Plate 3). Eroded and ulcerous lesions were found in the mouth, dental pad, tongue and foot/h hoof in the affected cattle (Plate 4, 5& 6). On a percentage basis, the mouth, foot/h hoof and udder/teats lesions were recorded in 98.87, 85.39, and 6.74 percent of animals, respectively (Table 19, Fig.6). Other clinical signs included anorexia, a decrease in ruminal movement and drop in milk yield. Mortalities included seven calves that had succumbed within 1-2 days of illness due to FMD.

Table 19: Clinical Lesions presented in FMD-affected cattle

Total No. of animals	Lesions				
	Mouth lesions	Foot/h hoof lesions	Udder/teat lesions	Salivation	Nose bleeding
89	88	76	6	89	1
Percentage	98.88	85.39	6.74	100.00	1.12

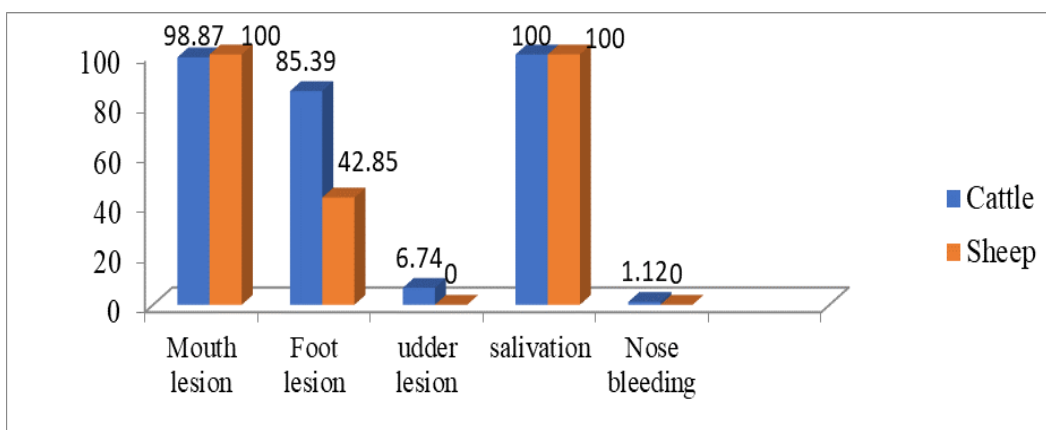


Fig. 6: Clinical lesions in FMD-affected cattle

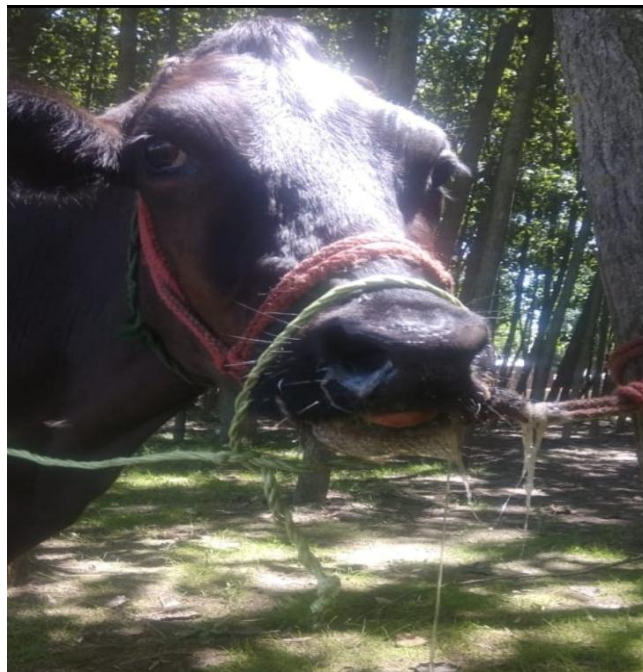


Plate 3: Excessive Salivation and nasal discharge in FMD affected animal



Plate 4: Eroded lesions present on dorsum of tongue



Plate 5: Linear erosions present on dental pad with fibrin



Plate 6: Necrosis of inter-digital skin with flap of skin in cattle

4.7.2 Clinical Lesions in FMD affected Sheep

In sheep, the lesions were seen in the mouth and hoof areas. The existence of these lesions in affected animals was 100 and 43 percent, respectively (Table 20, Plate 7). There was salivation in all the affected sheep (100%).

Table 20: Clinical Lesions in FMD affected Sheep

Total no. Animals	Lesions		
	Mouth lesions	Foot/h hoof lesions	Salivation
7	7	3	7
Percentage	100.00	42.85	100.00

4.7.3 Clinical Lesions in FMD affected Cattle with respect to different age groups

The lesions were recorded in different age groups in FMD-affected cattle (Table 21, Fig 7). Percentage existence of mouth lesions recorded in different age groups viz., <1-2 years, 2-4 years and >4 years were 13.48, 6.85 and 68.54 percent, respectively indicating the maximum frequency of mouth lesions in older age group (>4 years) (Plate 8, 9). Percentage existence of foot/h hoof lesions recorded in different age groups viz., <1 year, 2-4 years and >4 years were 12.36, 14.61 and 60.67 percent, respectively with higher percentage occurrence in older age group cattle (>4 years) (Plate 10). A total of 3.37 percent of cattle in age group 2-4 and >4 years manifested the udder lesions.

Table 21: Clinical Lesions in FMD affected Cattle with respect to different age groups:

Age category	(<1-2 years) Young	(2-4 years) Adult	(>4years) Older	Total
Mouth lesions	12 (13.48)	15 (16.85)	61 (68.54)	88
Foot lesions	11 (12.36)	13 (14.61)	54 (60.67)	78
Udder lesions	0	3 (3.37)	3 (3.37)	6
Salivation	13 (14.61)	15 (16.85)	61 (68.54)	89

(Figures in parenthesis refers to percentage)

