

**COMPARISON OF IMMUNOGENICITY OF FOOT AND MOUTH
DISEASE VACCINES IN SHEEP AND GOAT**



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BY

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This is to certify that the thesis entitled, **“COMPARISON OF IMMUNOGENICITY OF FOOT AND MOUTH DISEASE VACCINES IN SHEEP AND GOAT”**, submitted by **Dr. Yogesh Kumar**, enrollment no. **V-1249/13** in partial fulfillment of the requirements of the award of the **Master of Veterinary Science in Veterinary Microbiology** of the **Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura (UP)**, India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

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(Rashmi Singh)

Major Advisor and Chairman

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We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which being presented by him for the award of the degree of the University.

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ABBREVIATIONS

%	Percent
°C	Centigrade/ Degree celcius
µl	Microliter
Ag	Antigen
Ab	Antibody
BEF	Bovine ephimeral fever
BQ	Black quarter
<i>C. chauvoei</i>	<i>Clostridium chauvoei</i>
CFT	Complement fixation test
DDW	Double distill water
DIVA	Differentiation in vaccinated and infected animal
ELISA	Enzyme linked immuno sorbent assay
<i>et al.</i>	<i>et alii/alii</i>
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
g	gram(s)
h	Hour(s)
HRPO	Horse reddish peroxidase
HS	Hemorrhagic septicaemia
LPB	Liquid phase blocking
LPS	Lipopolysaccharide
mg	Milligram
NSP	Non structural protein
OD	Optical density
OPD	Orthophenylenediamine dihydrochloride
PBS	Phosphate buffer saline
PBST 0.05%	Phosphate buffer saline containing 0.05% tween-20
PBST 0.1%	Phosphate buffer saline containing 0.1% tween-20
PDFMD	Project Directorate on foot and mouth disease
<i>P. multocida</i>	<i>Pasteurella multocida</i>
RNA	Ribonucleic acid
RP	Rinderpest
rpm	Revolution per minute
S/C	Subcutaneous

SP	Structural protein
VNT	Virus neutralization test
min	minute(s)
sec	second(s)

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CHAPTER-I

INTRODUCTION

Small ruminants are an important and integrated component of the agricultural production system in Asia. In India, small ruminants are reared by the poorest sections of rural population with very small holdings. Approximately, 59.68% and 41.94% of the total world population of 861.9 million goats and 1078.2 million sheep, respectively are found in Asia (Aziz, 2010). India, with 135.17 million goats and 65.06 million sheep population, ranks second in the world (19th Livestock Census, 2012).

Foot and mouth disease (FMD) is a highly contagious disease of domestic and wild cloven hoofed animals including cattle, sheep, goat, deer and pig (Parida, 2009). FMD virus (FMDV) is a non-enveloped virus with icosahedral symmetry that belongs to the *Aphthovirus* genus of the *Picornaviridae* family (Regenmortel *et al.*, 2000). FMDV particles are about 30 nm in diameter and are composed of 60 copies of each of four capsid proteins termed VP1, VP2, VP3 and VP4. VP1, VP2, VP3 are external and VP4 is internal in virion particle. The immunodominant site is located within the G–H loop of VP1 (Domingo *et al.*, 2002). There are seven serotypes of FMDV, which are O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3. Serotypes O, A, and C have been identified in Europe and South America while O, A, and Asia 1 are common throughout Asia. SAT 1 and SAT 2 are found throughout Africa while, SAT 3 is confined to Southern Africa. The serotypes found in the Middle East include A, O, Asia 1 and SAT 1. These serotypes are so defined because they do not cross-protect (Davies, 2002). In India, FMD outbreaks due to the serotypes O, A, C and Asia-1 have been reported but, since 1996 no outbreak due to serotype C has been reported (Depa *et al.*, 2012).

FMD causes great economic loss in animals including small ruminants. In terms of morbidity, it is about 97.64% and the rest, 2.36% is in terms of mortality. In India, the total estimated economic loss due to FMD is about 12,000 crore to 14,000 crore per annum and total losses per infected animal due to FMD are ₹ 2,023 and 3,046 in sheep and goat, respectively (Singh *et al.*, 2013).

In small ruminants, the disease is subclinical in nature and very few clinical signs are visible which are probably influenced by the virus strain, the breed of sheep or goat and to some extent by the environment (Geering, 1967). Incubation period is commonly between 3 and 8 days.

India, has a dense livestock population of cattle, buffalo, sheep and goat which are commonly grazed together under the system of extensive animal husbandry practices. The

epidemiological role of small ruminants in FMD outbreaks has been generally neglected. It was elucidated by Uppal *et al.* (1972) during investigation of FMD outbreak in an organized cattle farm, which was mainly due to small ruminant as source of infection. Here, FMD vaccination was followed regularly except in sheep, goats and pigs. As the clinical sign of the disease in sheep and goat were hardly visible, the disease remained unnoticed in these animals. FMDV isolated from the heart muscle of lambs was typed as 'O'. The possible risks of spread of FMD from sheep and goats have gained prominence by the work of Sharma (1981); Pay (1988) and Barnett and Cox (1999).

Most of FMD outbreaks in 1990s, within and around the European Union member states have involved sheep (Kitching, 1996; Ferguson *et al.*, 2001). In North Africa, a definite predilection for sheep in FMD outbreak was reported (Mackay, 1994). In Turkey, 18.5% of the total FMD cases reported in 1996 were associated with small ruminants (Taylor and Tufan, 1996). In Greece, 5000 sheep and goats were destroyed during the outbreak of FMD in 1996 (Kitching, 1996). In the 2001 epidemic of FMD in Great Britain, the first species infected on the affected farms was almost always sheep (53%) or cattle (45%) rather than pigs (1%) (Ferguson *et al.*, 2001). The health hazards to the small-ruminant population of the Middle East posed by the trade in live sheep and goats have also been pointed out by some researchers (Rapoport and Shimshony, 1997).

On serological investigation to detect FMDV circulation in the domestic small ruminant population of India, a total of 20.35% sheep and 13.60% goats were found to be positive for 3AB NSP antibodies providing an evidence of extensive viral activity. In LPB ELISA, only 4.54% sheep and 6.27% goats were found to have protective antibody ($\log_{10}$ titre of ≥ 1.8) against all three serotype strains in the vaccine, correlating with "no or sparse vaccination" scenario in these species in the country (Rout *et al.*, 2014 a).

According to OIE (2012), conventional live FMD vaccines are not acceptable due to the danger of reversion to virulence and as their use would prevent the detection of infection in vaccinated animals. Currently, available FMD vaccines are inactivated vaccines. FMD vaccine may be alum adjuvant based or oil adjuvant based. The oil adjuvant based FMD vaccines elicit higher immune response than alum adjuvant and the duration of immunity is more. These FMD vaccines may be polyvalent and monovalent. Monovalent vaccine contain inactivated antigen of only one serotype while the polyvalent vaccine contain inactivated antigen of multiple serotypes of FMDV. Again FMD vaccine can be Monovac (for FMD only) or Polyvac [combined vaccine for FMD with other diseases like haemorrhagic septicaemia (HS) and black quarter (BQ)].

Vaccination at repeated intervals is not only costly, but also causes stress to the animals and inconvenience to the farmer. Reddy *et al.* (1997) vaccinated the cattle with monovac FMD vaccine and combined vaccine (FMD, HS, BQ) and observed no significant difference in antibody titres of calves vaccinated with FMD vaccine and combined vaccine. The serological response to FMD antigens was unaffected when it was inoculated alone or in combination with other antigens (Joseph and Hedger, 1984; Myrian *et al.*, 2015; Palanisamy *et al.*, 1992; Srinivasan *et al.*, 2001; Bagoury *et al.* 2014) or it showed an elevated response in combined vaccine as reported by Chhabra *et al.* (2004).

Serum antibody titre is an indicator of the immune status as well as of the prevalence of the disease in animals (Hamblin *et al.*, 1987). Humoral immunity is known to be the most influential factor in preventing FMD (Saiz *et al.*, 2002). According to OIE (2012), serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs). The SP tests are serotype-specific and detect antibodies elicited by vaccination and infection. The examples are virus neutralisation test (VNT) (Golding *et al.*, 1976), the solid-phase competition ELISA (Mackay *et al.*, 2001; Paiba *et al.*, 2004) and the liquid phase blocking ELISA (Hamblin *et al.*, 1986; Hamblin *et al.*, 1987). These tests are serotype-specific and are highly sensitive, provided that the virus or antigen used in the test is closely matched to the strain circulating in the field.

The detection of antibody to the NSPs of FMDV can be used to identify past or present infection with any of the seven serotypes of the virus, whether or not the animal has also been vaccinated. Mohapatra *et al.* (2011) developed r3AB3 indirect ELISA. The diagnostic sensitivity of the assay was estimated to be 96%, while the diagnostic specificity varied between the naïve and vaccinates as 99.1% and 96.4%, respectively.

In India, sheep and goats are co housed and usually share common pastures with cattle and buffaloes in a mixed farming set up. Among these, regular vaccination is usually done in cattle against FMD while other susceptible small ruminants are vaccinated only in the face of an outbreak depending on the extent of infection in the animals. Although, FMD in small ruminants is usually milder compared to cattle or pigs, the subclinically infected sheep and goats have been incriminated in the transboundary spread of the disease on several occasions in the past (Kitching, 1996). Thus, airborne excretion of virus from sub-clinically infected small ruminants further contributes to the problem of control (Sharma, 1981; Pay, 1988). Small ruminants often migrate long distances in search of grazing lands or for sale through meat markets, thereby posing a serious risk of disseminating the virus (Madhanmohan *et al.*, 2011). To check silent amplification and dissemination of virus in a

mixed farming set up, small ruminants should be brought under the ambit of routine vaccination and surveillance programmes.

India, has got world's second largest population of goat and sheep, individual vaccination for each disease is not only cumbersome, labour intensive but financially putting heavy economic burden to the stakeholders as well as farmers. The better approach would be to incorporate various antigens in single vaccine dose to avoid such type of problems. The incorporation of more number of pathogen in the vaccine must be such that it should not result in lower immune response to the vaccine target. The comparison of vaccination with monovac and combined formulations in cattle has shown no significant difference between anti-FMD antibody titres of both the vaccines. There is no literature available in scientific domain on comparative study of monovac and combined FMD vaccines in small ruminants and hence present study was undertaken to fulfil the critical gap with the following objectives.

OBJECTIVES

1. Comparative evaluation of immunogenicity of FMD vaccines in sheep and goat.
2. Measurement of humoral immune response against FMD vaccines in sheep and goat.
3. Comparison of antibody titre against FMD antigen in monovac (FMD only) and combined vaccine in sheep and goat.

CHAPTER-II

REVIEW OF LITERATURE

2.1 Historical background

FMD in animals was first time described by Löffler and Frosch (1898) to be a disease caused by a filterable agent. Waldmann and Pape (1920) were able to produce FMD in guinea pig and reported lesions on the feet and tongue with general loss of condition. Suckling mouse was added to the guinea pig as a valuable research tool in FMD research by Skinner (1951). Valle'e and Carre (1922) first recognized the occurrence of two serotypes and named them after their place of origin, O for the Department of Oise in France and A for Allemagne. Waldmann and Trautwein (1926) discovered a third serotype which they called C. Three additional serotypes were described in Southern Africa and were named accordingly as Southern African Territories types SAT 1, SAT 2 and SAT 3 (Galloway *et al.*, 1948; Brooksby, 1958). In 1954, serotype Asia 1 was discovered from Pakistan (Brown, 2003). Bedson *et al.* (1927) found that two isolates of a serotype A virus could be differentiated by cross-neutralization tests. The importance of this difference in the field became clear during the major epidemic in Mexico during 1946-1954 in which field strains belonging to serotype A broke through the immunity induced by a vaccine made from another strain of the same serotype (Galloway *et al.*, 1948). Within serotypes, many strains can be identified by biochemical and immunological tests (OIE, 2012).

FMD virus is a non-enveloped icosahedron belonging to genus *Aphthovirus* of the family *Picornaviridae* (Regenmortel *et al.*, 2000). Its genome is a single stranded RNA molecule of about 8500 nucleotides of positive polarity. Particles are about 30 nm in diameter and are composed of 60 copies of each of four capsid proteins termed VP1, VP2, VP3 and VP4. VP1, VP2, VP3 are external and VP4 is internal. One copy of each capsid protein assembles to produce a protomer; in turn, five protomers form a pentamer and 12 pentamers complete the capsid (Domingo *et al.*, 2002).

Valle'e *et al.* (1925) prepared first experimental FMD vaccine using formaldehyde inactivated vesicular fluid from tongue epithelium of infected calves. Frenkel (1947) showed large scale production of the virus by using surviving tongue epithelium from cattle. Espinet (1951) first described use of adjuvants with inactivated FMD antigen preparations and aluminum hydroxide was supplemented with a second adjuvant, saponin. Freund (1956) described oil based adjuvants and Michelsen reported the use of incomplete oil adjuvants for vaccination of pigs (Michelsen, 1961). Brown and Crick (1959) described use of aziridine,

acetyleneimine, as a first order inactivant of the FMDV. Capstick *et al.* (1962) adapted growth of FMDV in baby hamster kidney (BHK) monolayer cell suspension cultures. The advantages of either monolayers or suspensions derived from a cell line from pig kidney or BHK cells replaced the use of tongue epithelium in vaccine formulation (Mowat and Chapman, 1962; Capstick *et al.*, 1962). The work of many authors including Mussgay (1959) Bachrach (1960) and Wild and Brown (1967) demonstrated the labile nature of the virus to mild pH (6.5), heat (56.8°C), trypsin etc. and confirmed the presence of essential immunogenic component of FMD vaccine in 146S particle of which the VP1 protein appeared to be the most relevant constituent (Laporte *et al.*, 1973). Brown *et al.* (1963) and Bahnemann (1973) introduced the ethyleneimines for FMDV antigen inactivation.

2.2 Foot and mouth disease

Foot and mouth disease is highly contagious disease of domestic and wild cloven hoofed animals, including cattle, sheep, goats, deer and pigs (Parida, 2009). The incubation period of FMD is highly variable, 2-14 days and depends on the strain and dose of virus, the route of transmission, the animal species and the husbandry conditions (Alxenandersen and Mowat, 2005).

Cattle with FMD often have severe clinical signs. They usually become febrile and develop lesions on the tongue, dental pad, gums, soft palate, nostrils and/or muzzle. The vesicles on the tongue often coalesce, rupture quickly, and are highly painful. The animal becomes reluctant to eat. Profuse salivation and nasal discharge are common in this specie; affected animals become lethargic, may loose condition rapidly. Hoof lesions, with accompanying signs of pain, occur in the area of the coronary band and interdigital space. Young calves may die of heart failure without developing vesicles (Aftosa, 2014).

In small ruminants, the disease has a wide spectrum of clinical signs (Geering, 1967; Littlejohn, 1970; Sharma, 1981; Pay, 1988) which are probably influenced by the virus strain, the breed of sheep or goat and to some extent by the environment (Geering, 1967). The disease in naturally acquired infections often takes on a milder form in small ruminants than in cattle and pigs. Incubation period is commonly between 3 and 8 days. The animal may show fever, anorexia and lassitude and lameness occurs in the presence or absence of foot lesions (Pay, 1988; Shukla *et al.*, 1974). Foot lesions are in the interdigital cleft, along the coronary bands and on the bulb of the heels. Hoof shedding sometimes happens in sheep. Oral lesions are less common than foot lesions and often disappear by the time foot lesions appear. They occur mostly on the posterior aspects of the dorsum of the tongue rather than the tip, being more necrotic than vesicular, or on the dental pad (Barnett and Cox., 1999). Mouth lesions more likely occur in goats (Olah *et al.*, 1976) and disease in goats is even milder.

Mortality is often seen in lambs and kids in the absence of any clinical signs and is generally the result of myocarditis or myocardial lesions (Geering, 1967; Pay, 1988).

FMDV can cause a persistent infection and duration of persistence is higher in cattle (about 3.5 years) than sheep (9 months) and goat (4 months) (Moonen and Schrijver, 2000).

2.3 Role of small ruminants in epidemiology and transmission of FMD

FMD in sheep and goat is subclinical in nature, their role as virus carriers represent a reservoir for further infection and spread of disease but the epidemiological role of small ruminants in FMD outbreaks has been generally neglected (Patil *et al.*, 2002). Several outbreaks of FMD within and around the European Union member states have involved sheep (Donaldson and Doel 1992; Ferguson *et al.*, 2001; Kitching, 1996; Taylor and Tufan, 1996) and in North Africa, a definite predilection for sheep has been reported (Mackay, 1994). In Turkey, 18.5% of the total FMD cases reported in 1996 were associated with small ruminants (Taylor and Tufan, 1996) and 39 outbreaks of FMD type O also occurred in Greece during 1996, probably as a consequence of the illegal importation of live sheep from Turkey (Kitching, 1996). In the 2001 epidemic in Great Britain, the first species infected on the affected farms was almost always sheep (53%) or cattle (45%) rather than pigs (1%) (Ferguson *et al.*, 2001). The health hazards to the small ruminant population of the Middle East posed by the trade in live sheep and goats have also been pointed out by some researchers (Rapoport and Shimshony, 1997). They revealed that all of the most recent outbreaks of FMD within and around the European Union member states have involved sheep. Kitching (1996) reported that Tunisia which was previously free from FMD got the disease in cattle with the importation of sheep and goat imported from the Middle East during 1989. Subsequently, the disease spread into Algeria and Morocco. Blanco *et al.* (2001) tested 598 sheep sera using a liquid-phase blocking ELISA (LPBE) to detect antibodies against FMDV structural proteins in Morocco. The study confirmed the presence of FMDV specific antibodies in 77 clinically normal sheep, indicating that unrecognized FMDV-infected sheep could represent a potential risk of FMD dissemination. Hyera *et al.* (2006) collected a total of 250, 142 and 134 goat sera originating respectively, from Kasane, Maun and Shakawe districts (in northwestern Botswana) and they were tested for FMDV antibodies against the three SAT serotypes by the LPBE and 10.4%, 3.5% and 13.4% were positive either to SAT 1 or SAT 3, or to both serotypes, respectively. None of the goat's sera was positive to SAT 2 serotype. All sheep sera tested negative against all three serotypes of the virus.

In India, sheep and goats are co housed and usually share common pastures with cattle and buffaloes in a mixed farming set up. Among these, regular vaccination is usually done in cattle against FMD while other susceptible small ruminants are vaccinated only in the

face of an outbreak depending on the extent of infection in the animals. Although, FMD in small ruminants is usually milder compared to cattle or pigs, the subclinically infected sheep and goats have been incriminated in the transboundary spread of the disease on several occasions in the past (Kitching, 1996). Thus, airborne excretion of virus from sub-clinically infected small ruminants further contributes to the problem of control (Sharma, 1981; Pay 1988). Small ruminants often migrate long distances in search of grazing lands or for sale through meat markets, thereby posing a serious risk of disseminating the virus (Madhanmohan *et al.*, 2011). Uppal *et al.* (1972) associated a FMD outbreak in one of the organized cattle farm in northern India due to small ruminants as source of infection. The cattle in farm were vaccinated regularly except sheep, goats and pigs. The clinical sign of the disease in sheep and goat were hardly visible and remained unnoticed. Serotype 'O' FMDV was isolated from the heart muscle of lambs. Singh and Sharma (1980) reported a very severe outbreak of FMD at the Central Sheep and Wool Research Station, Pashulok, in the Doon valley of the sub Himalayas. Two hundred fifty eight of 506 sheep in 5 paddocks were affected and the disease took three weeks to spread to all 5 paddocks. Mortality was 1.16%. Morbidity was highest in animals more than 1 year old (57.2% compared with 20.7% in those 6-12 months old and 32.65% in sheep less than 6 months old) and was 58.7% in females and 14.7% in males. When breeds were compared, morbidity was 77.9% in Nali, 47.6% in Rambouillet and 46.3% in cross-breeds. This outbreak was much more severe than usually found in sheep. The FMD virus isolated was identified as type 'O'. Singh *et al.* (1994) investigated a severe outbreak of FMD in an unvaccinated flock of small ruminants revealing overall mortality in lambs 20.89%, case fatality rate 38.55%, proportional mortality rate 56%, attack rate 54.47%, 50% and 30.76% for Muzaffarnagri lambs, Barbari and Jamunapari kids, respectively. FMDV type 'O' was isolated from heart muscles. Ranabijuli *et al.* (2010) conducted serological investigations to generate estimates of antibody prevalence in goats of Orissa state to both non-structural (NSP-Ab) and structural proteins (SP-Ab) of FMD. The apparent overall NSP-Ab and SP-Ab seroprevalences were 38% and 20.7%, respectively, which signifies a very high level of FMD virus circulation in the goat population despite the lack of clinical signs in this species.

Lazarus *et al.* (2012) conducted preliminary serological investigation in a flock of sheep and goats from Tafawa Balewa, Bauchi State, Nigeria using the FMD 3-ABC ELISA assay. Results indicated the evidence of antibody to FMDV in sheep and goats was 28.7 %. Saleh *et al.* (2013) collected 241 sheep sera from 13 suspicious infected sheep flocks with FMD from two major areas in Basra (Abulkhaseeb and Alzubair). All these samples were examined by ELISA to virus infection associated (VIA) antigen. It was found that 71.9% of the total tested sheep sera had specific VIA antibodies against FMDV, 91.7% of the clinically

infected sheep gave positive result and 66.8% of the clinically non-infected sheep were negative. The higher rate of seropositivity in both Abuelkhaseeb and AL-Zubair areas was in the age between 3.5 – 4.5 year (80%) and (81.8%), respectively. The high prevalence of seropositivity to VIA could be due to sub clinical infection or to carrier state and the disease in sheep is mild and go un-noticed but important because of transmission to cattle. Hemanth *et al.* (2014) collected samples and reported the FMD in small ruminants in nearby villages in Kadapa district in Andhra Pradesh. They also performed the histopathology of different organs of small ruminants that died after the infection. Rout *et al.* (2014 a) conducted serological investigation to detect FMDV circulation in the domestic small ruminant population of India. A total of 4407 and 4035 serum samples from sheep and goats, respectively were collected at random covering majority of the states across the country during 2010–2012. These samples were analyzed for antibodies against the NSP of FMDV in an indirect 3AB NSP ELISA and against SP in a LPB - ELISA. A total of 20.35% sheep and 13.60% goats were found to be positive for 3AB NSP antibodies providing a serological evidence of extensive viral activity. In LPB ELISA, only 4.54% sheep and 6.27% goats were found to have protective antibody ($\log_{10}$ titre of ≥ 1.8) against all three serotype strains in the vaccine, which correlates with “no or sparse vaccination” scenario in these species in the country. Rout *et al.* (2014 b) assessed FMDV in traditionally managed goats from different villages of Andaman & Nicobar Islands during September 2014. A total of 81 serum samples from goats were collected and subjected to 3AB NSP - ELISA. Only 1 out of 81 animals was found to be positive suggesting absence of FMD virus circulation in the islands during the recent past.

2.4 Foot and mouth disease vaccines

Currently available FMD vaccines are inactivated vaccines. According to OIE (2012), conventional live FMD vaccines are not acceptable due to the danger of reversion to virulence and as their use would prevent the detection of infection in vaccinated animals. Current FMD vaccines are produced by infecting BHK- 21 cells with virulent FMDV, under biosecure conditions, followed by chemical inactivation with binary ethyleneimine (BEI) and purification by ultrafiltration (Kitching *et al.*, 2007). Some vaccine manufacturers also use polyethylene glycol (PEG) precipitation and ultrafiltration to purify the antigen. In some advanced vaccine companies, the relatively crude bulk of inactivated antigens are subjected to chromatography to obtain highly purified antigens, which are usually stored in a gaseous phase of liquid nitrogen until vaccine formulation (Doel, 2003).

At the time of vaccine formulation, the concentrated antigen is diluted with buffers and then blended with a suitable adjuvant, either oil or aluminum hydroxide/saponin. Oil emulsion vaccines are now widely used for the immunization of pigs, sheep, goats and cattle;

whereas, aluminum hydroxide/saponin- adjuvanted vaccines are only used for ruminants (Parida, 2009). The immunity elicited by aqueous $\text{Al}(\text{OH})_3$ /saponin vaccine is short lived and antibody concentration rapidly falls after administration (Anderson *et al.*, 1971b; Barnett *et al.*, 1996; Hunter, 1996) and that stronger immunity is generated using oil adjuvanted formulations (Anderson *et al.*, 1971a; Bahnemann and Mesquita, 1987). Ready to formulate oil adjuvants have been introduced which contains esters of octadecenoic acid and anhydromannitol. These adjuvants readily form double or mixed (water-in-oil-in-water) emulsions, which are both, stable and have low viscosity, suggesting promising candidates for the new generation FMD vaccines (SEPPIC, 1994).

In India, cattle and buffalo population is vaccinated with a trivalent inactivated FMD vaccine containing O, A and Asia 1 serotypes, while small ruminants are not covered under such state aided vaccination programmes (Annual Report, Project Directorate on Foot and Mouth Disease, PDFMD, 2011-2012).

Literature on use and evaluation of immune response after FMD vaccination is sparse in case of small ruminants.

Patil *et al.* (2002) evaluated polyvalent FMD vaccine in goats. FMDV quadrivalent double oil emulsion (Montanide ISA 206) vaccine was tested in goats at reduced doses. The oil adjuvant elicited superior immune response at any given period than aluminium hydroxide gel (AGS) vaccine and the rapidity of development of response was quicker. The duration of immunity also appeared to be maintained for long period. The differences in immune response between two adjuvant groups were statistically significant ($P < 0.05$) indicating possible universal usage of double oil emulsion vaccines for disease control programs irrespective of species of animals. Selim *et al.* (2010), studied the immune response in sheep vaccinated with bivalent inactivated aluminum hydroxide gel ($\text{Al}(\text{OH})_3$) and oil adjuvant (Montanide ISA206) FMD vaccine by ELISA and SNT. Vaccination of sheep with bivalent inactivated oil adjuvant vaccine gave higher long lasting immunity than $\text{Al}(\text{OH})_3$, suggesting replacement of the commercial aluminum hydroxide vaccine by oil adjuvant vaccine.

Madhanmohan *et al.* (2011) vaccinated FMD seronegative Nellore sheep ($n = 30$) and Osmanabadi goats ($n = 30$) with different payloads of O1 Manisa vaccine (0.45–5 μg). Vaccinated and sero-negative unvaccinated sheep ($n = 6$) and goats ($n = 6$) were challenged intradermally into the coronary band with O1 Manisa virus. The sheep and goats were monitored for signs of FMD and samples were collected for measuring viraemia and virus associated with nasal swabs and probang samples. Vaccines containing antigen payload up to 0.94 μg protected sheep and goats against challenge. Sheep and goats vaccinated with 0.45 μg antigen payload were poorly protected against challenge suggesting an antigen payload of

0.94 µg sufficient to offer complete protection and also absence of carrier status. Sheep and goats with no vaccination or with poor seroconversion to vaccination showed sub-clinical infection and became carriers. The results of the study suggest that vaccination offers protection from clinical disease even at a low payload of 0.94 µg and hence one-half of cattle dose of the oil-based vaccine formulations is sufficient to induce protective immune response in sheep and goats.

Combined vaccines of FMD

Use of combined vaccines in large ruminants has been reported where FMD vaccine was given simultaneously or in combined form with other bacterial or viral vaccines and is reviewed here. Literature on use of combined vaccine in sheep and goat is not available.

Joseph and Hedger (1984) divided trial group of 104 cattle into three groups to ascertain that FMD and HS vaccines can be given simultaneously. Group 1 animals received FMD and HS both vaccines simultaneously, group 2 animals received only FMD vaccine and group 3 animals received only HS vaccine. The serological response to vaccinations was monitored at 0, 21 and 35 days by the virus neutralisation test for FMD and the mouse-protection and indirect haemagglutination tests for HS. The simultaneous administration of the two inactivated vaccines produced no adverse effects and the serological response did not differ from the response to either vaccine given separately, thus indicating that cattle may be safely and effectively vaccinated simultaneously in this way. The simultaneous administration of FMD vaccine with other vaccine or use of combined vaccine in cattle produced no ill effects and the serological responses did not differ significantly from the response to either vaccine given separately as observed in case of rinder pest vaccine (Hedger *et al.*, 1986), anthrax vaccine (Myrian *et al.*, 2015), FMD + rabies vaccine (Palanisamy *et al.*, 1992), FMD + rabies + *Pasturella multocida* + *Clostridium chauvoei* (Srinivasan *et al.*, 2001) and FMD + bovine ephemeral fever (Bagoury *et al.*, 2014).

Reddy *et al.* (1997) vaccinated the cattle with FMD alone and FMD, HS, BQ combined vaccine and observed that there was no significant difference in mean antibody titres of calves vaccinated with FMD vaccine and combined vaccine. The serological response to FMD antigens was unaffected when it was inoculated alone or in combination with other antigens. Chhabra *et al.* (2004) used combined vaccine (FMD + HS) and FMD vaccine alone in different groups of buffalo calves and evaluated humoral immune response by monitoring the serum antibody titres. Higher LPB ELISA antibody titres and virus specific IgM titres were observed in calves receiving combined vaccine as compared to calves immunized with FMD vaccine alone. In another study in rabbits, combined vaccine (FMD+HS) produced better antibody titer that persisted for longer time (Altaf *et al.*, 2012).

2.5 LPB- ELISA and DIVA ELISA

Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs) (OIE, 2012).

The SP tests are serotype-specific and detect antibodies elicited by vaccination and infection; examples are the virus neutralisation test (VNT) (Golding *et al.*, 1976), the solid-phase competition ELISA (Brocchi *et al.*, 1990; Chenard *et al.*, 2003; Mackay *et al.*, 2001; Paiba *et al.*, 2004) and the liquid-phase blocking ELISA (Hamblin *et al.*, 1986; Hamblin *et al.*, 1987). These tests are serotype-specific and are highly sensitive, providing that the virus or antigen used in the test is closely matched to the strain circulating in the field. McCullough *et al.* (1985) developed liquid phase ELISA to detect antigen/antibody reactions in liquid phase. They found that this liquid phase ELISA was same efficient, both qualitatively and quantitatively as the double sandwich or antigen trapping ELISA but six to eight times more sensitive than indirect ELISA. Hamblin *et al.* (1986) developed a liquid phase blocking sandwich ELISA for quantification of antibody against FMDV. In this test constant amount of antigen was incubated with a range of test serum dilutions in liquid phase. The assay was found rapid, more sensitive, more specific, relatively simple to perform, economic and could be completed in shorter duration of time as compared to SNT. In liquid phase ELISA, the VP1 protein was well expressed on surface of virus of FMD, thus, antibody reactivity in liquid phase could be directly related to functional serological assay such as virus neutralization test and immune status or protection.

Hamblin *et al.* (1987) described that the serum antibody titre is an indicator of the immune status as well as of the prevalence of the disease in animals particular geographic region and solid phase. McCullough *et al.* (1992) observed the antibody response after vaccination against FMD virus using SNT, sandwich ELISA, sandwich competition ELISA, liquid phase blocking ELISA phase blocking competition ELISA. The competition ELISAs (in particular the “blocking” ELISA) was the most effective in detecting antibody in the serum sample. Neidbalaski *et al.* (1994) developed LPB sandwich ELISA for serological study of antibody to FMDV. It was found that the assay was sensitive, specific and precise. They found it easy and rapid to perform. The test was useful for large scale serological tests. Saha and Sen (1995) raised hyperimmune sera in rabbits or guinea pigs to purify infective virus particles (146S). Serotype O, A₂₂, C and Asia 1 were used to test with sandwich ELISA, LPB, ELISA, CFT and SNT on 25 samples of infected bovine tongue epithelium stored between 1975 to 1992. Among them the blocking ELISA was found more sensitive than sandwich ELISA or SNT.

The potential use of the nonstructural proteins (NSPs) for differential detection of animals infected with the FMD virus (FMDV) in the vaccinated herds (DIVA) is well established. Several NSPs such as 2B, 2C, 3A, 3AB, 3B, 3ABC and 3D have been exploited as antigens for this purpose. A reasonable consensus has emerged in favour of the polyprotein 3ABC or its derivatives such as 3AB as the most appropriate antigens for use in the screening tests (De Diego *et al.*, 1997; Mackay *et al.*, 1998; Sorensen *et al.*, 1998). Chung *et al.* (2002) demonstrated that the antibody response against polyprotein 3AB (3AB-Ab) could be used as a reliable serological marker for DIVA since the 3AB-Ab in animals persisted up to 3.5 years post-outbreak and the animals, even after 10 rounds of vaccination, were found negative for 3AB-Ab. Mohapatra *et al.* (2011) developed r3AB3 DIVA- ELISA. They expressed the FMD virus NSP 3AB in a prokaryotic system and an indirect ELISA (r3AB3 I-ELISA) was developed and validated as a screening assay for detecting virus in vaccinated bovines. The diagnostic sensitivity of the assay was estimated to be 96%, while the diagnostic specificity varied between the naïve and vaccinates as 99.1% and 96.4%, respectively.

Hedger *et al.* (1986) divided a total of 118 local cattle into three groups, one which received a trivalent inactivated FMD vaccine, another which received attenuated tissue culture rinderpest (RP) vaccine and a third which received both vaccines simultaneously at separate sites on opposite sides of the neck. The serological response to vaccination was monitored at day 0, 21 and 42 days in virus neutralisation tests. The simultaneous administration of the two vaccines produced no ill effects and the serological responses did not differ significantly from the response to either vaccine given separately.

Palanisamy *et al.* (1992) vaccinated three groups of crossbred calves with FMD vaccine only, combined FMD + rabies vaccine and rabies vaccine alone. Efficacy of the vaccines was determined by serum antibody assay at different intervals post-vaccination. The results of the study indicated that the immune response of animals to a single inoculation of FMD vaccine only as well as combined FMD + rabies vaccine was unsatisfactory due to maternally derived antibodies to FMD virus antigen. However, two inoculations elicited a satisfactory antibody response to FMD virus antigens in both group of animals. Rabies antigen alone as well as combined FMD + rabies antigen induced satisfactory serum antibody titers to rabies antigen. There were no significantly difference in serological responses in animal administered with FMD vaccine only, rabies vaccine only or combined FMD + rabies vaccine.

Srinivasan *et al.* (2001) vaccinated five groups of ten animals (cattle) with a combined vaccine containing FMD, rabies, *P. multocida* and *C. chauvoei* antigens and individual component vaccines containing FMD, rabies, *P. multocida* and *C. chauvoei* antigens, respectively. Serological response of the calves was assayed on days 21 and 90 post

vaccination. There was no significant variation in the serological response elicited by individual component vaccines and combined vaccine containing all four antigens.

Altaf *et al.* (2012) studied efficacy of FMD+HS vaccine to FMD and HS vaccines alone in rabbits. Previously isolated and characterized FMD virus “O” serotype and *P. multocida* were used in the experimental design. Alum precipitated FMD, HS and FMD+HS vaccines were prepared and injected in experimental rabbits (6 months of age) at 0 and 14th day. The serum samples were collected on 0, 14, 28, 35 and 48 days post-priming. Antibody titer was measured by VNT for FMD “O” virus and Indirect IHA test for lipopolysaccharide (LPS) of *P. multocida*. It was concluded that combined vaccine produced better antibody titer that persisted for longer time.

Bagoury *et al.* (2014) prepared an inactivated combined vaccine containing three FMD virus serotypes O, A, SAT2 and bovine ephemeral fever (BEF) antigens adjuvanted with Montanide oil ISA 206. The prepared vaccine proved to be sterile and safe and induced humoral immune response in calves after single dose administration. Serum neutralizing antibody reached the protective titers at 3rd week post vaccination for FMD virus serotypes O, A and SAT 2 and at 2nd week post vaccination for BEF virus. Serum neutralizing antibody maintained at the protective titers for up to 32 and 42 weeks post vaccination for FMD and BEF viruses, respectively without inducing any adverse reactions. In conclusion, animals could be safely vaccinated with combined inactivated FMD and BEF viruses vaccine without impairing the immune response against these antigens.

Myrian *et al.*, (2015) evaluated if the simultaneous application of a *Bacillus anthracis* live vaccine with a commercial tetravalent oil-based FMD vaccine. Two groups of 16 heifers with comparable liquid phase blocking ELISA (LPBE) titers were immunized with the FMD alone or simultaneously with a commercial attenuated bovine anthrax Sterne strain vaccine (ABV). Serum samples were obtained at 0, 25, 60 and 90 days post vaccination (dpv) and specific antibodies against two FMDV vaccine strains were assessed by LPBE, avidity and IgG-isotype ELISAs. Bovines immunized with FMD + ABV responded with a boost in the LPBE antibody titers and avidity at 25 dpv, and remained within similar levels up to the end of the study. Animals vaccinated with FMD + ABV had significantly higher LPBE titers at 25 dpv, compared to those immunized with FMD alone; which was due to an increase in IgG2 titers. They concluded that the simultaneous application of a live anthrax vaccine with the current FMD tetravalent in cattle previously immunized against FMD, did not counteract the serological response induced by FMD vaccination.

CHAPTER –III

MATERIALS AND METHODS

3. 1 Materials

In this study, liquid phase blocking ELISA (LPBE) and recombinant 3AB₃ nonstructural protein (NSP) based indirect ELISA was used. The kits were kind gift by Project Directorate on FMD, Mukteswar. LPB ELISA was used to detect serum antibody against serotype O, A and Asia-1. R3AB₃ DIVA kit was used in differentiating the infected animals from vaccinated animals.