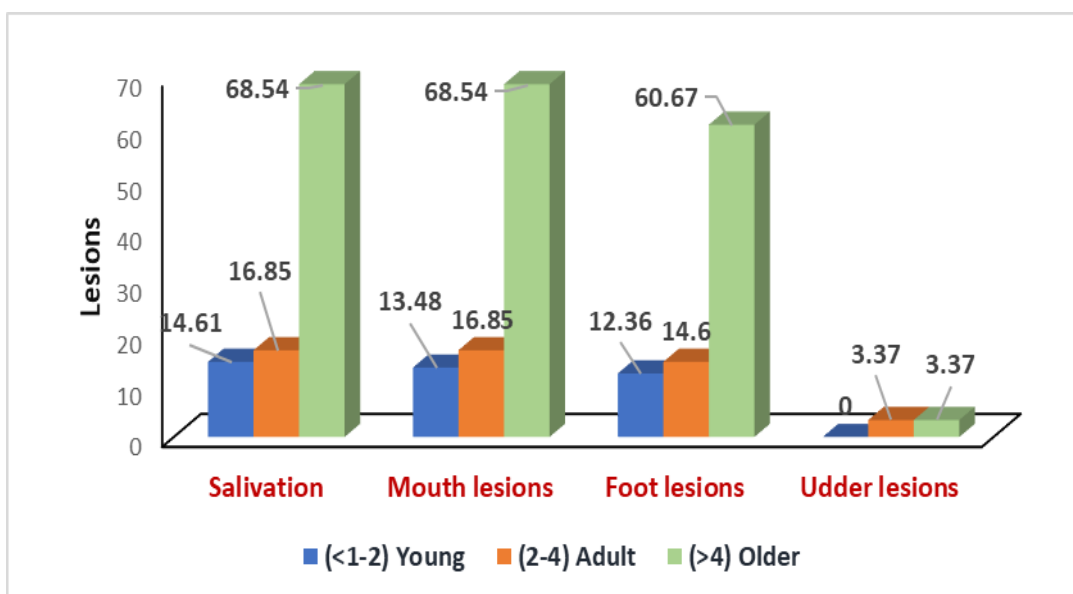


Fig. 7: Age wise clinical lesions in FMD affected Cattle



Plate 7: Eroded and ulcer lesions present on dental pad in sheep



Plate 8: Eroded and ulcer lesions present on dental pad in cattle



Plate 9: Eroded and ulcer lesions present on hard palate in cattle



Plate 10: Ruptured inter-digital vesicles with flap of necrotic skin in Cattle

4.7.4 Clinical Lesions in FMD affected Sheep with respect to different age groups

The lesions in different age groups in FMD-affected sheep were recorded (Table 22). The percentage of mouth lesions in age groups <1-2 years and >2-4 years were 57.14 and 42.86 percent, respectively with a higher occurrence of mouth lesions in young animals. The percentage presence of foot/hoof lesions in age groups <1-2 years and >2-4 years were 14.29 and 28.57 percent, respectively. The occurrence of foot lesions was found to be higher in the <2-4 years age group as compared to another age group.

Table 22: Clinical Lesions in FMD affected Sheep with respect to different age groups

Age Category	<1-2 Years (Young)	2-4 Years (Adults)	Total
Mouth lesions	4 (57.14)	3 (42.86)	7
Foot/hoof lesions	1 (14.29)	2 (28.57)	3
Salivation	4 (57.14)	3 (42.86)	7

(Figures in parenthesis refers to percentage)

4.8 Therapeutic study in clinically FMD-affected cattle

The observations with respect to treatment regimens of clinical cases were made in different groups of animals as shown in table 23.

The rate of recovery in days (16.17 ± 1.14) was evident with the normalization of clinical signs in animals given the prescribed treatment regimen (antibiotic-gentamicin, antipyretic, antiallergic) and an immunomodulator (tissue aid). The recovery was visibly evident from the 7th day onwards in four cases and from the eighth day onward in two cases. However, all the affected cattle had recovered completely by 14-20 days.

The rate of recovery in days (12.5 ± 0.76) was evident with the normalizing of clinical symptoms in the animals given the prescribed treatment regimen (antibiotic-enrofloxacin, antipyretic, antiallergic) and an immunomodulator (Restobal). After 12-18 days all the cattle recovered fully.

The rate of recovery in days (21.00 ± 1.06) was visible in animals that were given the treatment regimen (antibiotic-enrofloxacin, antipyretic, antiallergic) without an immunomodulator from the 15th day onwards with the normalizing of clinical signs. Although the rate of recovery in terms of days of resolution of clinical signs was delayed, however, by day 18th -25th all the animals recovered.

Therapeutic study trials on clinical cases revealed significantly ($P \leq 0.05$) better results considering the number of days taken for the resolution of clinical signs in animals treated with antibiotics and immunomodulators compared to those treated with therapeutic regimens without immunomodulators.

Table 23: Therapeutic study in clinically FMD-affected cattle

Group	Treatment	Number of calves		Average No. of days taken for recovery Mean \pm SE
		Treated	Cured	
Group I (N=6)	Enrofloxacin @ 2.5mg/Kg b.wt. x I/M for 5 days Meloxicam & Paracetamol @ 10ml x I/M for 5 days Pheniramine maleate @10 mL x I/M for 5 days Boro-glycerine Paste Potassium Permanganate (1% mouthwash)	6	6	21.00 \pm 1.06 ^c
Group II (N=6)	Gentamicin @ 2.5 mg/Kg b.wt. I/M for 5 days Meloxicam & Paracetamol @ 10ml x I/M for 5 days Pheniramine maleate @10 mL x I/M for 5 days	6	6	16.17 \pm 1.14 ^b

	Boro-glycerine Paste Potassium Permanganate (1% mouth wash) Immunomodulator (Tissue aid @ 2 boli/day)			
Group III (N=6)	Enrofloxacin @ 2.5mg/Kg b.wt. I/M for 5 days Meloxicam & Paracetamol @ 10ml x I/M for 5 days Pheniramine maleate @10 mL x I/M for 5 days Boro-glycerine Paste, Potassium Permanganate (1% mouthwash) Immunomodulator (Restobal @ 50 mL/animal)	6	6	12.5±0.76 ^a

4.9 Post FMD complications

Affected cattle were observed to have post-FMD complications like heat intolerance/panting, hypertrichosis and mastitis. The three major post-FMD problems recorded were heat intolerance/panting, hypertrichosis and mastitis, occurring in 21.34, 7.80, and 6.74 percent of affected cattle, respectively (Table 24; Fig. 8).