3. 1. 1 LPB ELISA Kit

- i. Lyophilized ready to use type specific anti-146S FMDV anti-FMDV coating serum
- ii. Lyophilized ready to use type specific anti-146S FMDV anti-FMDV tracing serum
- iii. Lyophilized ready to use inactivated type O, A, and Asia-1 antigens
- iv. Rabbit/ goat anti-guinea pig immunoglobulin HRPO conjugate (DAKO)
- v. Substrate: OPD (Orthophenylenediamine dihydrochloride, Sigma)
- vi. Lyophilized blocking buffer
- vii. Phosphate buffer saline
- viii. Tween-20
- ix. Double distilled water
- x. 1M H₂SO₄
- xi. 30% hydrogen peroxide

3. 1. 2 R3AB₃ DIVA Kit

- i. Freeze dried recombinant 3AB₃ protein: Each vial was dissolved in 11 ml of carbonate – bicarbonate coating buffer, pH 9.5. After reconstitution, the vials were immediately used without storing further.
- ii. Freeze dried positive and negative control sera: There were different positive and negative control sera for sheep and goat samples. Each vial was dissolved in 160 µl of distilled water and then distributed into single use aliquots for storage at -20°C. Working dilution of 1:50 in diluent buffer for control serum was used.
- iii. *E. coli* lysate: Each vial of *E. coli* lysate was reconstituted with 70 µl of PBS, pH 7.4 and stored at -20°C.

- iv. Lapin anti caprine- HRP conjugate
- v. Lapin anti ovine- HRP conjugate
- vi. Skimmed milk powder (Merck)
- vii. Horse serum (Hyclone): stored at -20 °C
- viii. Salts for PBS, pH 7.4
- ix. Tween-20 (Merck)
- x. Carbonate- bicarbonate coating buffer (Sigma): 1 capsule for preparing 100 ml of buffer
- xi. Phosphate- Citrate substrate buffer (Sigma): 1 tablet for preparing 100 ml of buffer
- xii. OPD (Sigma): 50 mg, stored at -20°C

3. 1. 3 Equipments

- i. 96-well maxisorp ELISA plates (Nunc)
- ii. Multi channel micropipette (Eppendorf): 100 – 1000 µl
- iii. Single channel micropipettes along with disposable tips (Eppendorf): 100 – 1000 µl
- iv. Immunowasher (Nunc)
- v. Wash bottle
- vi. ELISA plate reader (Tecan)
- vii. Refrigerator (Blue star)
- viii. Incubator (Vaiometra)
- ix. pH paper strips of various ranges (Qualigens)
- x. Flasks (Borosil)
- xi. Graduated cylinders (Borosil)
- xii. Graduated pipettes (Borosil)
- xiii. Syringes: 5 ml, 10 ml
- xiv. Cryovials: 2 ml (Axiva)
- xv. Eppendorf tubes (Axiva)
- xvi. Refrigerated Centrifuge machine (Remi)
- xvii. Deep well plates
- xviii. Absortive pad
- xix. Double distill water assembly (J- Sil)

3.1.4 Experimental animals

Forty three apparently healthy sheep (n=16) and goats (n= 27) aged more than 4 months were selected for the study. The animals were maintained in the Department of Physiology, DUVASU, Mathura. The animals were fed by grazing and supplementation with green and concentrate.

3.1.5 Vaccines

Two types of commercially available FMD vaccines procured from the market were used in the present study.

- i. Combined Triovac-oil adjuvanted vaccine against FMD, HS and BQ containing inactivated *Pasteurella multocida*, *Clostridium chauvoei* and FMDV (serotype O, A and Asia 1) from Indian Immunologicals (Raksha Triovac)
- ii. Monovac-oil adjuvanted vaccine against FMD containing inactivated FMDV (serotype O, A and Asia 1) from Indian Immunologicals (Raksha Ovac)

3.2 Methods

3.2.1 Deworming of animals

Deworming of all the animals was carried out with fenbendazole @ 10 mg per kg body weight three weeks before the start of vaccination schedule.

3.2.2 Experimental design and vaccination

The animals (sheep and goats) were divided in three groups each. Group 1 received combined vaccine (FMD + HS + BQ) and group 2 received monovac vaccine (FMD alone) as given in detail in table 1. Group 3 animals served as control. One ml dose of each vaccine was given subcutaneously (s/c).

Table 1: Different groups of sheep and goat receiving combined and monovac vaccine against FMD

Sheep				Goat		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
No of animals	6	7	3	12	12	3
Vaccine	Combined	Monovac	No vaccine	Combined	Monovac	No vaccine
Dose	1 ml	1 ml	-	1 ml	1 ml	-
Route of vaccination	S/C	S/C	-	S/C	S/C	-

3.2.3 Collection of blood samples

Blood samples were collected on 0, 30, 60, and 90 days of vaccination. Zero day sample was collected prior to vaccination while 30, 60 and 90 days were post vaccination samples. Blood samples were collected aseptically from jugular vein in plain vacutainer with the help of 5 ml syringes with 20 gauge needle.

3.2.4 Separation of serum

Serum was collected from blood samples by placing tubes in slanting position for 1 h at room temperature followed by 3-4 h of incubation at 4°C. The tubes were centrifuged at 1000 rpm for 15 min. A clear supernatant was collected as serum and stored in aliquots at -20°C till use. The collected serum samples were analyzed by LPB- ELISA and R3AB₃ DIVA ELISA.

3.2.5 Liquid phase blocking ELISA

The test was performed on the serum samples collected on 0, 30, 60 and 90 days of vaccination of sheep and goat as per the instructions provided with the kit.

- i. **Coating of ELISA plates:** The ready-to-use lyophilized serotype specific coating antibodies was reconstituted with 18 ml of double distilled water (DDW) and three ELISA plates were coated by adding 50 µl of the reconstituted solution in each wells of ELISA plate and they were named as O, A, Asia-1 as per serotype of coating serum. The ELISA plates were incubated at 37°C for 1 h followed by incubation at 4°C overnight.
- ii. **Preparation of Ag-Ab mixture:** Two-fold dilutions (4 dilutions starting from 1:16) of test serum samples were prepared in a low binding perspex plate with PBS, pH 7.4 containing 0.05% tween-20 (PBST-0.05%). For it, in first row and in fifth row, 750 µl of PBST-0.05% was taken and in rest of rows, 400 µl of PBST-0.05% was taken. Then in first and fifth row, 50 µl of test serum sample was taken in each well (except in the wells of control). It was mixed and serial double dilution was made from first to second, from second to third, from third to fourth row by transferring 400 µl and from 4th row 400 µl of mix was discarded. In the same way, dilution was made from 5th to 6th, from 6th to 7th, from 7th to 8th and from 8th row 400 µl of mix was discarded. The serum samples were analyzed in duplicate. Now, 50 µl of diluted serum was transferred to three different deep well plates labeled as O, A, Asia-1. Respective, antigen vial was reconstituted with 18 ml of PBST-0.05% and equal volume of diluted antigen (50 µl) was added to all the dilutions of test serum samples. For antigen control, equal volume of PBS-0.05% was added to the already diluted

antigen. The diluted antigen and antibody mixture was incubated at 4°C overnight without disturbance.

- iii. **Washing of ELISA plates:** The wells of ELISA plates were washed by adding washing buffer (200-300 µl) using immune-wash and the contents of wells were discarded by abrupt downward hand motion. Washing was done for 3 times with 3 min of hold period between each wash. The inverted microplate was slapped 3-4 times onto a dust free absorbent pad to remove all residual contents in the wells.
- iv. **Dispensing of antigen-antibody (Ag-Ab) mixture:** 50 µl of the overnight incubated Ag-Ab mixture was dispensed in duplicate to the ELISA plates. The plates were incubated at 37°C for 1 h. 50 µl of the reconstituted blocking buffer was added to the wells of background control. The ELISA plates were washed.
- v. **Tracing:** Tracing serum vial was reconstituted with 18 ml PBST- 0.1% and the 50 µl of reconstituted serotype specific tracing serum was added to each serotype specific plate. The plates were incubated at 37°C for 1 h. The plates were washed.
- vi. **Conjugate:** Each vial of anti-guinea pig-HRPO conjugate was reconstituted with 18 ml of PBST- 0.1% and 50 µl were added to all the wells. The plates were incubated at 37°C for 1 h. Then the plates were washed.
- vii. **Substrate:** Substrate vial were reconstituted just before use with 18 ml DDW and 9.6 µl of 30% H₂O₂ solution was added and 50 µl were added to each well of the plate and the plates were incubated at 37°C for 15 minutes.
- viii. The colour reaction was stopped by adding 50 µl of stopping solution. The OD values were taken in the wells at 492 nm in ELISA reader.
- ix. The OD sheet data were converted into MS Excel format and results were analyzed and interpreted using PDFMD ELISA Analyst v2.0.

The percentage inhibition in each well was calculated in relation to antigen control using the following formula:

$$\text{Percentage inhibition} = 100 - \frac{(\text{OD of test well} - \text{Background OD})}{(\text{OD of antigen control} - \text{Background OD})} \times 100$$

The reciprocal log₁₀ dilution corresponding to 50 % inhibition was considered to be titre of the serum.

Layout of LPB- ELISA plate

AC	AC	TS1 1:32	TS1 1:32	TS2 1:32	TS2 1:32	TS3 1:32	TS3 1:32	TS4 1:32	TS4 1:32	TS5 1:32	TS5 1:32
AC	AC	TS1 1:64	TS1 1:64	TS2 1:64	TS2 1:64	TS3 1:64	TS3 1:64	TS4 1:64	TS4 1:64	TS5 1:64	TS5 1:64
AC	AC	TS1 1:128	TS1 1:128	TS2 1:128	TS2 1:128	TS3 1:128	TS3 1:128	TS4 1:128	TS4 1:128	TS5 1:128	TS5 1:128
BG	BG	TS1 1:256	TS1 1:256	TS2 1:256	TS2 1:256	TS3 1:256	TS3 1:256	TS4 1:256	TS4 1:256	TS5 1:256	TS5 1:256
TS6 1:32	TS6 1:32	TS7 1:32	TS7 1:32	TS8 1:32	TS8 1:32	TS9 1:32	TS9 1:32	TS10 1:32	TS10 1:32	TS11 1:32	TS11 1:32
TS6 1:64	TS6 1:64	TS7 1:64	TS7 1:64	TS8 1:64	TS8 1:64	TS9 1:64	TS9 1:64	TS10 1:64	TS10 1:64	TS11 1:64	TS11 1:64
TS6 1:128	TS6 1:128	TS7 1:128	TS7 1:128	TS8 1:128	TS8 1:128	TS9 1:128	TS9 1:128	TS10 1:128	TS10 1:128	TS11 1:128	TS11 1:128
TS6 1:256	TS6 1:256	TS7 1:256	TS7 1:256	TS8 1:256	TS8 1:256	TS9 1:256	TS9 1:256	TS10 1:256	TS10 1:256	TS11 1:256	TS11 1:256

TS- Test serum BG- Background control AC- Antigen control

3.2.6 R3AB₃ NSP based- Indirect ELISA

The test was performed on serum samples collected zero day before vaccination in sheep and goat as per the instructions provided with the kit.

- i. The freeze dried recombinant protein in the vial was dissolved with 1 ml of coating buffer and another 10 ml of coating buffer was added to it (one vial was sufficient for coating two 96 well ELISA plates). The reconstituted protein vial was used immediately without storing. The freeze dried vials of positive (one month post infection serum) and negative (one month post vaccination serum) control serum was dissolved with double distilled water and were stored at -20°C after reconstitution in single use aliquots. The positive and negative serum samples were different for sheep and goat.
- ii. The content of freeze dried vial carrying *E. coli* lysate was dissolved in 70 µl of PBS, pH 7.4 and stored at -20°C after use.
- iii. 96-well ELISA plates was coated with the diluted recombinant protein @ 50 µl per well. (~40 ng of purified recombinant protein per well). The plate was incubated at 4°C overnight.
- iv. The plate was removed from the refrigerator and it was thawed at 37°C for 15 minutes.
- v. In a low protein binding perspex plate, the test serum, negative and positive serum sample were diluted 1:50 in diluent buffer. 122.5 µl of diluent buffer and 2.5 µl of serum sample were used so that 100 µl of diluted serum sample can be transferred to coated ELISA plates. On a coated ELISA plate, 43 test serum sample (16 from sheep, 27 from goat were used), two positive (for sheep and goat different), two negative control serum (for sheep and goat different) and two background controls were used. In background controls only 100 µl of diluent buffer were dispensed without any serum.
- vi. Three continuous wash were given to overnight coated ELISA plates without hold time.
- vii. 100 µl of serum and diluents buffer mixture was transferred from Perspex plate to ELISA plate and the plate was incubated for one hour at 37°C with 20- 30 rpm.
- viii. After incubation the ELISA plates were washed for three times with 3 minutes of soak period of each.
- ix. Anti-caprine-HRP and anti-ovine-HRP conjugate were diluted in diluent buffer @1:20,000 (0.3 µl of conjugate and 6 ml of diluent) and they were dispensed @ 50 µl per well. Plate was incubated for 1 hour at 37°C.
- x. Three continuous wash was given with 5 min of soak period of each.

- xi. Freshly prepared substrate solution was added @ 50 μ l per well and it was incubated at 37°C without shaking.
- xii. The colour reaction was stopped by adding 1 M H_2SO_4 .
- xiii. Optical density was measured at wavelength of 492 nm with ELISA reader.

Performance and interpretation

The test was considered valid only if the mean absorbance of positive control wells was not less than 0.8 and the plate was rejected if the mean absorbance of negative control was more than 0.3. The O.D. in back ground control wells was less than 0.1.

To reduce inter-run variation due to differences in absolute absorbance between runs, final results for each test serum was expressed as the PP value (percent positivity). PP value was calculated by dividing the reaction of the test serum by that of the positive control serum and then multiplied with 100. The results interpreted based on the following cut off zones:

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

CHAPTER-IV

RESULTS

In the present study, comparative evaluation of immunogenicity of FMD vaccines (monovac and combined) was studied in sheep and goat. Measurement of humoral immune response was done by LPB-ELISA and to check infection status of animals DIVA (R 3AB₃ NSP based- Indirect ELISA) test was performed.

4. 1 R 3AB₃ NSP based- Indirect ELISA

43 test serum samples (16 from sheep and 27 from goat) collected on zero day before vaccination, were analyzed for seroreactivity to 3AB₃ NSP by DIVA ELISA. The test was considered valid only if the mean absorbance of positive control wells was not less than 0.8 and the plate was rejected if the mean absorbance of negative control was more than 0.3. The O.D. in back ground control wells was less than 0.1.

To reduce inter-run variation due to differences in absolute absorbance between runs, final results for each test serum were expressed as the percent positivity (PP value). PP value was calculated by dividing the reaction of the test serum by that of the positive control serum and then multiplied with 100. The results interpreted based on the following cut off zones:

- 1) 3 AB₃ NSP reactivity positive: If PP value was more than 40%
- 2) 3 AB₃ NSP reactivity negative: If PP value was less than 40%

Out of total 16 sheep samples, two samples were found positive having PP value more than 40%. Among goat samples, none of sample was found positive, for antibody against 3AB₃ NSP. The two sheep found positive in DIVA ELISA were excluded from the experimental vaccination study.

4. 2 LPB - ELISA

The humoral immune response against the two different FMD vaccines (monovac and combined triovac) was compared by LPB-ELISA. Combined triovac vaccine contained inactivated *P. multocida*, *C. chauvoei*, and FMDV. Monovac contained only FMDV. Both the vaccines contained three common prevailing serotypes in India (O, A, and Asia1).

4.2.1 Antibody titre against FMDV in different vaccine groups in sheep and goat

The antibody titre of serum samples against all three FMDV serotypes were deduced from the final plate OD value with the help of PD-FMD analyst software. Antibody titre for

different FMDV serotypes at day 0 (pre vaccination) and 30, 60 and 90 days of post vaccination are given in table 2 and 3.

4.2.2 Comparison of mean antibody titre against different serotypes of FMDV in monovac vaccine in goat

Mean antibody titre against the serotypes (O, A and Asia 1) of FMDV was maximum at day 30 and then it remained almost constant at day 60 and after that at day 90 it decreased. Immune response was highest for serotype Asia 1 as compared to serotype O and serotype A during the whole course (Table 4; Figure 1 a and 1 b)

4.2.3 Comparison of mean antibody titre against different serotypes of FMDV in monovac vaccine in sheep

For serotype O and Asia 1, antibody response was maximum at day 30 and then at day 60 and 90 it decreased for both the serotypes but the fall in the mean antibody titre was more for serotype O than for Asia 1. The mean antibody titre against serotype A was maximum at day 60 and then it decreased at day 90. The immune response was maximum against serotype O throughout the whole course in monovac vaccine in sheep as compared to serotype A and Asia 1 (Table 5; Figure 2 a and 2 b).

4.2.4 Comparison of mean antibody titre against different serotypes of FMDV in combined vaccine in goat

Mean antibody titre against serotype A and serotype Asia-1 was maximum at day 30, then it remained constant at day 60 and after that it decreased at day 90. Mean antibody titre against serotype O increased from day zero after vaccination and remained almost constant from day 30 to day 60 (slightly higher at day 60 than day 30) and after that it decreased on day 90. Mean antibody titre was highest against serotype Asia 1 as compared to serotypes O and A in combined vaccine in goat (Table 4; Figure 3 a and 3 b).

4.2.5 Comparison of mean antibody titre against different serotypes of FMDV in combined vaccine in sheep

Against serotype A and Asia-1 antibody response was highest at day 60 and then it decreased at day 90. For serotype O, immune response was maximum at day 30 and then it decreases slightly at day 60 and at day 90 a rapid decrease was seen. Mean antibody titre was highest against serotype O during the course than serotype A and Asia 1 (Table 5; Figure 4 a and 4 b).

Table 4: Comparison of mean antibody titre against serotype A, O and Asia 1 of FMDV in monovac and combined vaccine in goat

Serotypes	Groups	Antibody titre			
		Day 0	Day 30	Day 60	Day 90
A	Group 1 (Combined vaccine)	1.0448 ^a	2.1026 ^b	2.0494 ^b	1.9908 ^b
	Group 2 (Monovac vaccine)	1.0733 ^a	2.1508 ^b	2.114 ^b	1.9229 ^b
	Group 3 (Control)	1 ^a	1 ^a	1 ^a	1 ^a
O	Group 1 (Combined vaccine)	0.9896 ^a	2.1912 ^b	2.2093 ^b	1.9563 ^b
	Group 2 (Monovac vaccine)	1.1348 ^a	2.1544 ^b	2.1122 ^b	1.889 ^b
	Group 3 (Control)	0.8511 ^a	1 ^a	1 ^a	1 ^a
Asia 1	Group 1 (Combined vaccine)	1.038 ^a	2.3551 ^b	2.2946 ^b	1.8348 ^b
	Group 2 (Monovac vaccine)	0.9726 ^a	2.3306 ^b	2.2886 ^b	1.8459 ^b
	Group 3 (Control)	1 ^a	1 ^a	1 ^a	1 ^a

Table 5: Comparison of mean antibody titre against serotype A, O and Asia 1 of FMDV in monovac and combined vaccine in sheep

Serotypes	Groups	Antibody titre			
		Day 0	Day 30	Day 60	Day 90
A	Group 1 (Combined vaccine)	1 ^a	2.199 ^b	2.242 ^b	1.9663 ^b
	Group 2 (Monovac vaccine)	1 ^a	1.888 ^b	2.0135 ^b	1.8077 ^b
	Group 3 (Control)	1 ^a	1 ^a	1 ^a	1 ^a
O	Group 1 (Combined vaccine)	1 ^a	2.3327 ^b	2.3054 ^b	1.9783 ^b
	Group 2 (Monovac vaccine)	0.9809 ^a	2.115 ^b	2.0633 ^b	1.7914 ^b
	Group 3 (Control)	1 ^a	1 ^a	1.2373 ^a	1 ^a
Asia 1	Group 1 (Combined vaccine)	1.0838 ^a	2.171 ^b	2.3799 ^b	1.8253 ^b
	Group 2 (Monovac vaccine)	1 ^a	1.9546 ^b	1.9098 ^b	1.706 ^b
	Group 3 (Control)	1 ^a	1 ^a	1 ^a	1 ^a

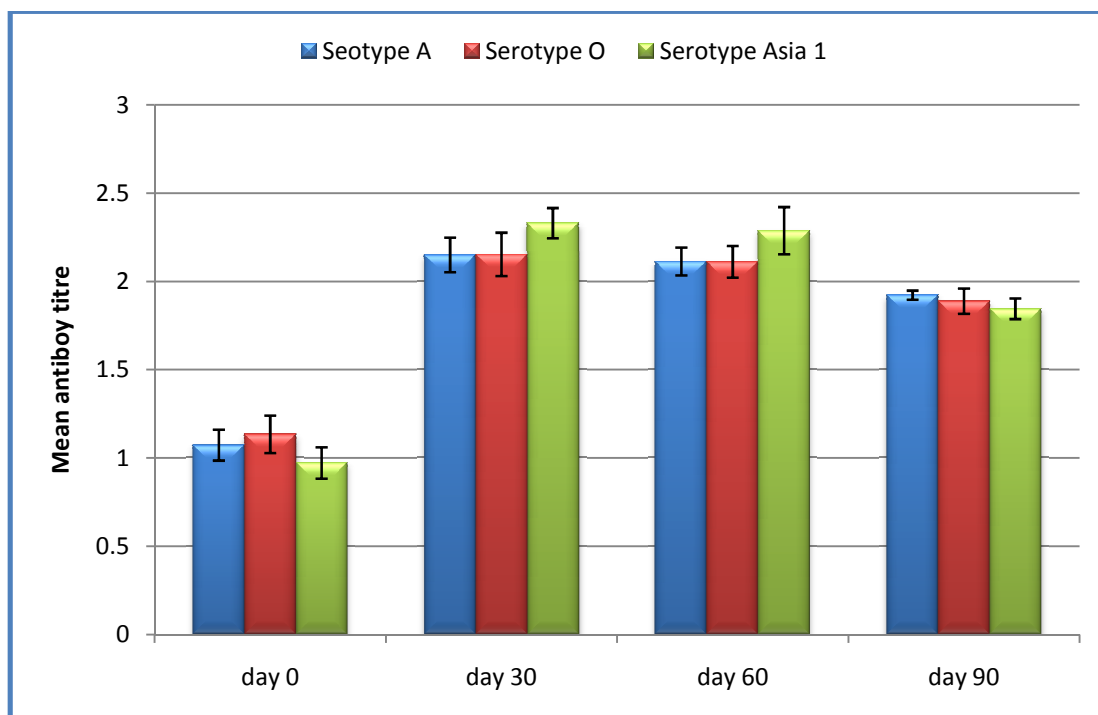


Figure 1 a: Comparison of antibody titre against different serotypes of FMDV in monovac vaccine in goat

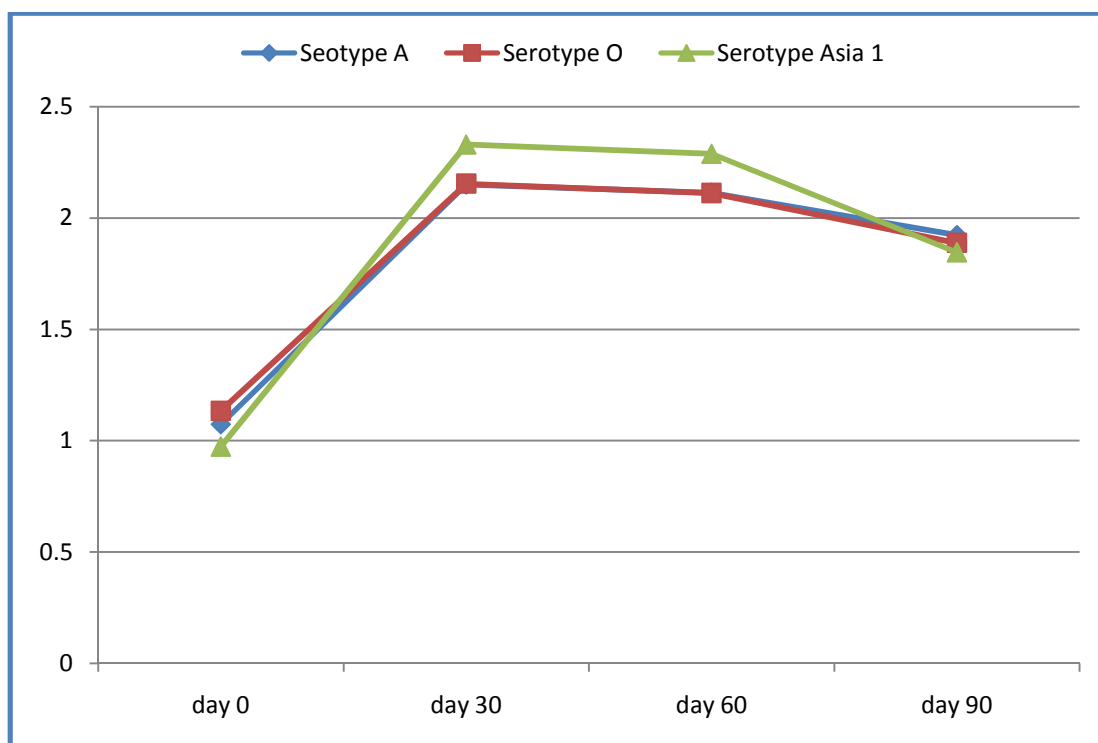


Figure 1 b: Comparison of antibody titre against different serotypes of FMDV in monovac vaccine in goat

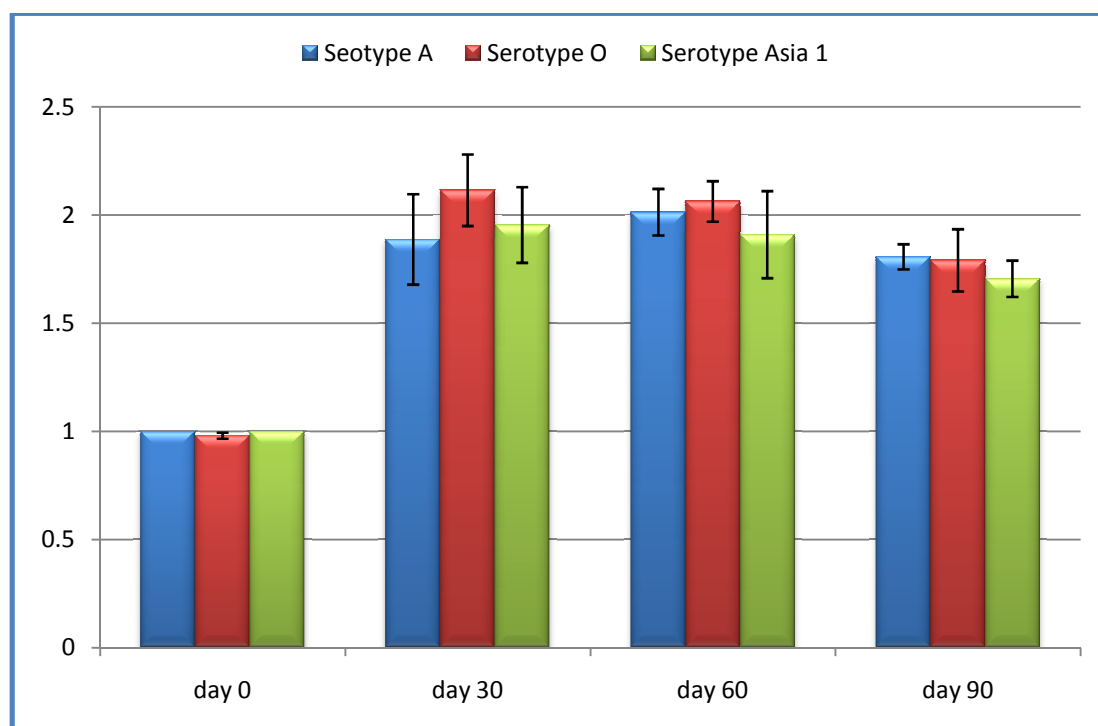


Figure 2 a: Comparison of antibody titre against different serotypes of FMDV in monovac vaccine in sheep

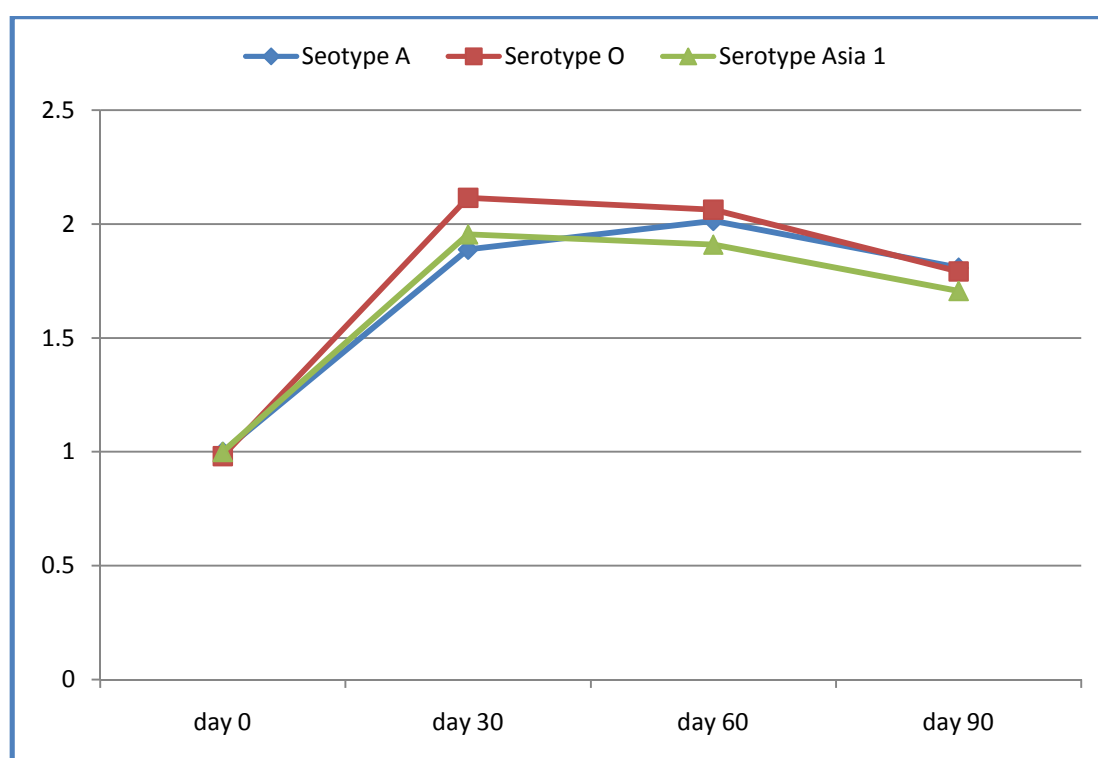


Figure 2 b: Comparison of antibody titre against different serotypes of FMDV in monovac vaccine in sheep

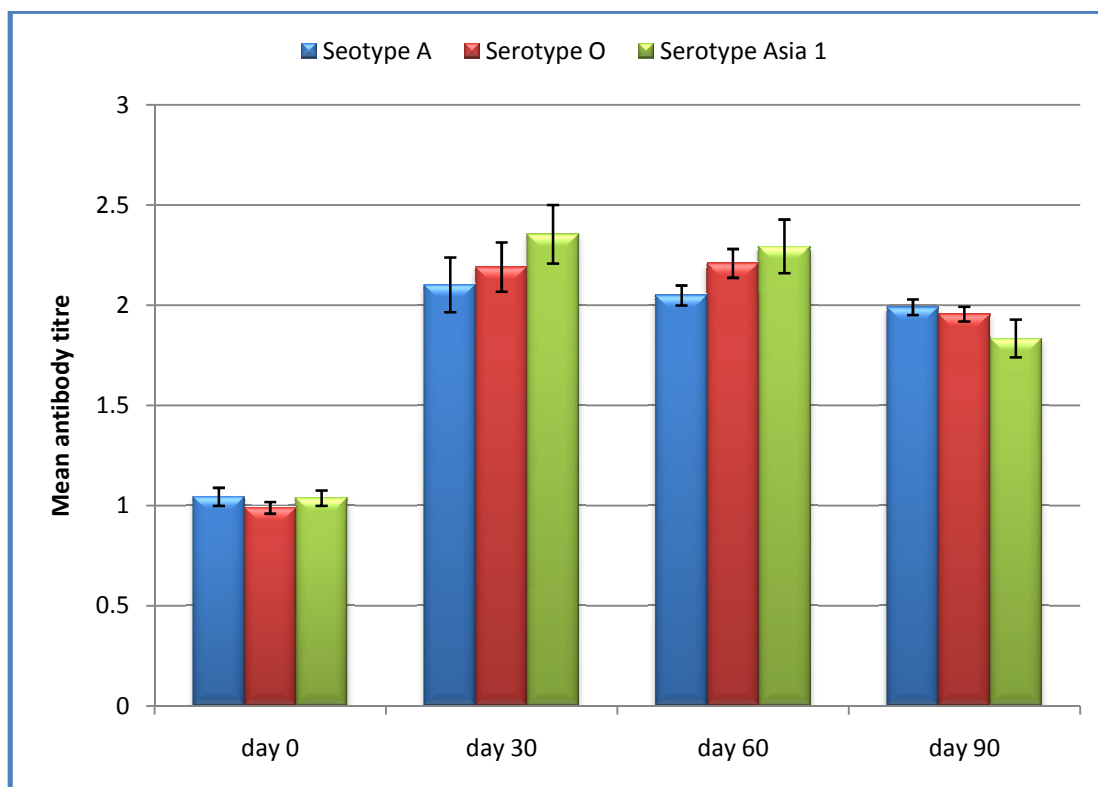


Figure 3 a: Comparison of antibody titre against different serotypes of FMDV in combined vaccine group in goat

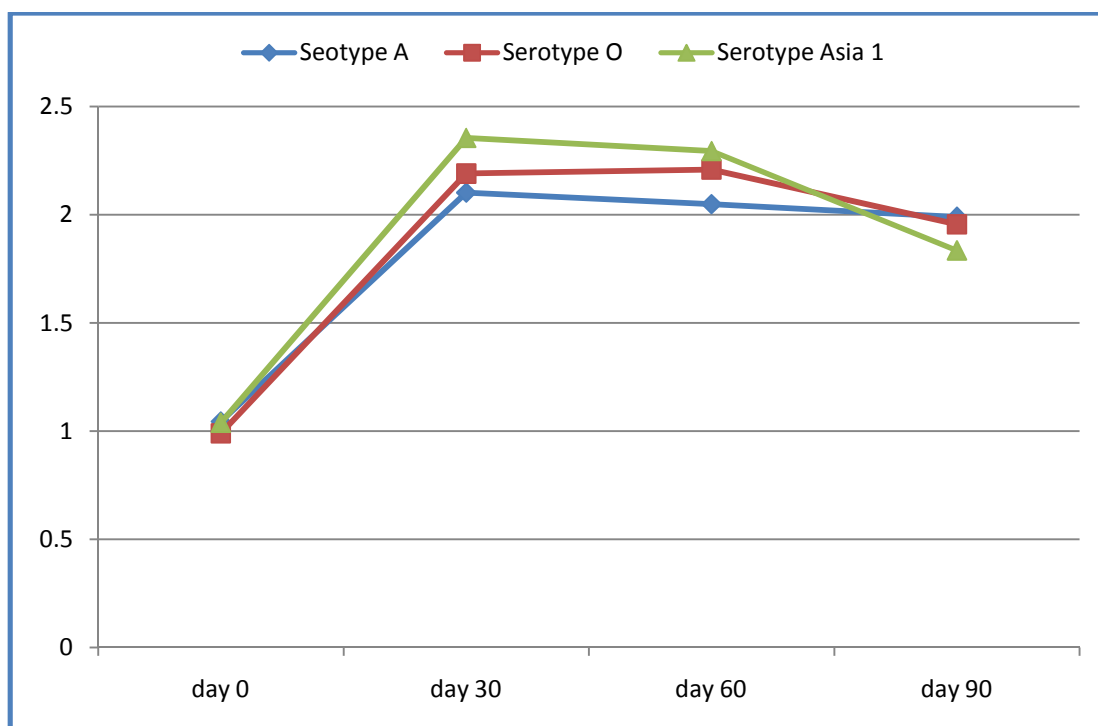


Figure 3 b: Comparison of antibody titre against different serotypes of FMDV in combined vaccine group in goat

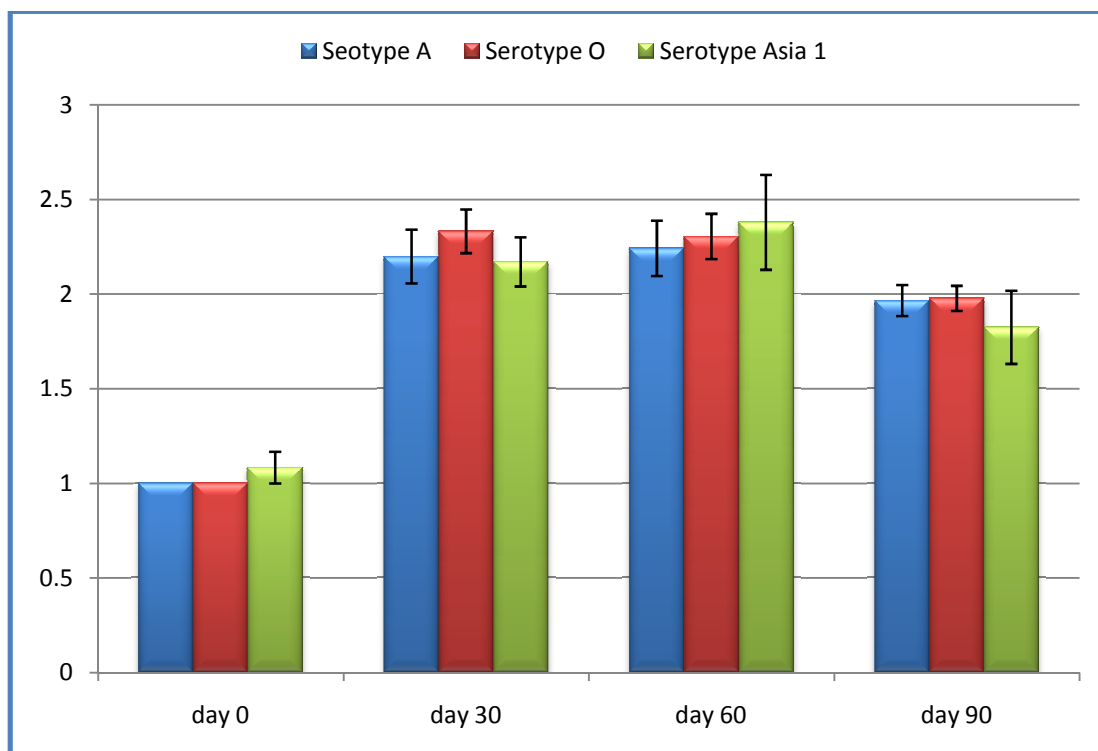


Figure 4 a: Comparison of antibody titre against different serotypes of FMDV in combined vaccine in sheep