Table 24: Post FMD complications

S. No.	Heat intolerance/ Panting	Hypertrichosis	Mastitis
No. of Animals	19	7	6
Percentage	21.35	7.87	6.74

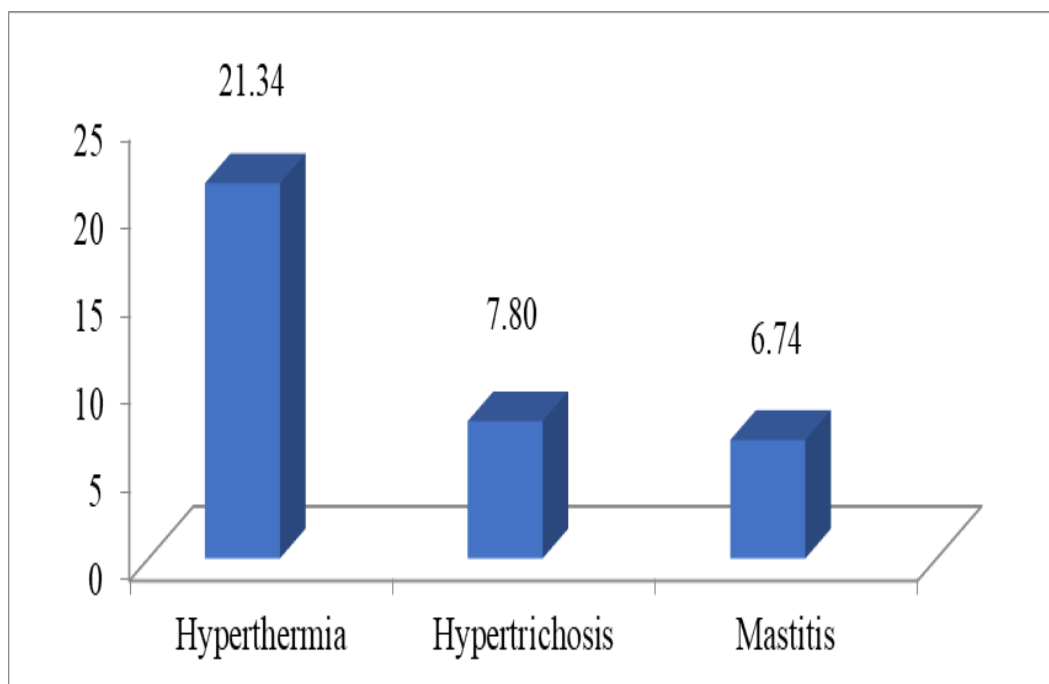


Fig. 8: Post-FMD complications

4.10 Molecular Detection by Polymerase Chain Reaction

4.10.1 Molecular detection of FMD virus by PCR in cattle

Sixty-five (73.03%) samples were found positive for foot and mouth disease virus (FMDV) out of 89 samples by polymerase chain reaction targeting 5-untranslated region (5-UTR) gene fragment (Table 25, Fig 9, Plate 11). PCR amplification of 1301 bp and 863-866 bp amplicons identified serotypes O and A of the FMD virus. However, after sequencing the representative samples, serotype O was identified and confirmed from 65 (73.0%) FMD-positive samples (Plate 12).

Table 25: Detection of FMDV by PCR in cattle

No. of animals	FMD positive	Percent Positive
89	65	73.03

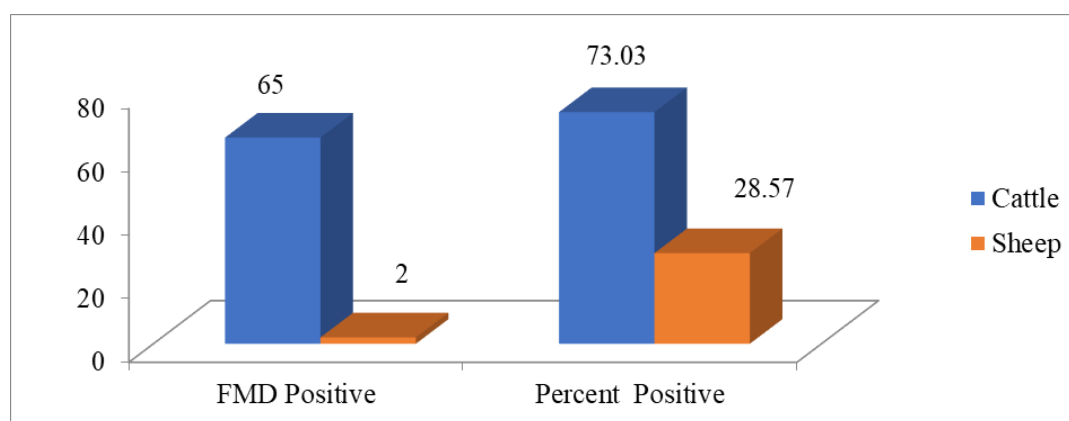


Fig. 9: FMD positive Cattle and Sheep on PCR detection

4.10.2 Detection of FMDV by PCR in Sheep

Out of seven samples, two (28.57%) samples from sheep were positive for FMDV by polymerase chain reaction targeting 5-untranslated region (5-UTR) gene fragment. Serotype O was identified in two positive (28.57 %) samples (Table 26).

Table 26: Molecular detection of FMD positive Sheep

No. of animals	PCR positive	Percent Positive
7	2	28.57

4.11 Sequencing of representative positive samples

Sequencing results confirmed serotype O in four representative FMDV-positive samples. Serotype O identified in the present study confirmed close genetic proximity with FMDV serotype O - PanAsia strain of Pakistan. Four