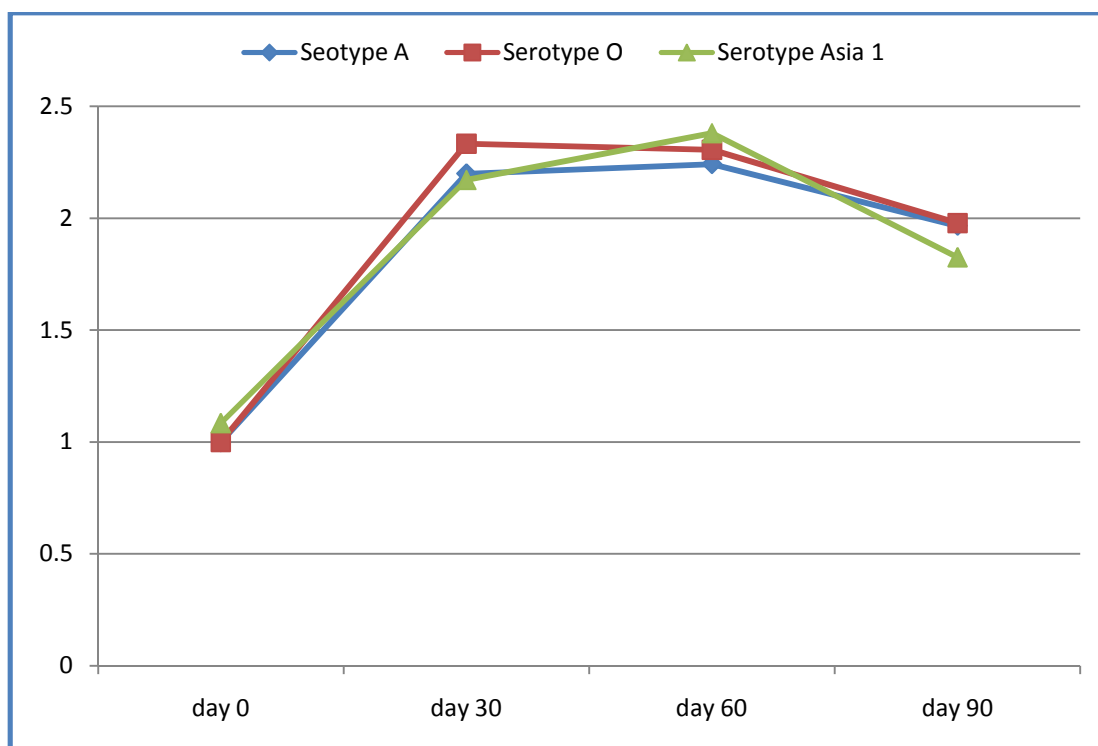


Figure 4 b: Comparison of antibody titre against different serotypes of FMDV in combined vaccine in sheep

4.2.6 Comparison of mean antibody titre against different serotypes of FMDV in monovac vaccine and combined vaccine in goat

On day zero, there was no significant difference in mean antibody titre against all the serotypes (O, A and Asia 1) of FMDV in monovac, combined triovac and control group ($p < 0.05$) as seen in table 3. However, on day 30, 60 and 90 a significant rise in antibody titre was noticed in both monovac and combined groups in comparison to control group ($p < 0.05$). There was no significant difference between mean antibody titre against all serotypes of FMDV (O, A and Asia 1) in monovac and combined vaccine at day 30, 60 and 90 (Table 4).

While comparing the seroconversion pattern of combined triovac and monovac vaccine in goats (Figure 5-7), it was found that both the vaccines showed maximum immune response at day 30 and it was nearly constant up to day 60 and after that there was gradual decrease in antibody titre for all these serotypes under the study.

4.2.7 Comparison of mean antibody titre against different serotypes of FMDV in monovac vaccine to combined vaccine in sheep

The seroconversion pattern in sheep was found to be in unison to the pattern of the goat. There was no significant difference between the mean antibody titre against all the serotypes (O, A and Asia 1) of FMDV in combined vaccine, monovac vaccine and control groups ($p < 0.05$) in sheep on day zero (Table 5).

However, on day 30, 60 and 90 there was significant rise in mean antibody titre between the vaccinated group and the control group ($p < 0.05$) for all the three serotypes. No significant difference between the mean antibody titre against all the three serotypes (O, A and Asia 1) of FMDV was observed in combined and monovac vaccine group ($p < 0.05$) in sheep on day 30, 60 and 90.

In comparison to goat, mean antibody titre increased rapidly from day 0 to 30 for both the group. A slight increase in mean antibody titre was noticed at day 60 for serotype A while mean antibody titre for serotype O and Asia 1 showed peak response at day 60 (Figure 8-10).

4.2.8 Comparison of mean antibody titre against different serotypes of FMD of monovac vaccine in sheep and goat

Comparison of mean antibody titre of monovac vaccine between sheep and goat revealed no significant difference against all the three serotypes ($p < 0.05$) of FMDV (Table 6; Figure 11- 13).

4.2.9 Comparison of mean antibody titre against different serotypes of FMDV in combined vaccine in sheep and goat

At day 0 (pre vaccination) there was no significant difference between mean antibody titre against O, A and Asia 1 serotypes of FMDV in monovac vaccine group, combined vaccine group and control groups in sheep and goat. At day 30, 60 and 90 the mean antibody titre in monovac vaccine groups of sheep and goat was significantly higher from control groups. No significant difference between the antibody titre of monovac (FMD only) vaccine was observed in sheep and goat ($p < 0.05$) for serotype O, A and Asia-1 (table 7: figure 14-16).

Table 6: Comparison of mean antibody titre against different serotypes A, O and Asia 1 of FMDV of monovac vaccine in sheep and goat

Serotypes	Groups	Antibody titre			
		Day 0	Day 30	Day 60	Day 90
A	Combined vaccine in sheep	1 ^a	1.8882 ^b	2.0136 ^b	1.8078 ^b
	Combined vaccine in goat	1.0733 ^a	2.1508 ^b	2.114 ^b	1.9229 ^b
O	Combined vaccine in sheep	0.9809 ^a	2.115 ^b	2.0633 ^b	1.7914 ^b
	Combined vaccine in goat	1.1348 ^a	2.1544 ^b	2.1122 ^b	1.889 ^b
Asia 1	Combined vaccine in sheep	1 ^a	1.9546 ^b	1.9098 ^b	1.706 ^b
	Combined vaccine in goat	0.9726 ^a	2.3306 ^b	2.2886 ^b	1.8459 ^b

Table 7: Comparison of mean antibody titre against different serotypes A, O and Asia 1 of FMDV of combined vaccine in sheep and goat

Serotypes	Groups	Antibody titre			
		Day 0	Day 30	Day 60	Day 90
A	Monovac vaccine in sheep	1 ^a	2.1993 ^b	2.2421 ^b	1.9664 ^b
	Monovac vaccine in goat	1.0448 ^a	2.1026 ^b	2.0494 ^b	1.9908 ^b
O	Monovac vaccine in sheep	1 ^a	2.3327 ^b	2.3054 ^b	1.9664 ^b
	Monovac vaccine in goat	0.9896 ^a	2.1912 ^b	2.2093 ^b	1.9908 ^b
Asia 1	Monovac vaccine in sheep	1.0838 ^a	2.171 ^b	2.3799 ^b	1.8253 ^b
	Monovac vaccine in goat	1.038 ^a	2.3551 ^b	2.2946 ^b	1.8348 ^b

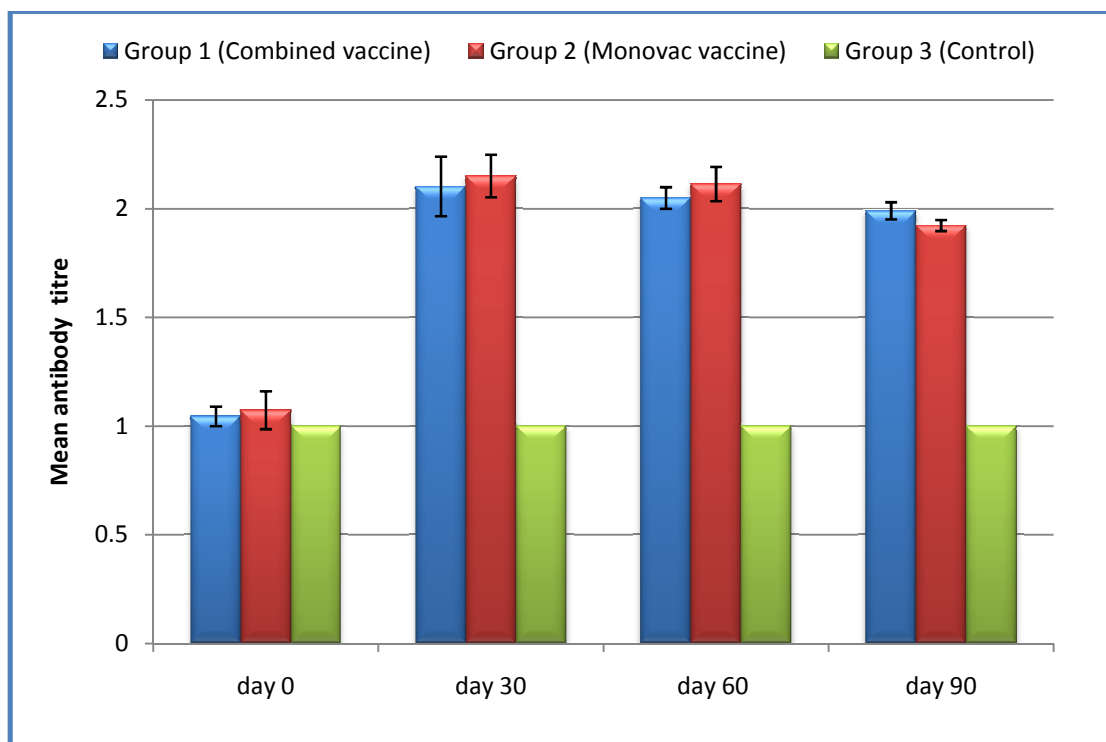


Figure 5 a: Mean antibody titre against serotype A of FMD in, group 1 (combined vaccine), Group 2 (monovac vaccine) vaccine and group 3 (control) in goat

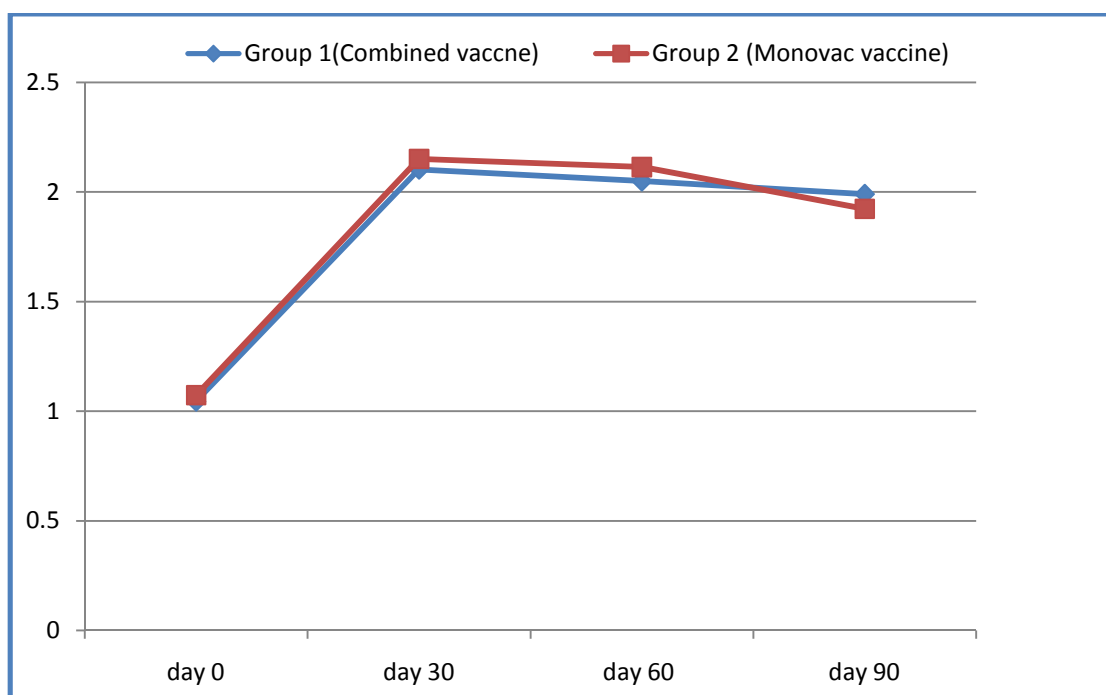


Figure 5 b: Comparison of mean antibody titre against serotype A of FMDV in group 1 (combined vaccine) and group 2 (monovac vaccine) groups in goat

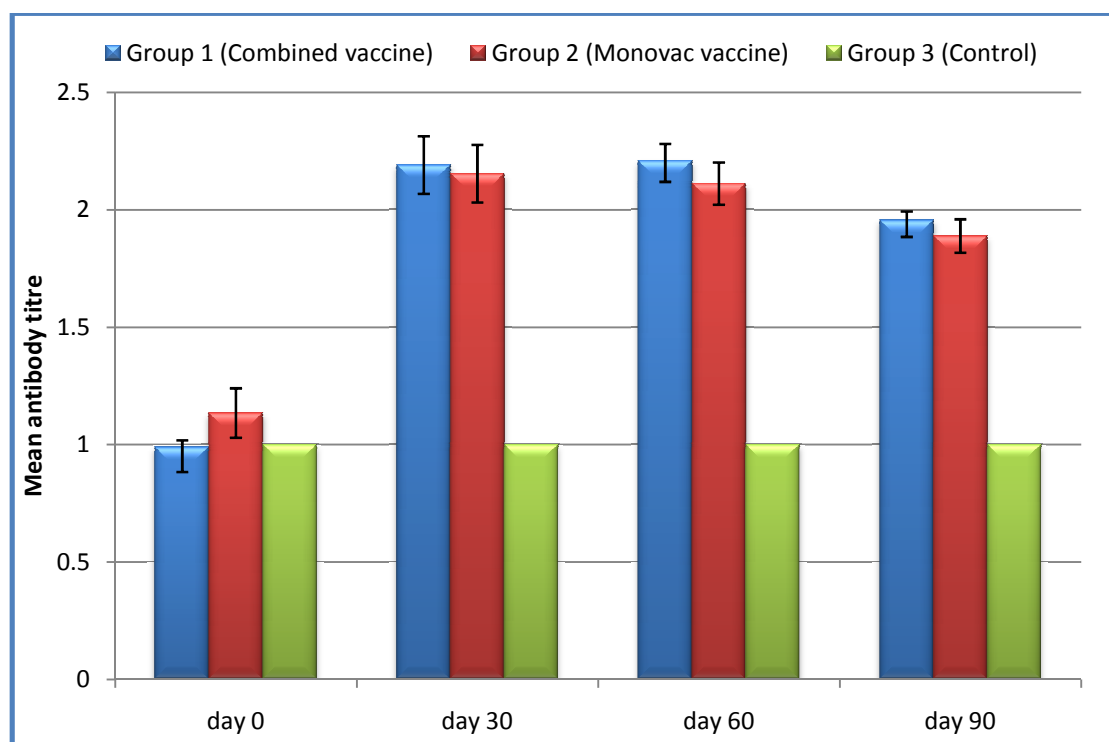


Figure 6 a: Mean antibody titre against serotype O of FMD in, group 1 (combined vaccine), Group 2 (monovac vaccine) vaccine and group 3 (control) in goat

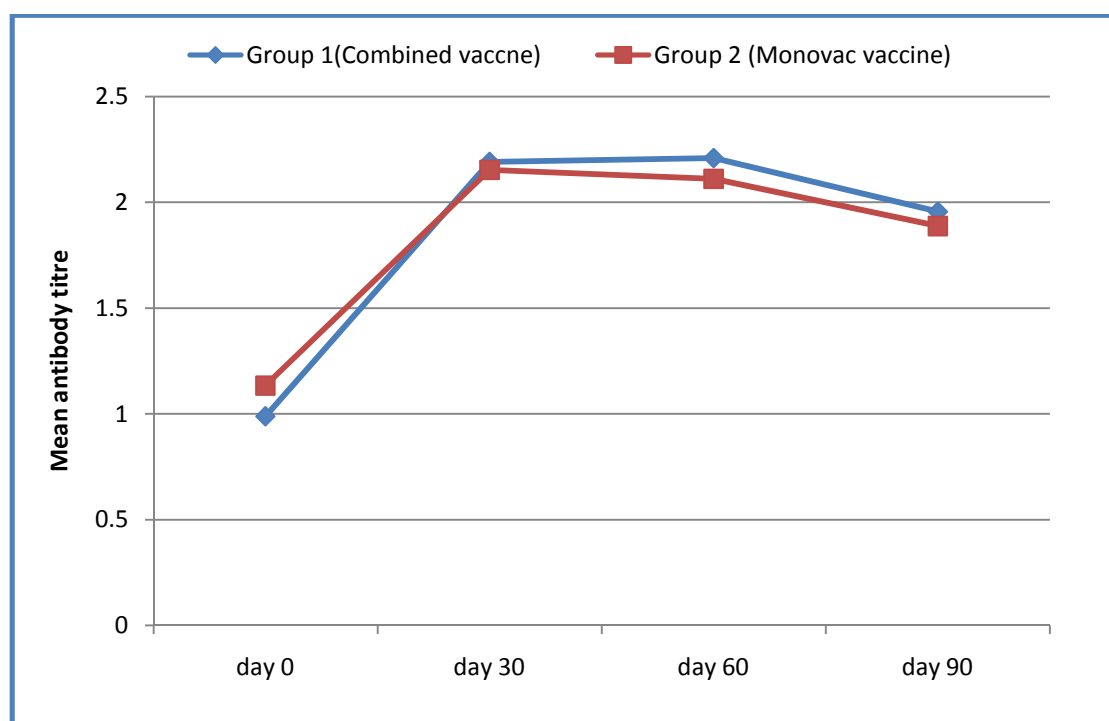


Figure 6 b: Comparison of mean antibody titre against serotype O of FMDV in group 1 (combined vaccine) and group 2 (monovac vaccine) groups in goat