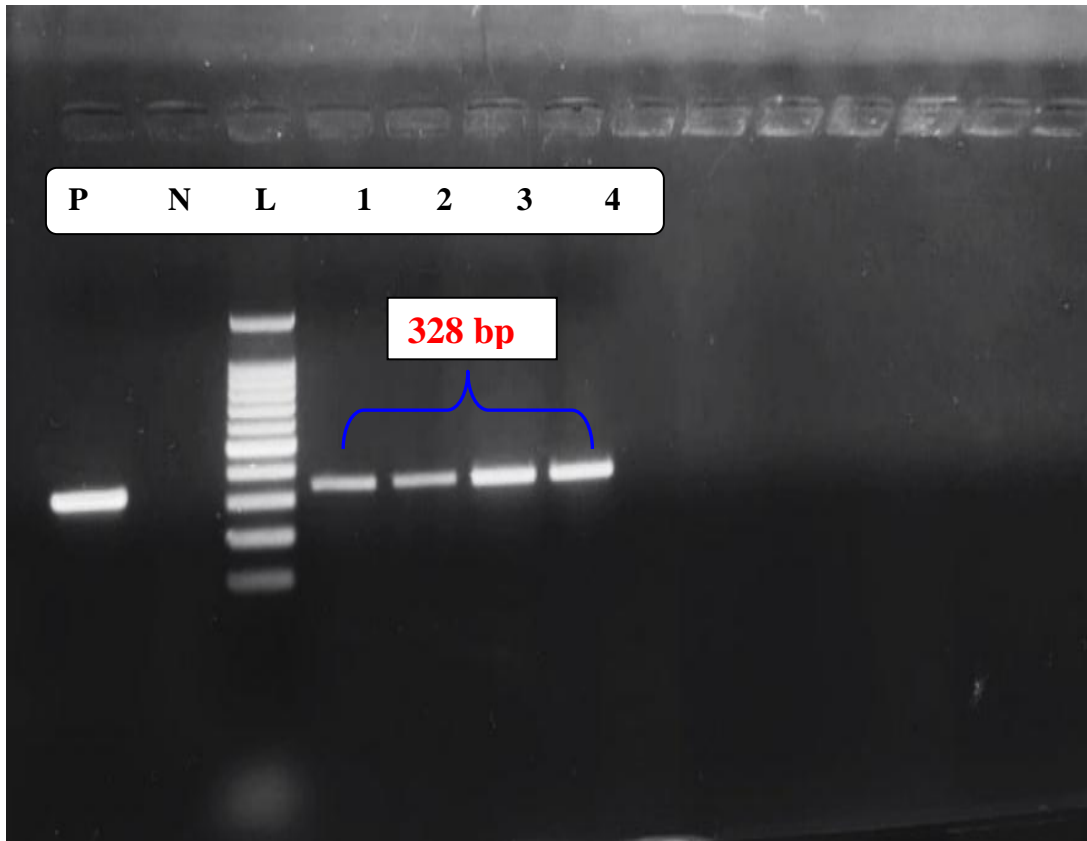


Plate 11: Detection of FMDV by Polymerase Chain Reaction

Lane P = Positive control

Lane N = Negative control

L = Ladder 100bp

1-4 = Samples

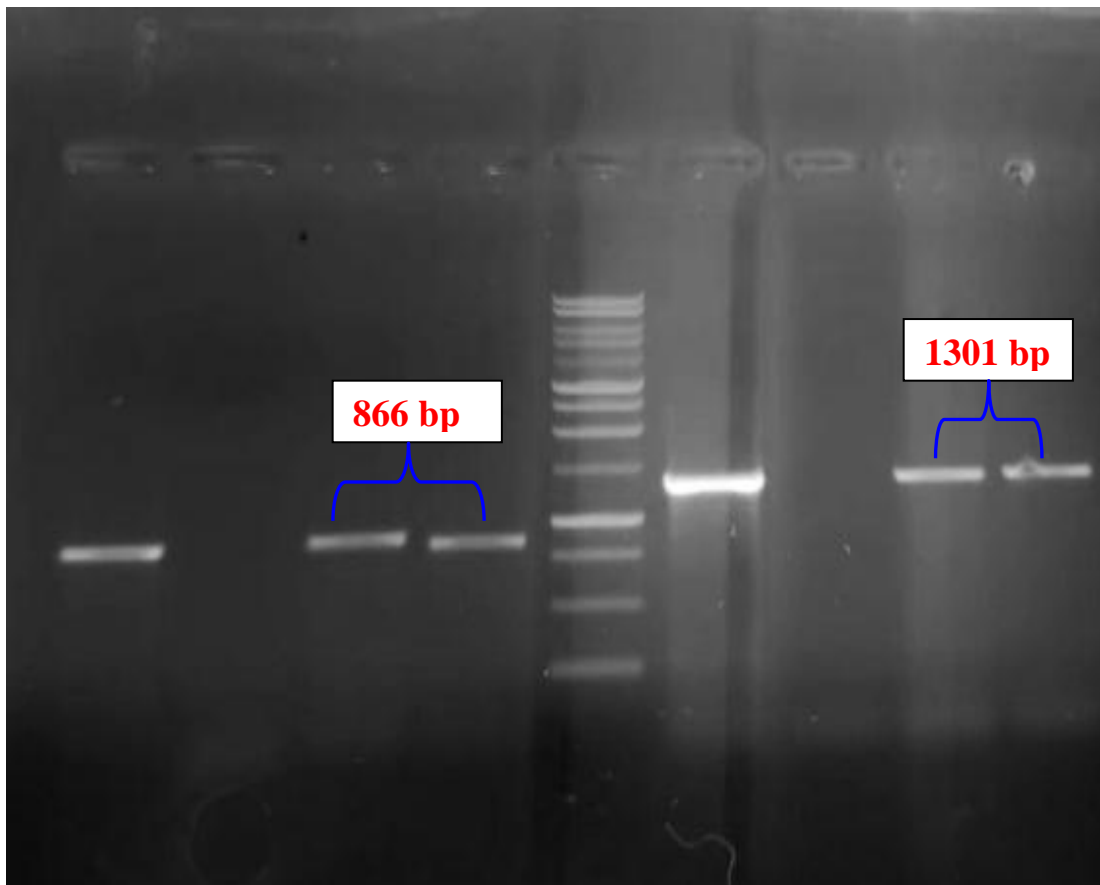


Plate 12: Serotyping of FMDV by Polymerase Chain Reaction

Lane P1= Positive control “A”

Lane P2 = Positive control “O”

L = Ladder 100bp

Lane N1 and N2 = Negative controls

Lane 1-2 and 3-4 = Samples

representative positive samples were cloned and these four representative sequences of FMDV serotype O of the present study were blasted to determine their identity. It was observed that three sequences (Acc. No.s OP189368; OP189370 and OP189371) showed sequence identity in the range of 91-93% with FMDV serotype O of Pakistan, while one of the sequences with accession number OP189369 showed 91.63% sequence identity with FMDV serotype O of Bhutan. All four sequences of this study were genetically distant from Indian FMDV serotype O strains as depicted in the phylogenetic tree. The multiple sequence alignments of these four representative amplicons are shown in (Plate 13). All four sequences were submitted to GenBank with accession numbers depicted in table 27.

Table 27: GenBank accession number of representative samples

S. No.	Sample No	GenBank accession number
1	PL “50”	OP189368
2	PL “110”	OP189369
3	PL “11A”	OP189370
4	PL “5A”	OP189371

4.11.1 Molecular Phylogenetic analysis by Maximum Likelihood method based on VP1 gene of FMDV

The evolutionary history was inferred by using the Maximum Likelihood method w/freq model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 75% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1292). The analysis involved 13 amino acid sequences. There was a total of 211 positions in the final dataset.

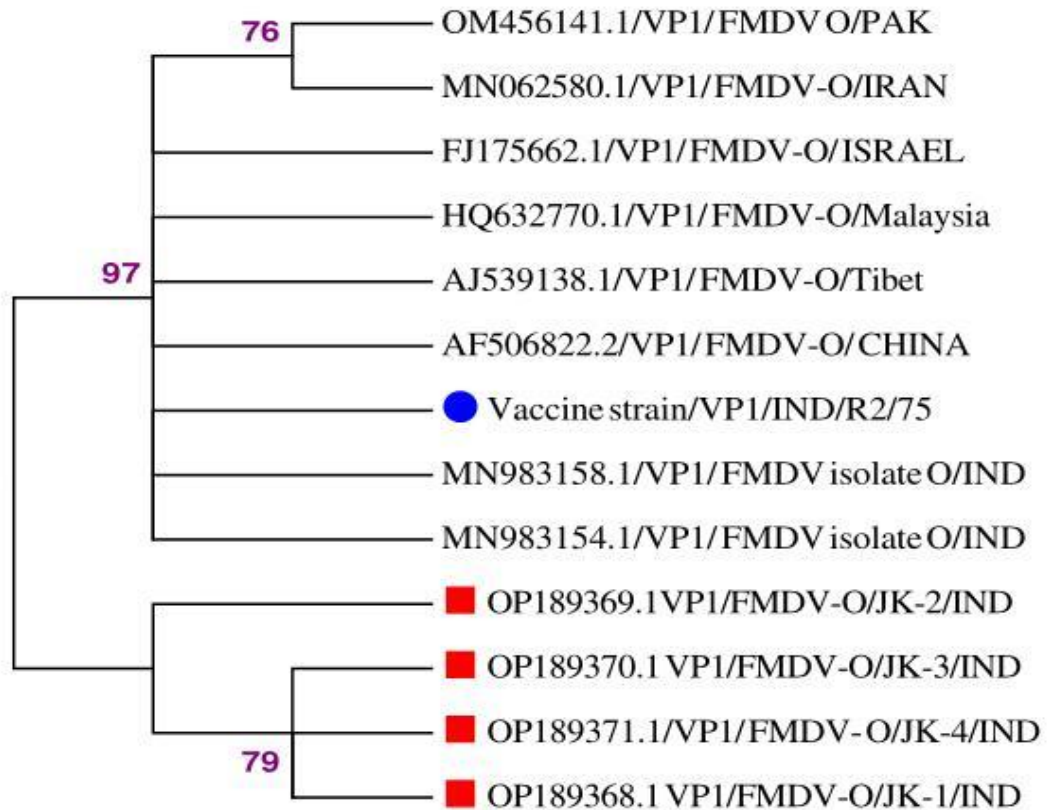


Plate 13: Molecular Phylogenetic analysis by Maximum Likelihood method based on VP1 gene of FMDV, bootstrap values above 75% are shown