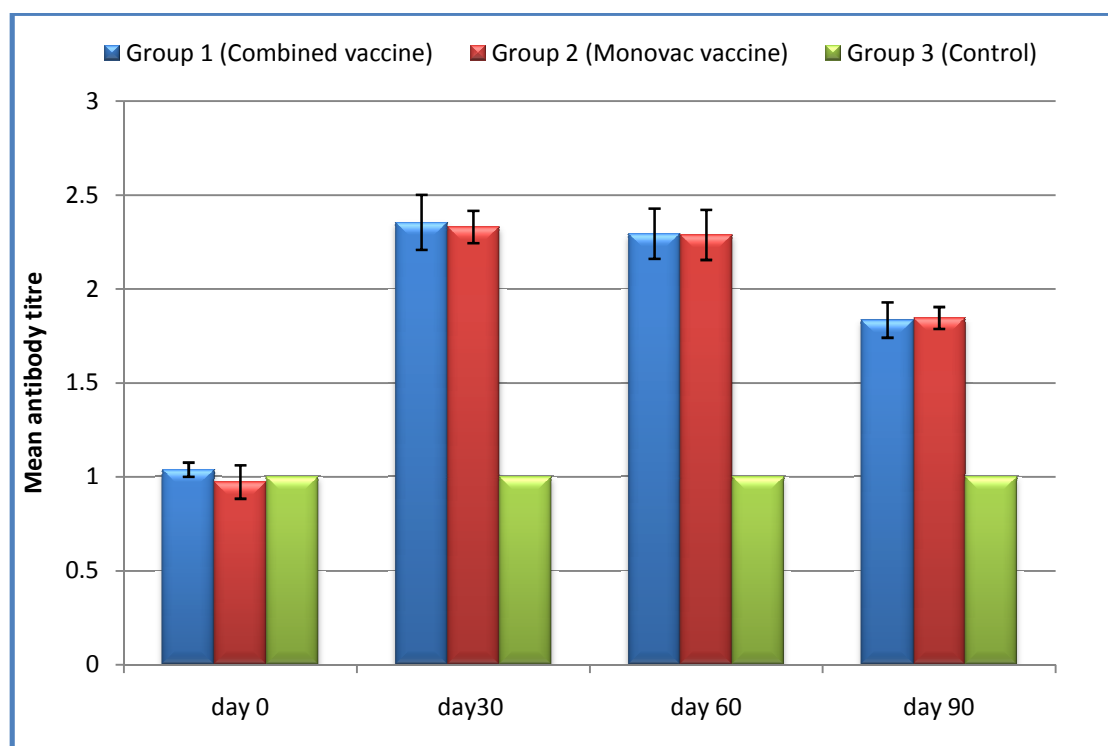


Figure 7 a: Mean antibody titre against serotype Asia 1 of FMD in, group 1 (combined vaccine), Group 2 (monovac vaccine) vaccine and group 3 (control) in goat

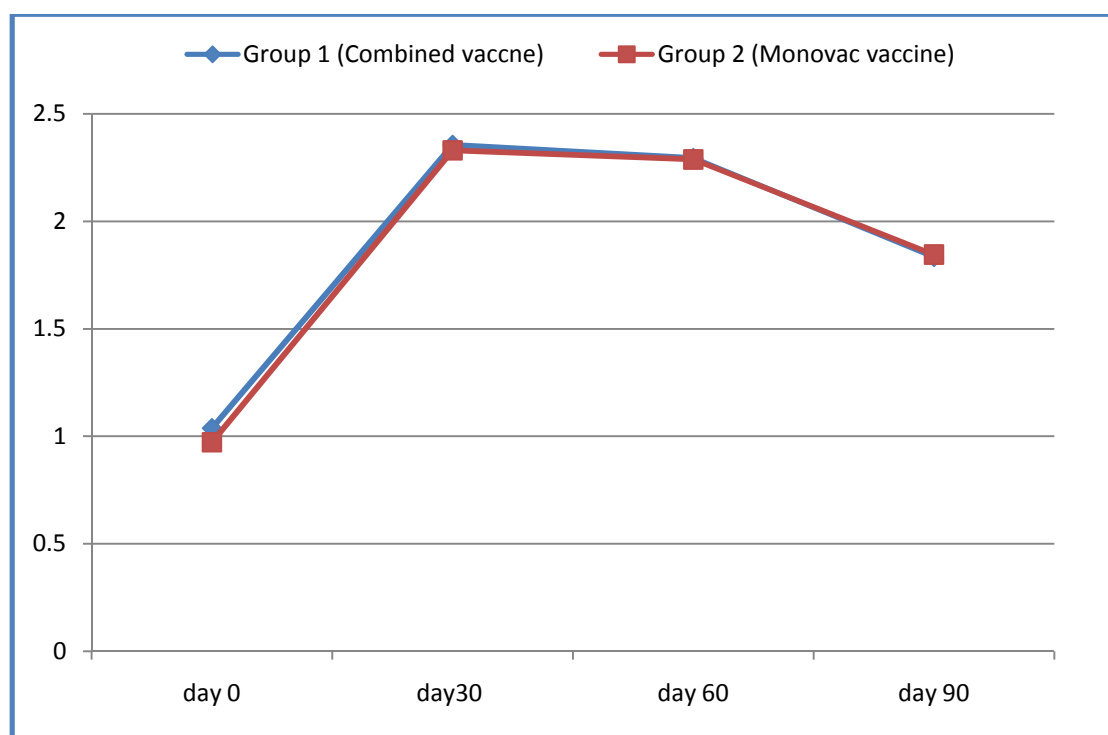


Figure 7 b: Comparison of mean antibody titre against serotype Asia 1 of FMDV in group 1 (combined vaccine) and group 2 (monovac vaccine) groups in goat

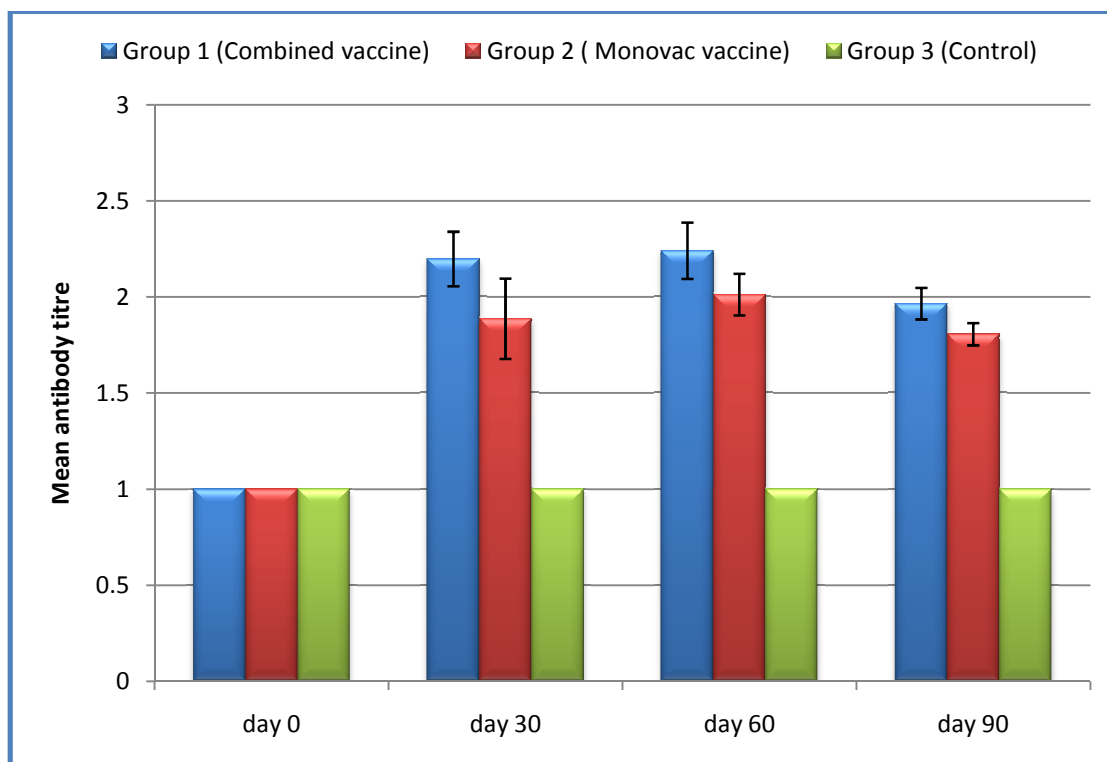


Figure 8 a: Mean antibody titre against serotype A of FMD in, group 1 (combined vaccine), Group 2 (monovac vaccine) vaccine and group 3 (control) in sheep

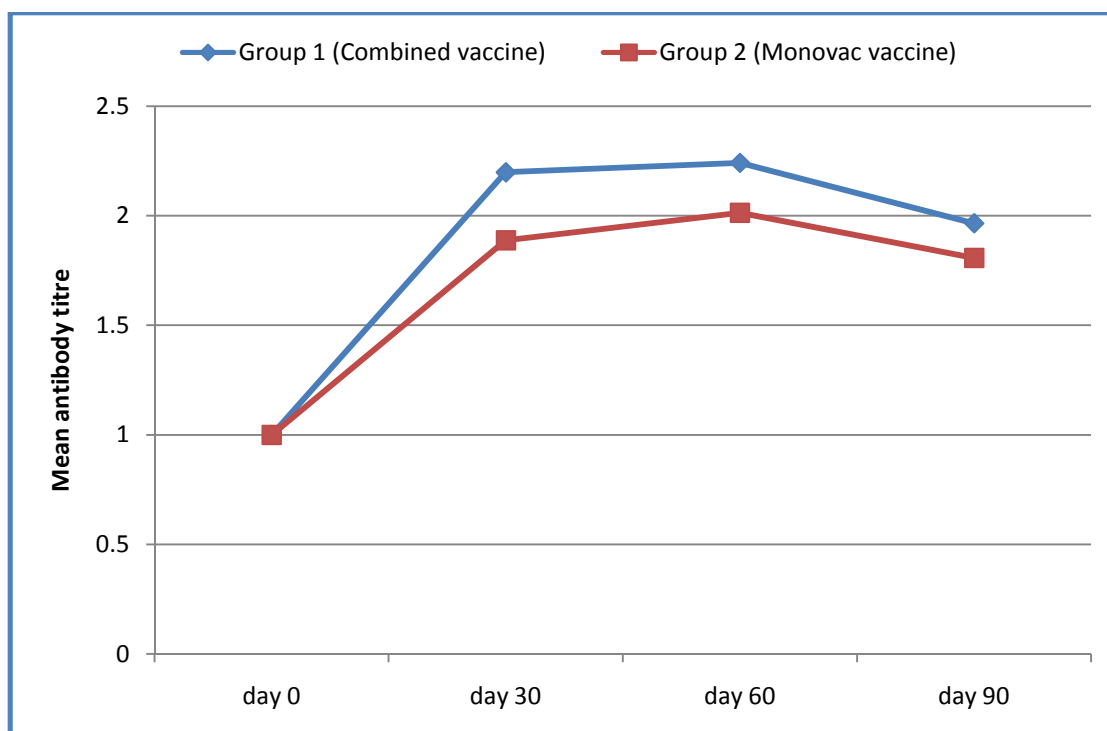


Figure 8 b: Comparison of mean antibody titre against serotype A of FMDV in group 1 (combined vaccine) and group 2 (monovac vaccine) groups in sheep

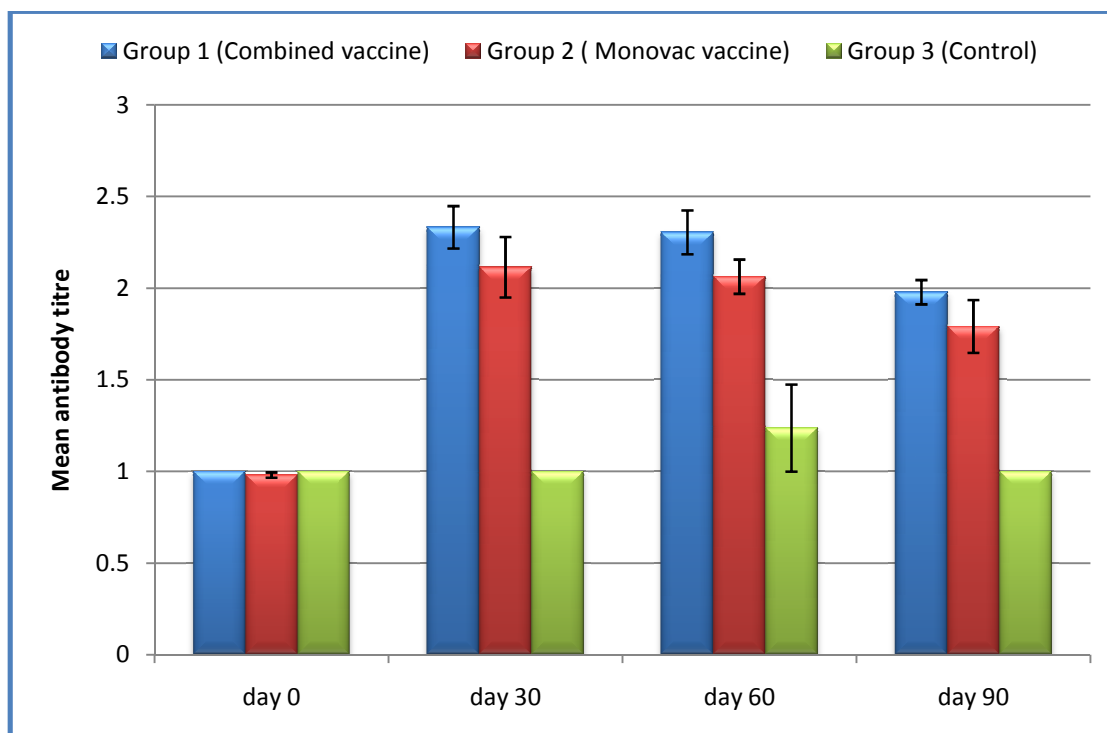


Figure 9 a: Mean antibody titre against serotype O of FMD in, group 1 (combined vaccine), Group 2 (monovac vaccine) vaccine and group 3 (control)

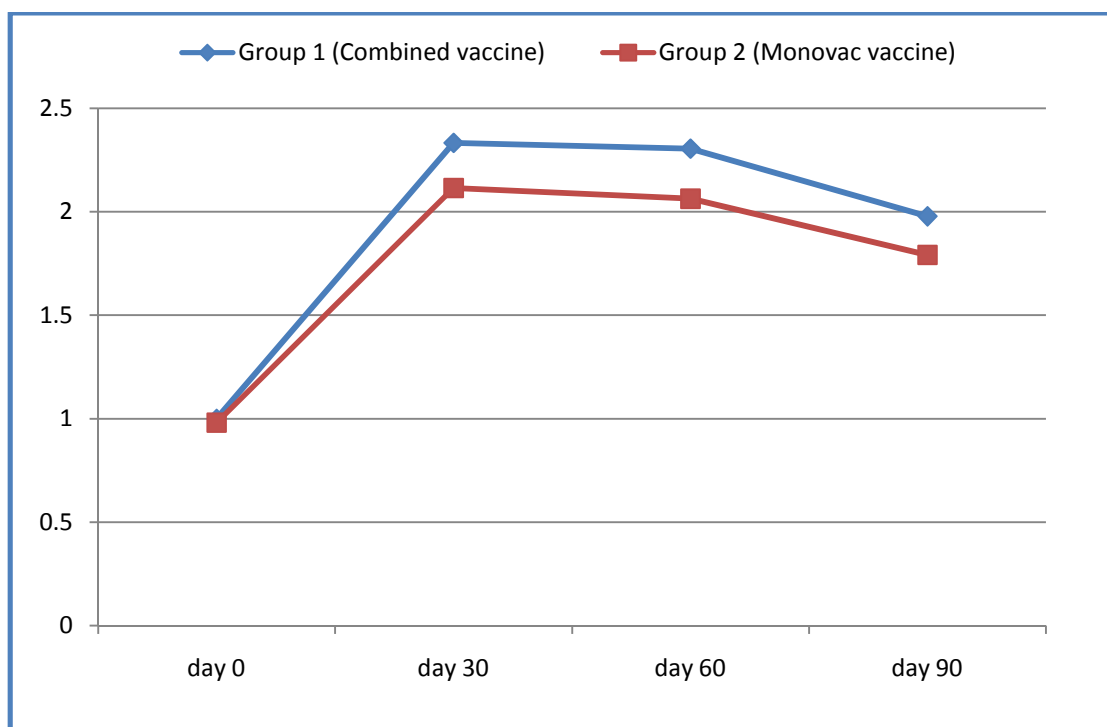


Figure 9 b: Comparison of mean antibody titre against serotype O of FMDV in group 1 (combined vaccine) and group 2 (monovac vaccine) groups in sheep

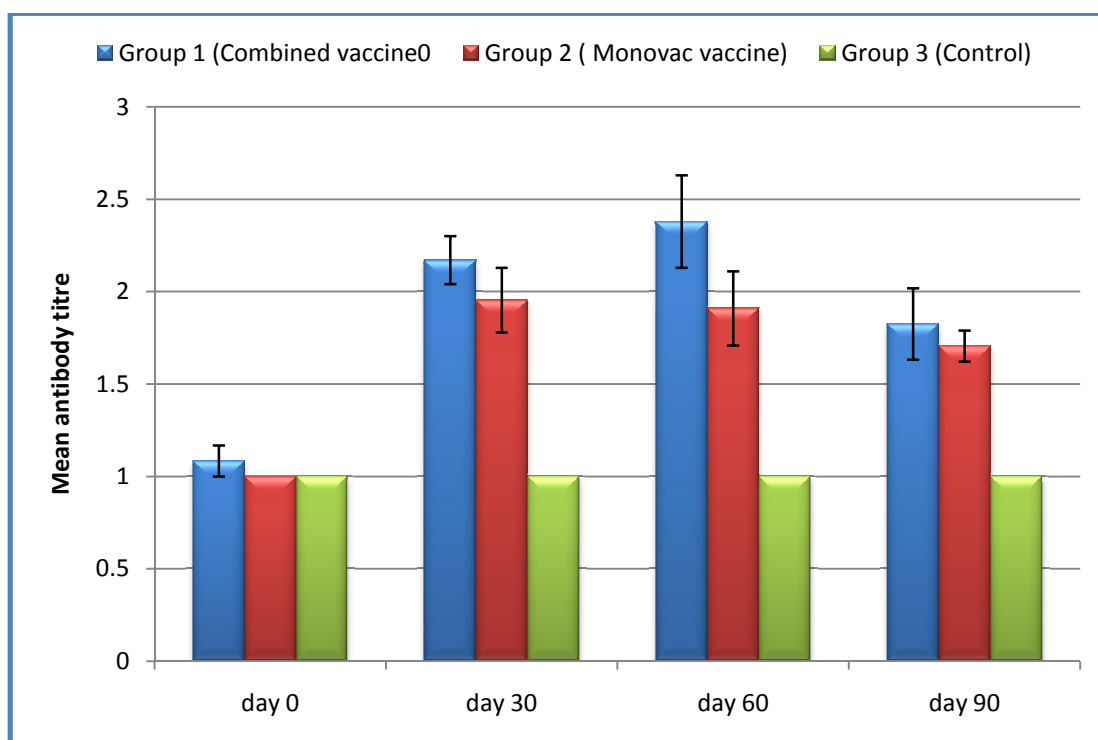


Figure 10 a: Mean antibody titre against serotype Asia 1 of FMD in, group 1 (combined vaccine), Group 2 (monovac vaccine) vaccine and group 3 (control) in sheep

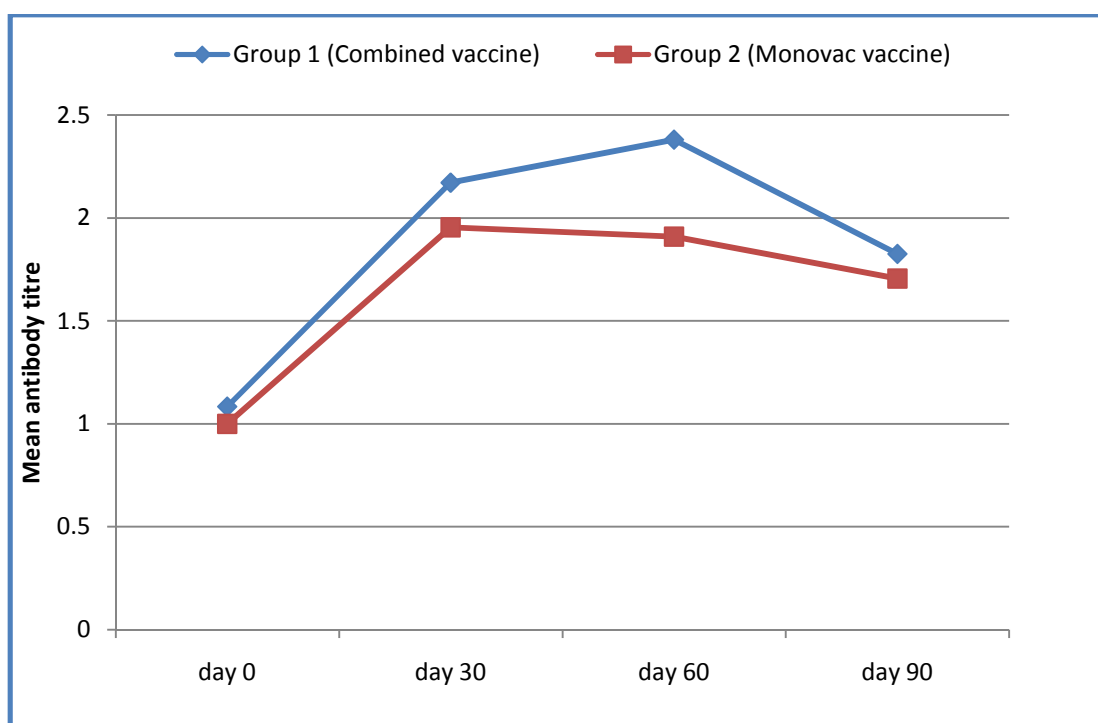


Figure 10 b: Comparison of mean antibody titre against serotype Asia 1 of FMDV in group 1 (combined vaccine) and group 2 (monovac vaccine) groups in sheep

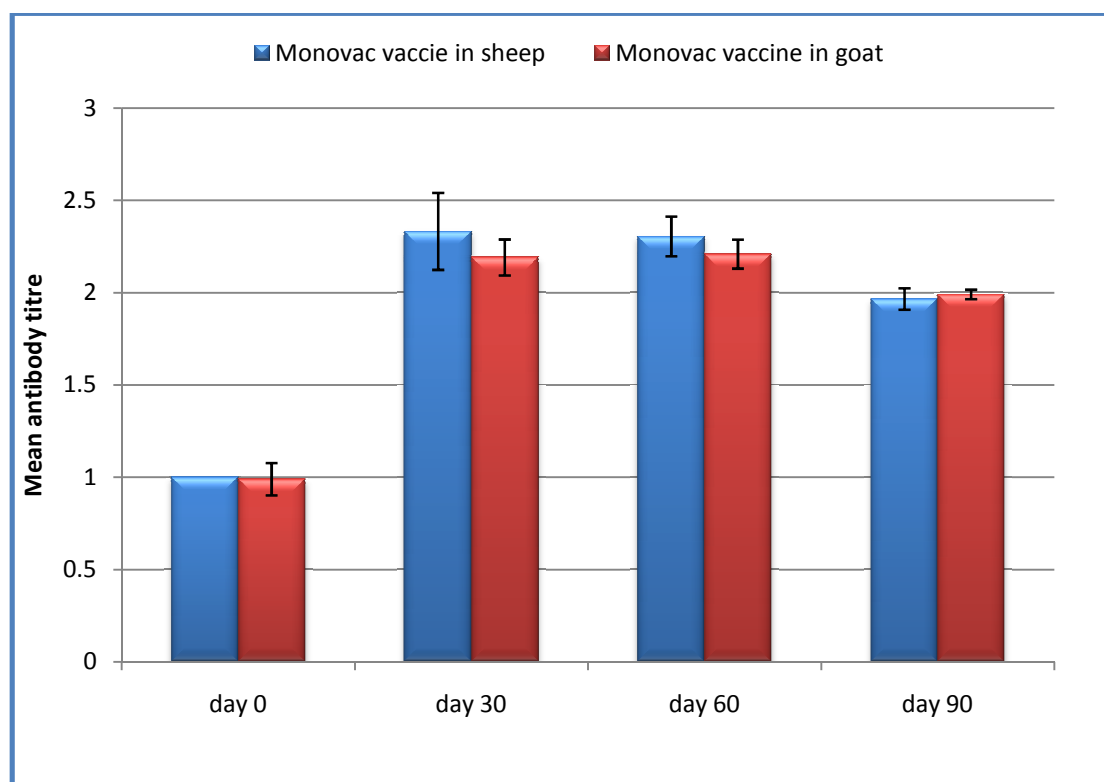


Figure 11 a: Comparison of mean antibody titre against serotype A of FMDV in monovac vaccine in sheep and goat

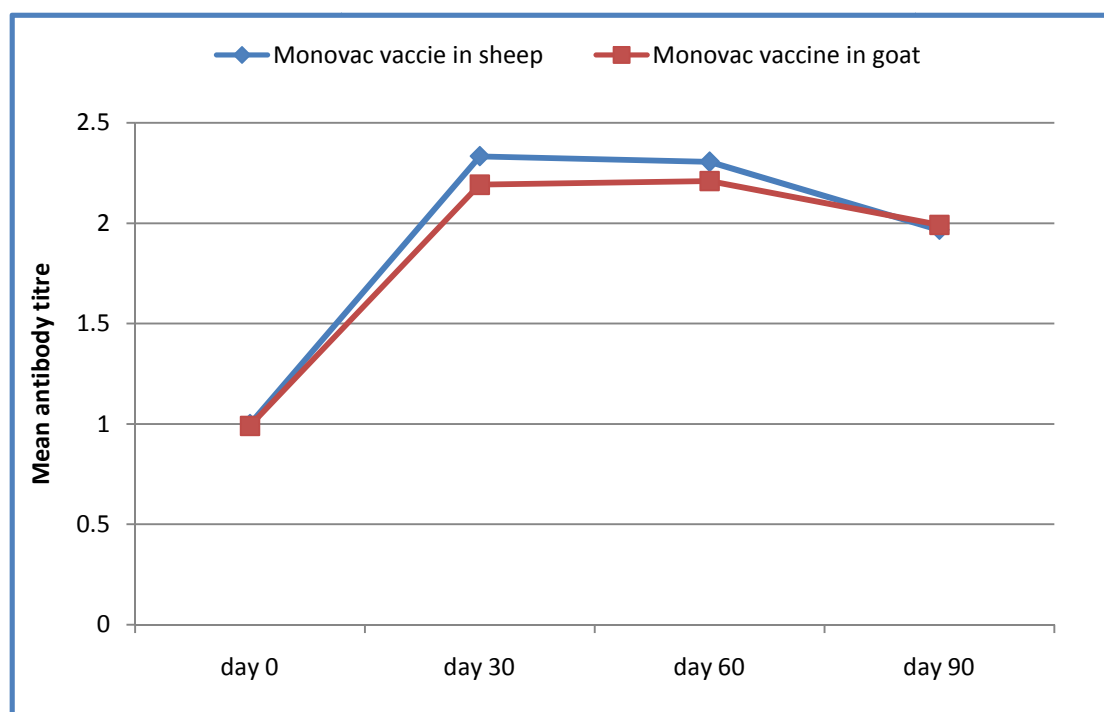


Figure 11 b: Comparison of mean antibody titre against serotype A of FMDV in monovac vaccine in sheep and goat

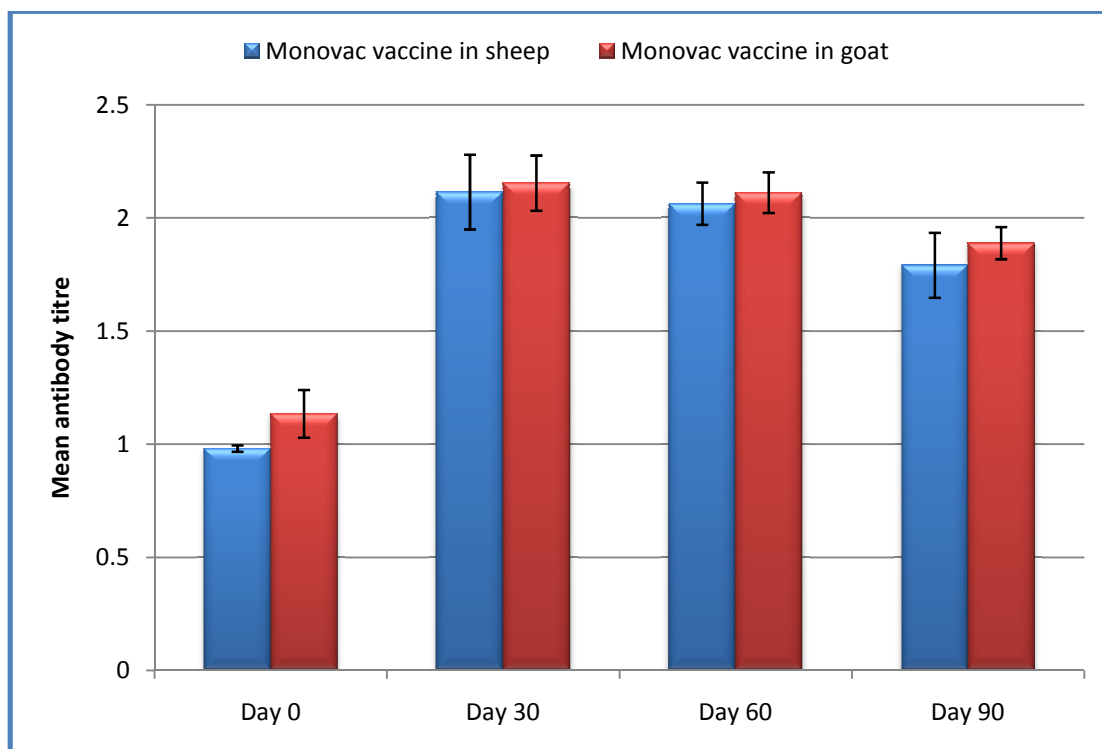


Figure 12 a: Comparison of mean antibody titre against serotype O of FMDV in monovac vaccine in sheep and goat

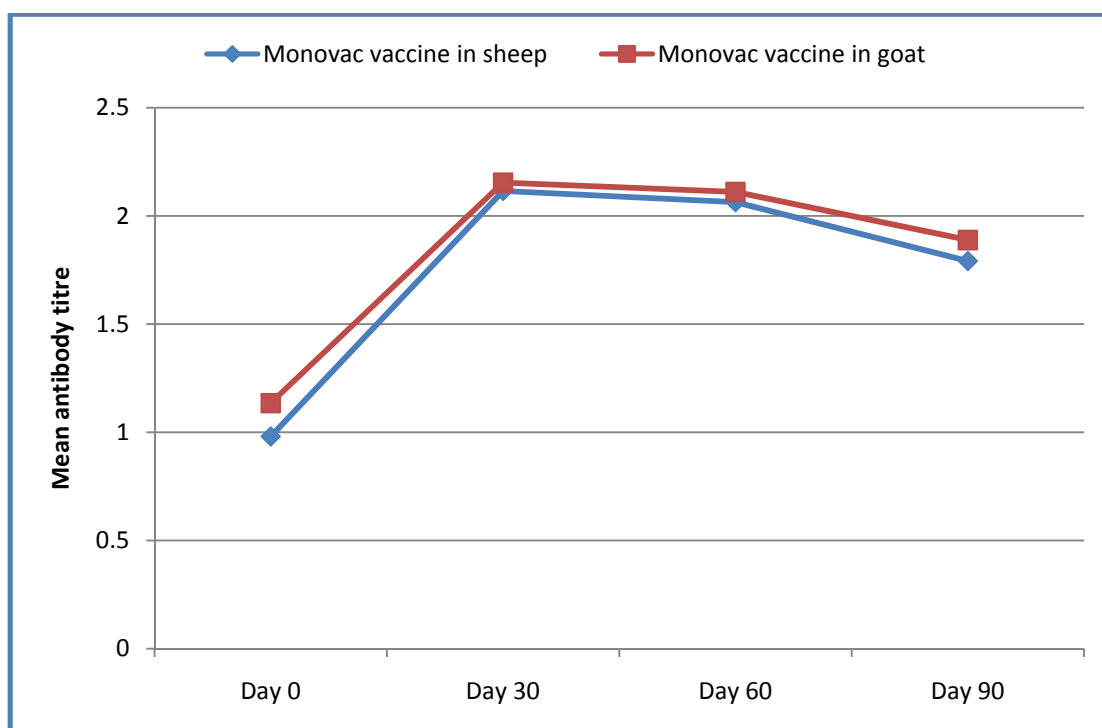


Figure 12 b: Comparison of mean antibody titre against serotype O of FMDV in monovac vaccine in sheep and goat

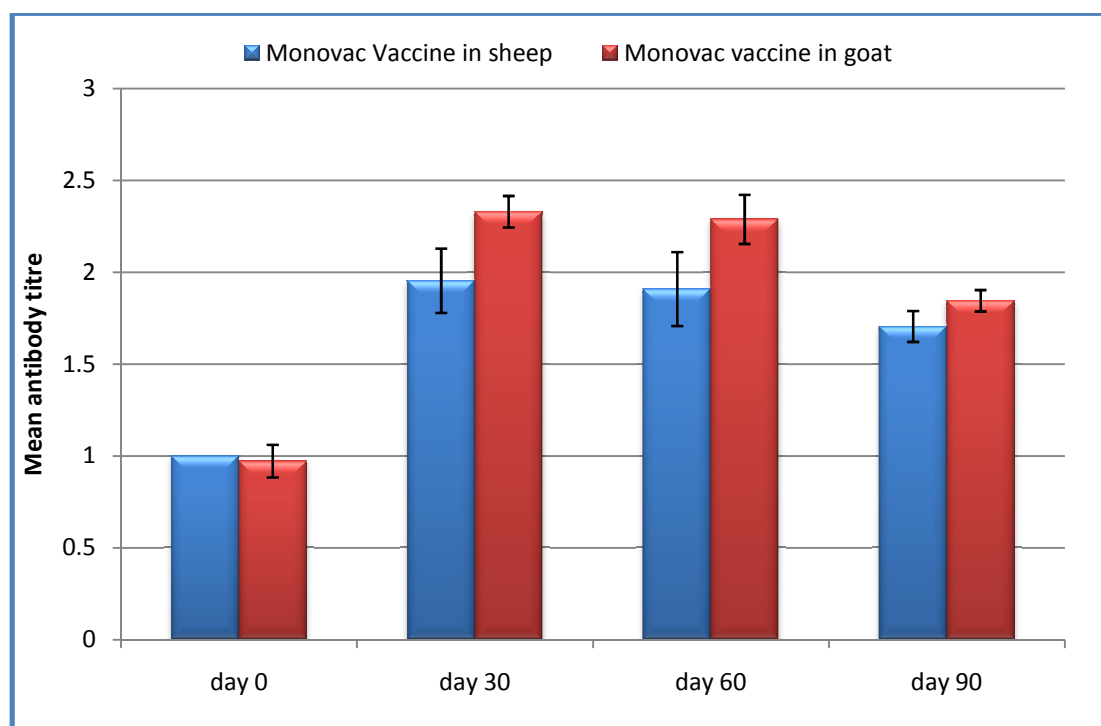


Figure 13 a: Comparison of mean antibody titre against serotype Asia 1 of FMDV in monovac vaccine in sheep and goat

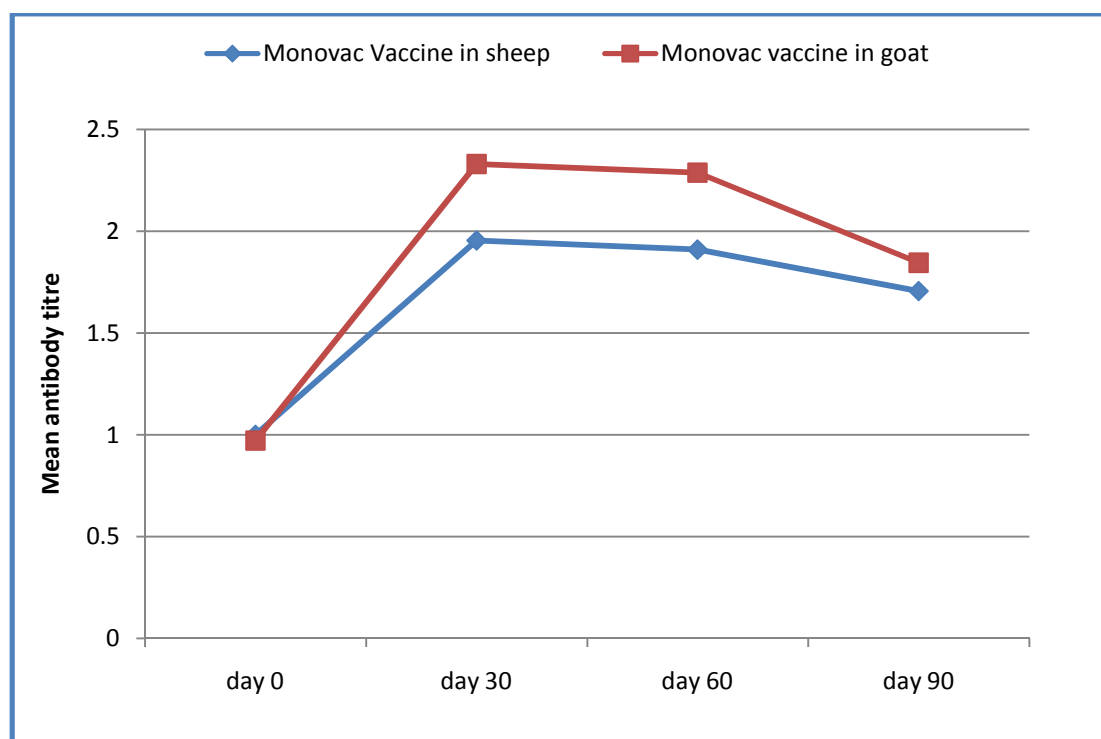


Figure 13 b: Comparison of mean antibody titre against serotype Asia 1 of FMDV in monovac vaccine in sheep and goat