DISCUSSION

Chapter-5

DISCUSSION

Foot and mouth disease (FMD) is a deleterious, highly contagious infectious disease responsible for high morbidity in domestic ruminants, primarily in cattle, buffaloes, sheep and goats (Alexandersen *et al.*, 2003; OIE, 2018; Thompson *et al.*, 2002; Yang *et al.*, 2020). Foot and mouth disease is endemic in many parts of the world (Anjum *et al.*, 2006; Jamal *et al.*, 2010; Farooq *et al.*, 2018; Yano *et al.*, 2018). The FMD outbreaks are usually associated with significant economic losses and consequences associated with the severity of clinical signs and mortality among affected cattle, mainly newly borne infected calves (Knight-Jones & Rushton, 2013).

The remarkable diagnostic assays that confirm the clinical determination of infection are based on clinical signs, macroscopic lesions on the tongue/foot, serological techniques and detection of antigen or isolation of FMD virus from clinical samples (Kitching, 2002). It is crucial to develop a diagnostic test that is sensitive, accurate and simple to use because of the highly contagious nature FMD virus and already various rapid diagnostic methods have been developed (Reid *et al.*, 2002 ; Yadav, 2009). There is no known cure for foot and mouth disease (Kim *et al.*, 2014). In endemic nations with no slaughter policy, treating FMD disease with antibiotics for secondary bacterial infections and dressing diseased areas to avoid secondary infections are the only recommendations (Admassu *et al.*, 2015). Control and prevention of disease in endemic countries mainly depend on annual vaccination to reduce disease occurrence and dissemination (Uddowla *et al.*, 2012). The results obtained are discussed as under:

5.1 Epidemiology

5.1.1 Age-wise FMD-affected Cattle:

In this study, FMD infection rates in the age group >4 years (68.53%) were found to be higher compared to animals of age groups <1-2 years (16.85%) and 2

years (14.6%) (young and adults). Older cattle (>4 years) were more likely to be exposed to the FMD virus as they frequently encounter other herds and wildlife during grazing, while for calves, the low prevalence could be due to the protective effects of maternally acquired antibodies that decrease as age increases, also young cattle are reared in an intimate farm environment and therefore have a lesser chance of exposure (Elnekave *et al.*, 2016). The findings of the study were in agreement with the findings of (Mostary *et al.*, 2018), who reported that older cattle (>4 years) were more susceptible to FMD (29.88%) compared to adult (2 to 4 years; 19.53%) and younger age group animals (less than two years old; 8.62%). (Tufani, 2013) also reported that older animals (64.41%) were more affected by FMD, followed by younger (32.20%) age group animals and only a few calves (3.39%) below one year of age were infected. (Sarker *et al.*, 2011) also reported an increased percentage of FMD-infected cattle with advanced age. (Mesfine *et al.*, 2019) found a statistically significant ($p < 0.05$) association between FMD positive percentage and the older age group of cattle. (Sorwar *et al.*, 2016; Real *et al.*, 2015) also found that clinical cases of FMD increased as the age of cattle increased. The findings of higher frequency of FMD-positive percentage in older animals are in concordance with the observations reported by other researchers (Megersa *et al.*, 2009; Bayissa *et al.*, 2011; Jenbere *et al.*, 2011; Habtamu *et al.*, 2011; Yahya *et al.*, 2013; Beyene *et al.*, 2015).

However, the higher frequency of FMD infections in older age cattle is in contrast with the findings of (Isholo *et al.*, 2011), who reported that young cattle are more prone to FMD than adults. This can be possibly attributed to the disproportional sample size numbers in the different age categories.

5.1.2 Age-wise FMD-affected Sheep

The frequency of FMD-infected sheep was found higher in the young age group (<1-2 years) as compared to adult age groups (2-4 years) which could be due to the asymmetrical sample size taken in the study. The findings are in agreement with (Sevidzem *et al.*, 2019), who reported that the younger age group of sheep was more affected by FMD than the adult age group. However, contrary to the present findings, the percentage of FMD infection in the older age group was

significantly higher than in younger sheep as reported by various other researchers (Al-Majali *et al.*, 2008; Rehman *et al.*, 2014). This percentage difference could be attributed to potential opportunities to get in contact with the disease. The older the animal, the higher the chance of being in contact with the diseased animal (Rehman *et al.*, 2014).

5.2.1 Gender-Wise FMD affected animals

In this study, the percentage frequency of FMD-affected females (68.53%) was more than FMD-affected males (31.46%). These findings agreed with that of (Mohamoud *et al.*, 2011; Esayas *et al.*, 2009; Megersa *et al.*, 2009; Kibore *et al.*, 2013; Abubakar *et al.*, 2017; Sevidzem *et al.*, 2019), who reported that female cattle were more affected than male cattle. Due to the economic importance of dairy cattle in production and reproduction, female animals usually undergo long-term husbandry procedures that increase their risk of infection compared to males (Susan and Asamays, 1998; Longjam *et al.*, 2011; Olabode *et al.*, 2013; Atuman *et al.*, 2020). The more significant percentage of FMD-infected females might be due to the physiological stresses that include estrus, pregnancy and lactation, which are known to affect their resistance to infection. (Hailu *et al.*, 2010) also documented a higher incidence rate of FMD infection in female (16.63%) cattle compared to male cattle (1.37%). (Tufani, 2013) also reported that females (89.83%) were more affected than males (10.17%). The findings of the present study were also in agreement with that of (Gelaye *et al.*, 2009), who reported a higher percentage of FMD among female animals (15.7%) than in males (8.27%). However, the results of the present study differed from that of (Remond *et al.*, 2002), who reported that male cattle were more affected than female cattle. (Rahman *et al.*, 2012; Real *et al.*, 2015) confirmed that male cattle showed the highest FMD infected percentage (25.23%) than females (16.61%). In contrast, according to (Jenbere *et al.*, 2011; Belina *et al.*, 2016) there were no significant differences in FMD infection rates between the sexes.

5.3 Vaccination status of the affected cattle

The present investigations revealed that only a small percentage of FMD-affected cattle (3.3%) were vaccinated. This could be attributed to Covid-19 pandemic which interrupted the FMD vaccine supply chain in Kashmir. Therefore, the cattle which were supposed to get vaccinated in February (2021) were left unvaccinated even at the end of the year. The researchers (Orsel *et al.*, 2009; Yoon *et al.*, 2012; Marcos and Perez, 2019) also reported that non-vaccinated animals were more affected by FMD and clinical signs were more prominent and severe than in vaccinated animals. A similar observation has been reported by (El-Brocchi *et al.*, 2006), who recorded that most of the cattle affected with FMD were non-vaccinated, while in vaccinated cattle, the percentage of FMD infection was considerably lower. (Kitching, 2002) also reported that transmission of the FMD virus within an unvaccinated herd is more rapid than in a vaccinated herd. Vaccinated animals when exposed to infection soon after the vaccination may become infected, recover, or can be carriers, but the clinical form is not severe as seen in unvaccinated animals (OIE, 2019; Alexandersen *et al.*, 2002).

Out of the seven sheep affected with FMD, none of the animals were vaccinated in the ongoing year. In a similar kind of study, (El-Brocchi *et al.*, 2006) reported the percentage of FMDV-affected sheep was lower in vaccinated sheep than in non-vaccinated and that non-vaccinated sheep were severely affected by FMD. (Parida *et al.*, 2008) reported that vaccinated sheep become sub-clinically infected with lower clinical signs and lesions due to FMD than un-vaccinated sheep.

5.4 Economic losses associated with Foot and Mouth Disease

In the present study, the total loss per infected animal due to FMD in cattle was found to be Rs.10,149.64 with a major loss due to reduced milk production. The result findings are in accordance with (Singh *et al.*, 2013), who reported that among different components of losses, the maximum loss of 49.83 percent was observed due to milk loss (direct and indirect), followed by opportunity cost (16.15%) and reduction in growth (12.20%) due to FMD in animals. (Mulei *et al.*,

2001) also calculated the economic loss due to FMD for 95 days and found the most significant impact due to milk losses (42.0%) followed by the purchase of additional feeds (13.6%). (Mathew and Menon, 2008) calculated the economic losses due to FMD in India by taking into consideration milk loss, losses due to abortion and treatment charges. The total economic loss was calculated at Rs. 313900, of which loss in milk production accounted for 80.68 percent. (Sobhy *et al.*, 2018) reported that mortality in calves was due to FMD-induced myocarditis. (Thirunavukkarasu and Kathiravan, 2005) estimated the economic losses in the livestock farms due to FMD outbreaks and found that the losses due to the treatment of affected animals have been estimated to be Rs 89.16 lakh and an amount of Rs 300.88 lakh is lost due to reduced milk yield. Foot and mouth disease is associated with substantial economic losses (Knight-Jones and Rushton, 2013; Ferrari *et al.*, 2014; Jemberu *et al.*, 2014). Around 25% loss of productivity of FMD-infected animals was recorded, including loss or reduction in animal milk production (Bradhurst *et al.*, 2019; Ferrari *et al.*, 2014). (Senturk *et al.*, 2008) reported that FMD-induced financial losses due to reduced milk output and draught power were Rs. 300.88 and 9.26 lakhs, respectively. Similarly, (EI-Hussein and Daboura, 2012) recorded the costs of the outbreak in terms of losses in milk production, costs of drugs and mortality. Total economic losses in India attributed to FMD range from dollar 1,800 to 2,100 million USD annually reported by (Singh *et al.*, 2013).

5.5 Clinical evaluation (Vital Parameters)

The mean vital parameters viz; heart rate (bpm), rectal temperature (°F), and respiration (rate/min) in FMD-affected animals were significantly in a higher range than in healthy control animals. The increased core body temperature might be due to endogenous pyrogens such as interleukins and tumour necrosis factor- α released in response to antigens. In agreement with our findings, (Rhaymah *et al.*, 2010; Lotfollahzadeh *et al.*, 2012; Mousa Galal, 2013; Alagmy *et al.*, 2022) reported a significant increase in the rectal temperature, respiration rate and heart rates in the FMD infected animals compared with control animals. In a similar study, (Jafarsab *et al.*, 2022) found that there was a significant increase in the rectal

temperature and respiratory rate, whereas heart rate and capillary refill time were within normal limits in FMD-infected cattle. (Aktas *et al.*, 2015) reported that respiration rate, heart rate and heart rhythm in FMD-affected animals were abnormal. The result of the present study also agreed with the findings of (Tufani, 2013), who reported a higher range of mean body temperature (°F) (103.631 ± 0.189), heart rate (bpm) (78.034 ± 1.757) and respiratory rate(/minute) (35.983 ± 1.524 bpm). (EI-Shoukary *et al.*, 2019/min) also reported a significant increase in physiological parameters like rectal temperature, heart rate and respiratory rate.

5.6.1 Clinical lesions in FMD-affected cattle

In this study, pathognomic clinical signs like salivation were recorded in all animals, followed by the presence of mouth lesions (98.87%), foot/hoof (85.39%) and udder/teats (6.74%) lesions in FMD-affected cattle. Initially, the affected animals had a high fever, anorexia, marked depression and a sharp drop in milk production, followed by acute painful stomatitis and profuse salivation. Lameness was the typical clinical feature when lesions were present in the foot region, with marked painful swelling of the coronet. Erosion of epithelial tissue of oral mucosa led to difficulty in feeding. The typical clinical findings including lesions (prominent in the buccal mucosa, tongue, dental pad, gums and soft palate, inter-digital space, coronary band and udder) and sudden onset and spread within a short period were also reported by (Tufani, 2013). (Ferrari, 2014) also reported the clinical signs of disease, like fever, drooling and lesions around the mouth and on the feet, but the mortality rate was lower except in young animals. In a similar finding, (Azeem *et al.*, 2020) reported clinical signs in FMD-affected cattle, such as fever, salivation and vesicular eruption on teats, feet and mouth. The main clinical signs in FMD-infected animals were high fever, sore feet and mouth, frothy salivation, lack of appetite, anorexia, loss of milk production, hoof dysfunction and lameness in cattle, as reported by (Fakhrul *et al.*, 2016; Ranjan, 2016).

The older animals showed more severe clinical lesions, the similar findings are also reported by (Gelaye *et al.*, 2009; Megersa *et al.*, 2009; Mohamoud *et al.*,

2011; Chepkwony *et al.*, 2012). On the other hand, (Esayas *et al.*, 2009) documented no significant association between FMD lesions and the age of cattle. Very rarely, FMDV causes death in adult animals but severe lesions in the myocardium of young animals are the primary reason for the higher mortality rates (Biswal *et al.*, 2012). However, the lower incidence of FMD recorded in young calves could be associated with a low frequency of exposure to the disease (Thrusfield, 2007). Apart from characteristic clinical features of high fever, excessive salivation, and lameness in chronic cases, anorexia, low conception rates, abortions, stillbirth and sore feet were reported by (Kandel, 2018). Similar clinical findings in animals affected with FMD were fever, lesions in the mouth, salivation, vesicular lesions on the epithelium of the oral cavity, tongue, gums and vesicular lesions at interdigital space causing lameness (Bozukluhan *et al.*, 2013; Sobhy *et al.*, 2018; Nikvand *et al.*, 2019). The results of the present study were in line with (El- Bayoumy *et al.*, 2014; Islam *et al.*, 2016), who also observed clinical signs in FMD-affected cattle started with high fever which persisted for 3-4 days, followed by anorexia, lesions on foot and mouth, hoof deformities and lameness. In a similar study, (Klein, 2009; Admassu *et al.*, 2015) reported that the mucosa of the lips, the dorsum of the tongue and the dental plate were severely involved due to FMD.

5.6.2 Clinical Lesions in FMD affected Sheep

All affected sheep salivated excessively and consistently. Mouth and hoof lesions were observed in 100 and 42.85 percent of sheep, respectively. The results of the present study were in agreement with (Tufani, 2013), who reported that the existence of salivation and mouth lesions were higher in FMD-affected sheep than hoof lesions. Similar findings of hypersalivation, fever, dullness, mouth lesions, anorexia, foot lesions and lameness were reported by (Watson, 2004). The findings were in accordance with (Muthukrishnan *et al.*, 2020) who confirmed clinical signs like fever, inappetence, lesions on the foot and mouth, and lameness in FMD-affected sheep. Elevated temperature, congested inter-digital skin and coronary band, and fresh lesions on the tongue, mouth and feet in FMD-affected sheep have been reported by (Parida *et al.*, 2008; El- Bayoumy *et al.*, 2014).

5.7 Therapeutic study in clinically FMD-affected cattle

The therapeutic trial study revealed significant results ($P \leq 0.05$) concerning the resolution of clinical signs in animals treated with antibiotic regimens and immunomodulator in comparison to those treated with antibiotic regimens without any immunomodulator. The antibiotics were given in clinical cases to effectively treat the secondary bacterial infection, particularly in animals with severe mouth and hoof lesions, to prevent lameness and enhance the recovery period. In addition to antibiotics, affected animals were given mild disinfectants and anti-inflammatory drugs. This conventional method involved using antibiotics, flunixin meglumine, and mild disinfectants for treating infected animals (Gakuya *et al.*, 2011). In a similar study, (Tufani, 2013) reported that FMD-affected animals treated with gentamicin and other supportive treatments showed higher recovery, followed by enrofloxacin and supportive treatment (91.30%). (Ambore *et al.*, 2009; Sivajothi *et al.*, 2018) reported that oral Restobal (a herbal product) acts as a potent immunomodulator and anti-stressor. Glycerine, boric acid and potassium permanganate (1% solution) when applied topically on the lesions, produce a soothing emollient effect. The combination has antiseptic effects and can reduce excessive salivation (Sil *et al.*, 1998). (Kandel *et al.*, 2018) reported that FMD-affected cattle with high fever were administered a meloxicam-paracetamol combination of 0.5 mg/kg body weight intramuscularly. The wounds on the oral cavity were cleaned using a 1% iodine solution and the oral mucosa was painted with boro-glycerine. The treatment of foot and mouth disease is nonspecific and somewhat challenging. The treatment must provide rapid and prolonged pain relief and prevent secondary bacterial infections (Radostits *et al.*, 2010). However, the use of antibiotics has its limitations and disadvantages and is even responsible for the financial burden. Therefore, necessitates the alternate substitutive FMD therapeutic paradigm (Windsor *et al.*, 2020).

5.8 Post FMD complications

The primary post-FMD complications recorded were heat intolerance/panting, hypertrichosis and mastitis in FMD-affected animals. The probable reason might be that the virus damages important hormone glands, including the pituitary, which regulates the body's metabolic processes. The resultant gland dysfunction may result in panting, anxiety and infertility (Radostitis *et al.*, 2010). At times the infection of udders and teats may progress to mastitis that causes permanent loss of teats and milk production. Similar post-FMD findings of mastitis, panting, lower fertility and abortion in were reported in affected animals by (Mdetele *et al.*, 2015). The findings of the present study were in agreement with (Tufani, 2013), who reported mastitis in 5.08% of animals and 16.95% of animals showed panting for a long time. Complications like heat intolerance and hair coat growth were also reported by (Bayoumi *et al.*, 2021). Similarly, (Mulei *et al.*, 2001) also found that some cattle developed chronic mastitis and abortions (1.4%). (Ghanem and Hamid, 2010) also observed complications like heat intolerance syndrome, hirsutism and reduced milk production in post-FMD-affected cattle.

5.9 Molecular (PCR) epidemiology of FMDV in cattle

Although all the clinical cases with the characteristic clinical picture were suggestive of FMD infection, 65 samples (73.0%) were detected positive for FMDV by polymerase chain reaction targeting 5-untranslated region (5-UTR) gene fragment. Further PCR amplification results of 1301 bp and 863-866 bp amplicons initially identified serotype O and serotype A in FMD-positive samples using primer sets ARS4/NK61 and A-1C562/NK61. However, after sequencing the representative samples, serotype O was identified and confirmed from all 65 (73.0%) FMD-positive samples, indicating that the primers used earlier in PCR amplification were nonspecific. The factors that might have led to sample discretion during laboratory testing, included a negligible amount of viral RNA in the initial samples or improper sample preservation and most importantly, RT-PCR with the 1F/1R primers had 85% of PCR efficacy in comparison with 87% by virus

isolation (Reid *et al.*, 2000). The identification of a universal primer set (1F/1R) located in the 5% untranslated region (UTR) of the FMD virus genome usually depicts the successful detection of FMD virus serotypes in clinical samples (Reid *et al.*, 2000). The findings of the present study were in agreement with (Rashtibaf *et al.*, 2012), who reported that among positive samples (96), 58 (60.4%) belonged to serotype O. There was no evidence for the presence of Asia-1 and A serotypes in the present study. (Diab *et al.*, 2019) also reported that out of 51 oral tissue samples collected from cattle in 13 farms suspected to be FMD (in Egypt), 44 (86.27%) were positive by RT-PCR using universal primers. Serotype O was predominant and detected in 31 samples (70.45%). (Dupka *et al.*, 2011) also reported that cattle were the most affected species among all other affected ruminant species during the outbreak period. Serotype O constituted 70.6% of the outbreaks, the most predominant serotype prevalent in Bhutan.

Similar to the findings of this study, (Hegde *et al.*, 2014) confirmed that cattle were the predominant species in most outbreaks caused by FMDV serotype O (64.04%). (Subramaniam *et al.*, 2013) also reported that serotype O was responsible for 80% of the confirmed outbreaks in India. (Ali *et al.*, 2017) reported that the prevalence rates for FMD serotypes in an outbreak in Pakistan were 70% for serotype O. (Abdela, 2017) also found that the most dominant serotype identified was O, accounting for 72% of the investigated outbreaks in Ethiopia. (Soltan, 2016) also reported that a foot and mouth disease outbreak with predominant serotype O was detected in 73.3% of herds. (Jemberu *et al.*, 2016) also confirmed that the most dominant serotype was O, accounting for 70% of all the collected samples that tested positive.

In sheep, only two (28.57%) samples were positive for FMDV serotype O out of seven samples by polymerase chain (PCR). The findings agreed with (Rehman *et al.*, 2014), who reported that the sequences showed maximum homology to FMD-type O isolates, also reported by (Waheed *et al.*, 2011), who detected 31 samples as FMD serotype O out of 32 samples in sheep. In a similar study, (El-Brocchi *et al.*, 2006) reported that in an outbreak of FMD in the sheep population, the only serotype identified was serotype O of FMDV.

In India, most of the outbreaks of FMD are due to serotype ‘O’ followed by serotype ‘A’ and serotype Asia-1 (Bhattacharya *et al.*, 2005; Verma *et al.*, 2008). In India, during the year 2016-17, 150 serotype-confirmed FMD incidences were recorded. The highest incidences were reported from the Northeastern region, the southern peninsula, and Karnataka. It is documented that Serotype O was responsible for all the incidences recorded during the year 2016-17 and for the first time, there was no incidence of both serotypes A and Asia1 in the country (FMD Annual Report, 2017).

5.9. Sequencing

Sequencing results confirmed serotype O in four representative FMDV-positive samples. The FMDV serotype O identified in the study confirmed its close genetic proximity with FMDV serotype O (the PanAsia strain of Pakistan). The results showed sequence identity in 91-93% with FMDV serotype O of Pakistan and 91.63% with sequence identity with FMDV serotype O of Bhutan. In consonance with published literature, (Le VP *et al.*, 2010; Brito *et al.*, 2017; Ferreira *et al.*, 2017) reported a predominance of serotype O and further sequencing of seventy-one O serotype viruses revealed that 65 of the Maximum Likelihood method based on the Kimura 2-parameter model. In a study, (Brito *et al.*, 2017) reported that the FMD viruses belonged to O/ME-SA/PanAsia lineage and only six were classified as O/SEA/Mya-98. The sequences identified were genetically distant from Indian FMDV serotype O strains. Thus, there was a possibility that FMDV serotype O involved in outbreaks in Kashmir valley might have been genetically distinct from the vaccine strain and perhaps not stimulated appropriate humoral response and could possibly be the cause of FMD outbreaks in the valley.



SUMMARY AND CONCLUSION

Chapter-6

SUMMARY AND CONCLUSION

The present study was undertaken to investigate Foot and mouth disease in affected ruminants (cattle and sheep) in four districts of UT Jammu and Kashmir from September 2021 to August 2022 with the objective to study the epidemiological pattern associated with economic losses, clinical features of the disease and identification of FMD virus serotypes prevalent in the Kashmir valley in domestic ruminants. A total of ninety-six FMD-infected ruminants (cattle and sheep) were included in the study. A higher number of cattle in the older age group (>4 years: 68.50%) were infected with FMD as compared to younger (<1-2 years: 14.60%) and adult (2-4 years: 16.85%) age groups whereas, young sheep (<1-2 years: 57.14%) were more affected with FMD than adult (2-4 year: 42.85%) sheep. The percentage of males and females infected with FMD cattle was 31.46 and 68.53 percent, respectively, out of the total number of FMD-infected animals. Out of the eighty-nine cattle infected with FMD, only a small percentage (3.3%) of FMD-infected cattle were vaccinated. The majority of the cattle (96.62%) infected with FMD in the year were not vaccinated against FMD.

The economic losses due to FMD were evaluated in terms of reduction in milk yield, treatment cost and mortality losses. In affected animals, the mean pre-FMD milk yield (10.57 ± 0.29 L) was significantly ($P \leq 0.05$) higher than the mean post-FMD milk yield (2.01 ± 0.21 L). The average treatment cost calculated was about Rs.1235.83 per FMD-infected animal and the average mortality cost was estimated at around 2014.29 per infected calf. The total losses calculated due to FMD were about Rs. 10,149.64 per infected cattle.

Infected animals had a significantly higher ($p \leq 0.05$) mean rectal temperature °F (102.44 ± 0.13) than the healthy control group animals (100.97 ± 0.05). The mean respiration rate (/min) recorded in FMD-infected animals (42.19 ± 1.82) was significantly ($p \leq 0.05$) higher in comparison to the healthy control (21.99 ± 0.15) group of animals. The mean heart rate (bpm) recorded in

FMD-infected animals (77.75 ± 1.53) was significantly ($p\leq 0.05$) higher in comparison to the healthy control (61.42 ± 0.14) group of animals. Hundred percent of the FMD-infected cattle and sheep exhibited salivation. The predominant clinical lesions in the mouth, foot/hof and udder/teats were manifested in 98.8%, 85.3% and 6.7% of affected animals, respectively. The older age group (>4 years) cattle showed higher existence of mouth lesions than young and adult ones. However, in sheep mouth lesions were seen more in the younger age group and the existence of hoof lesions was found higher in adults ones.

The three major post-FMD complications recorded were heat intolerance/panting (21.34 %), hypertrichosis (7.80%) and mastitis (6.74%) in affected cattle.

A total of 89 samples were subjected to polymerase chain reaction targeting 5-untranslated region (5-UTR) gene fragment and 65 samples (73.0%) were detected positive for FMDV. Further PCR amplification and sequencing results identified and confirmed serotype O in 65 samples (73.0%) FMDV-positive samples. The FMDV serotype O identified in the study confirmed its close genetic proximity with FMDV serotype O (the PanAsia strain of Pakistan).

The therapeutic trial study was also conducted on 18 animals to evaluate the number of days taken for the resolution of clinical signs in FMD-affected animals. The affected cattle treated with antibiotics and immunomodulators showed significantly better results compared to the animals treated with antibiotics excluding immunomodulators.

Conclusions:

On the basis of observations made in the present study, the following conclusions were drawn

1. Older age group cattle (>4 years) were more affected due to FMD than younger ($<1-2$) and adult (2-4) cattle.

2. The vaccination status was confirmed in only 3.30 % FMD affected cattle whereas, the majority (96.62%) of the cattle were unvaccinated.
3. Economic losses associated with the FMD were huge and a significant difference in pre-FMD and post-FMD milk yields was recorded. The total losses due to FMD were calculated and found around Rs. 10,149.64 per infected cattle.
4. Clinical lesions were more prevalent and severe in the older age group of affected animals compared to younger animals and the majority of animals exhibited salivations and mouth lesions followed by foot/h hoof lesions.
5. The prevalent post-FMD complications recorded were heat intolerance followed by hypertrichosis and mastitis.
6. A total of 73.0% samples were confirmed positive for FMDV on PCR detection and PCR amplification (sequencing) results confirmed serotype O (73.0%) in all FMD-positive samples. It could be concluded that FMD serotype O was responsible for the recent FMD outbreaks in ruminants in the Kashmir valley.
7. Serotype O identified in the study was closely related to the FMDV serotype O- PanAsia strain of Pakistan.

Recommendations:

1. The control of FMD in terms of timely vaccination in ruminants especially in Kashmir is highly significant for protecting the livestock and improving the livelihood of livestock farmers.



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CERTIFICATE

This is to certify that the corrections/modifications as suggested by External Examiner **Dr. R. K. Bhardwaj Associate Professor, Division of Veterinary Medicine, F.V.Sc & AH., R.S Pura, SKUAST-J** during viva voce examination held on **03-03-2023** has been incorporated in the manuscript entitled **“Epidemiological and Clinico-Diagnostic Studies on Foot and Mouth Disease in Ruminants”** submitted by **Aamina Dilawar (Regd. No. MSV-2020-475)**.

(Dr. Nuzhat Hassan)

Chairman Advisory Committee