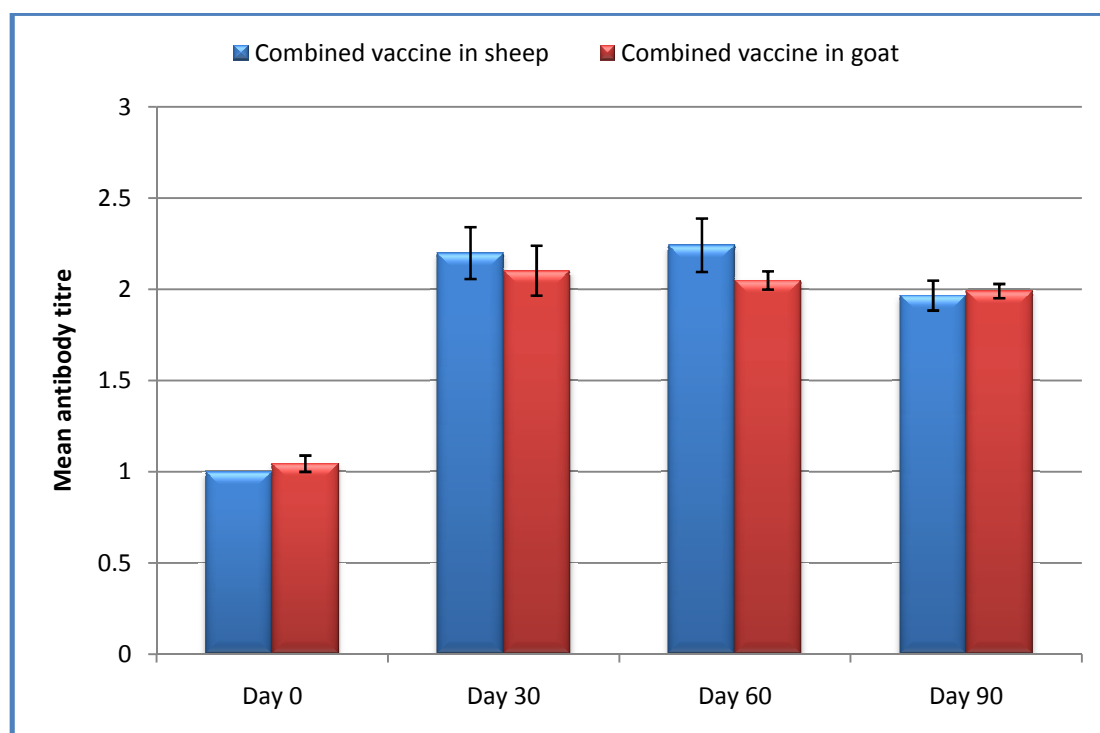


Figure 14 a: Comparison of mean antibody titre against serotype A of FMDV in combined vaccine in sheep and goat

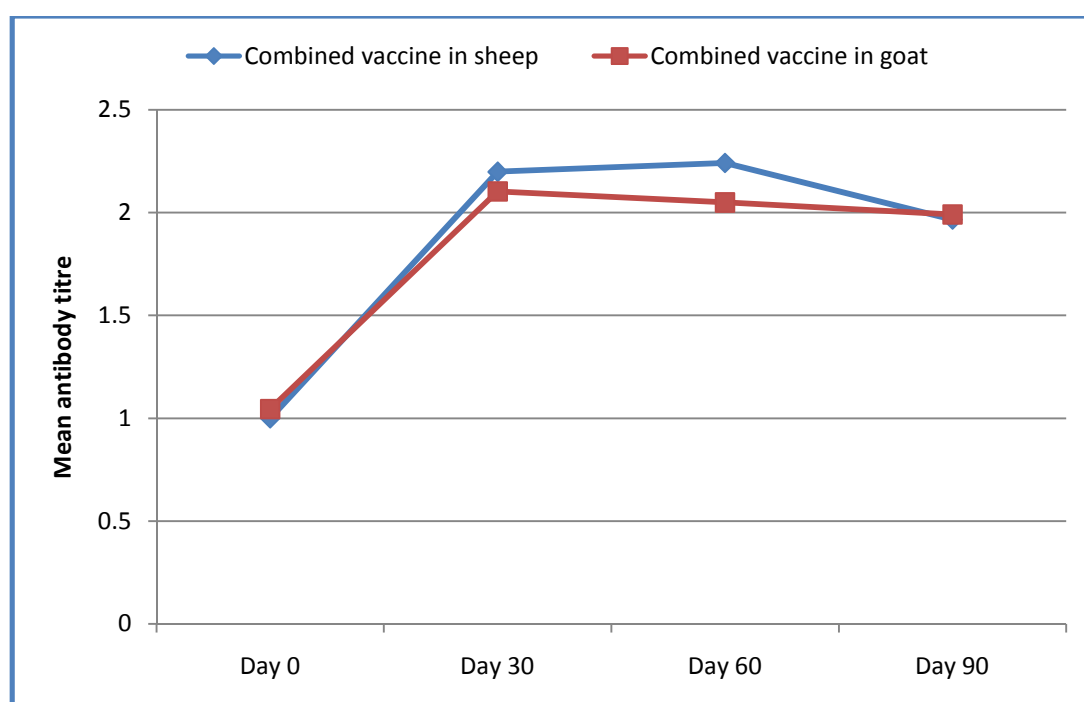


Figure 14 b: Comparison of mean antibody titre against serotype A of FMDV in combined vaccine in sheep and goat

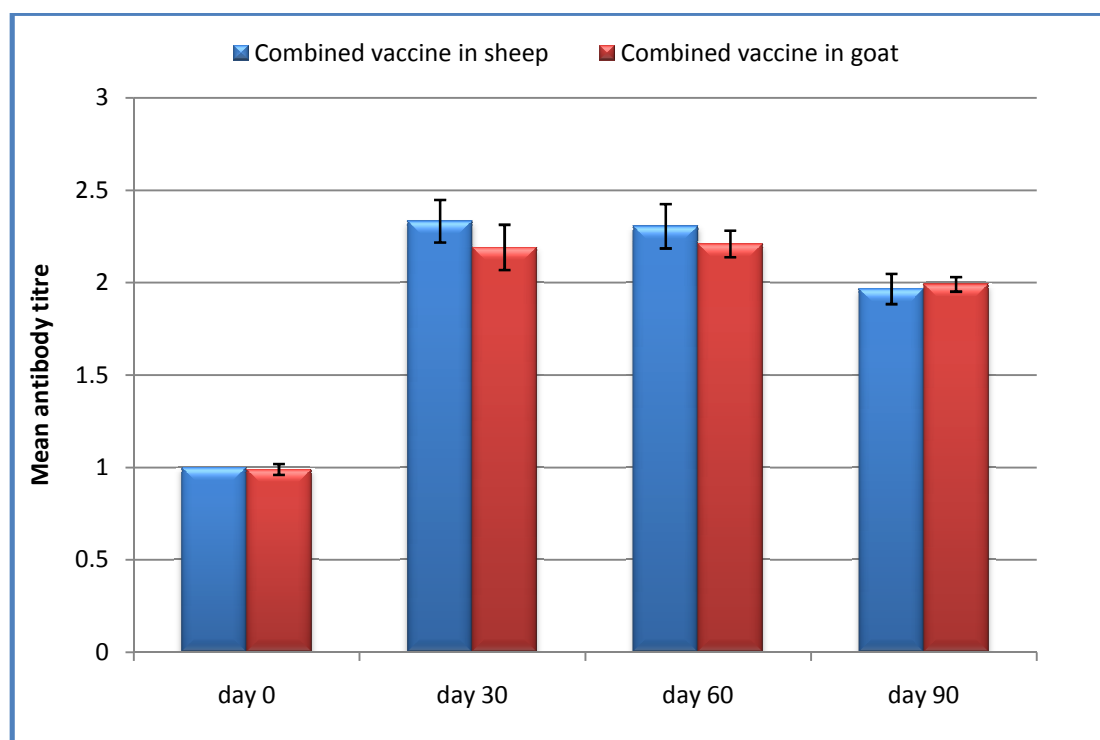


Figure 15 a: Comparison of mean antibody titre against serotype O of FMDV in combined vaccine in sheep and goat

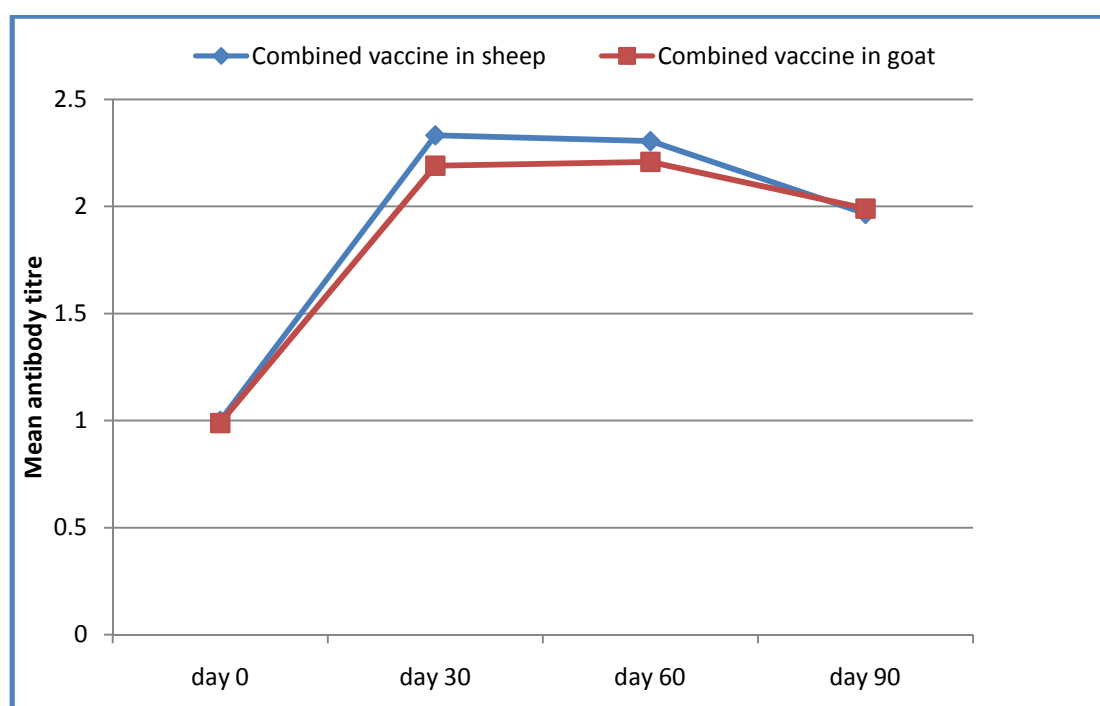


Figure 15 b: Comparison of mean antibody titre against serotype O of FMDV in combined vaccine in sheep and goat

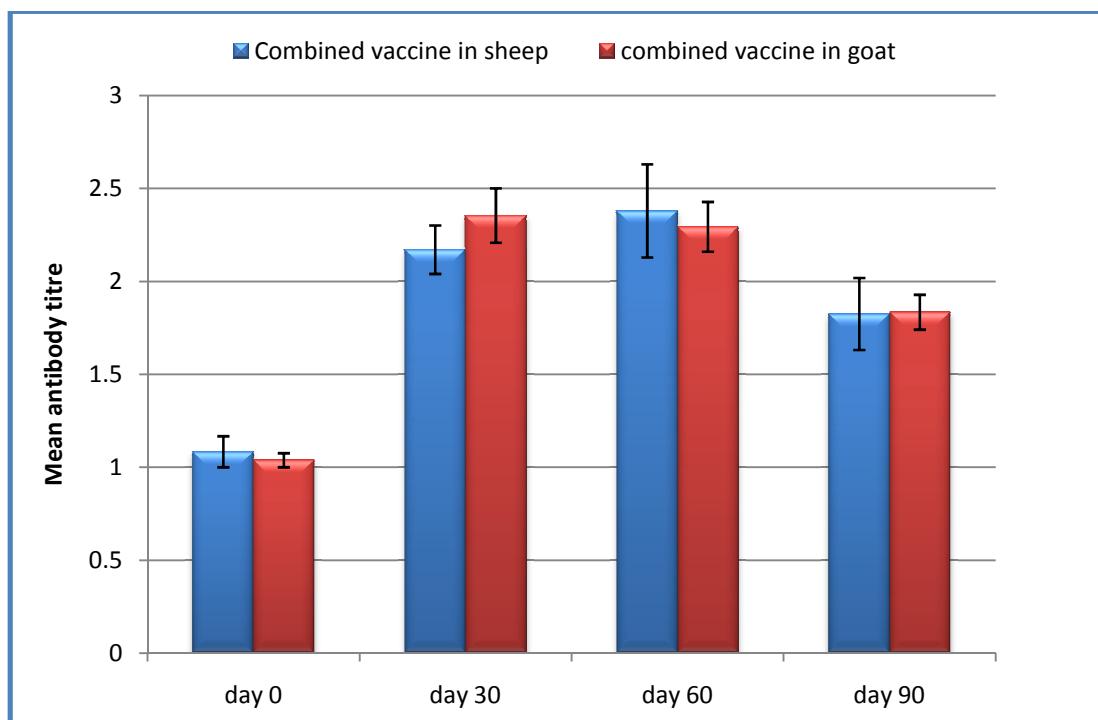


Figure 16 a: Comparison of mean antibody titre against serotype Asia 1 of FMDV in combined vaccine in sheep and goat

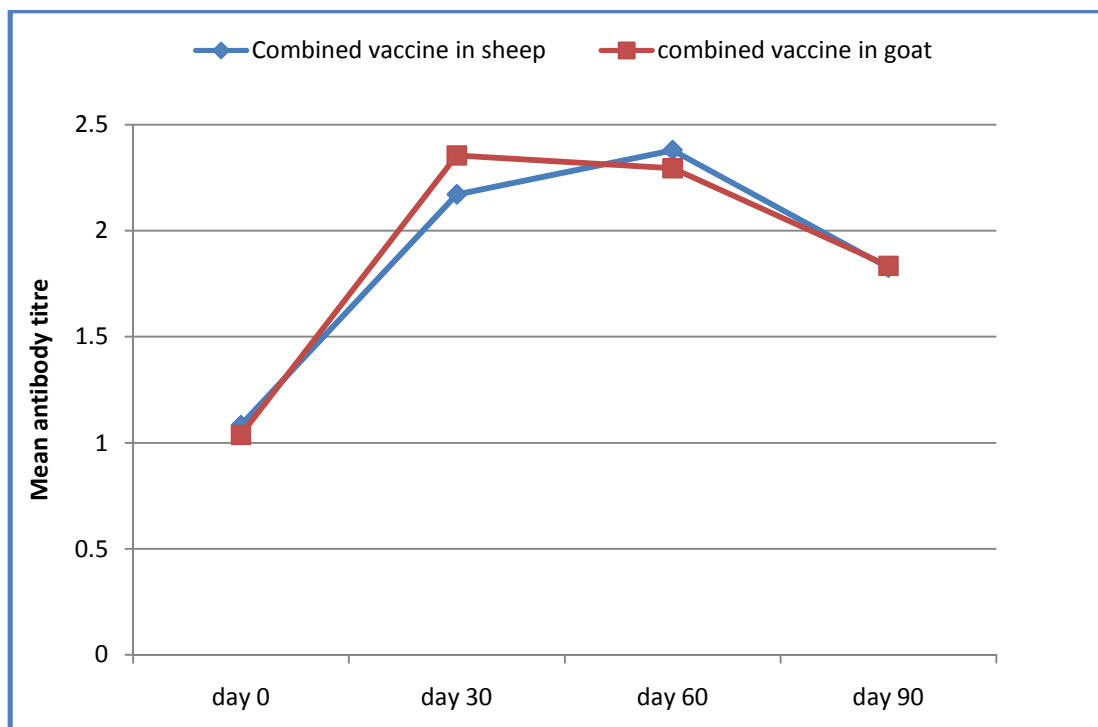


Figure 16 b: Comparison of mean antibody titre against serotype Asia 1 of FMDV in combined vaccine in sheep and goat

Table 2: Antibody titre against different serotypes (O, A and Asia1) of FMDV in different vaccine groups in goat

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CHAPTER-V

DISCUSSION

FMD is a highly infectious disease and affects all the cloven footed animals including cattle, sheep, goat and pig (Parida, 2009). There are seven prevailing serotypes of FMD worldwide. In India, FMD outbreaks caused by serotypes O, A, C and Asia-1 has been reported. Among them, FMD caused by serotype C has not been reported for the past 20 years (Depa *et al.*, 2012). FMD is generally subclinical in sheep and goat population, but is considered important in spreading the disease to other susceptible animals like cattle and buffaloes (Patil *et al.*, 2002). Vaccination is considered as the most appropriate preventive measure of FMD. In India, the FMD vaccination for cattle and buffalo is biannual while vaccination coverage to small ruminants is very poor and is not covered under FMD vaccination programmes. Furthermore, integrated system of farming involving sheep, goat and cattle leads to many episodes of FMD disease outbreak in other susceptible cloven footed animals (Uppal *et al.*, 1972).

While vaccination against FMD, the multivalent vaccine of FMD is used which contains the prevailing serotypes (O, A, and Asia 1). The FMD vaccine is either alum adjuvant based and/or oil adjuvant based. The immune response obtained due to oil adjuvant vaccine is better than the alum precipitated vaccine and the duration of immunity is more than that of alum adjuvant based FMD vaccine (Selim *et al.*, 2010).

In developing country like India, to make the vaccination programme more cost effective in terms of money and manpower, it will be better if two or more vaccines can be given as combined vaccine. A number of works carried out by different researchers suggest that FMD vaccine can be combined with other vaccine(s) or can be given simultaneously with other vaccine(s) without causing any difference in immune response of individual antigenic components (Reddy *et al.*, 1997; Chabra *et al.*, 2004).

The serological response of cattle to combined vaccine containing FMD, HS and BQ antigens in comparison to the individual vaccine alone was found to be similar (Reddy *et al.*, 1997).

Role of humoral immunity is important in prevention of FMD. Antibodies to structural proteins formed after vaccination against FMD vaccine are measured by LPB-ELISA. The samples showing log₁₀ titre of ≥ 1.8 against all three component serotypes such as O, A and Asia 1 in the vaccine are considered as having sufficient protective antibody.

Antibodies formed to NSP can be detected by DIVA test using 3AB3 NSP to identify the infected animals as in general the FMD vaccine in use is free from the NSP.

There are various NSPs which have been used in differentiation of vaccinated to infected animals like 2B, 2C, 3A, 3AB, 3B, 3ABC and 3D. A reasonable consensus has emerged in favour of the polyprotein 3ABC or its derivatives such as 3AB as the most appropriate antigens for use in the screening tests (De Diego *et al.*, 1997; Mackay *et al.*, 1998; Sorensen *et al.*, 1998., Mohapatra *et al.*, 2011). The diagnostic sensitivity of the assay is estimated to be 96%, while the diagnostic specificity varies between the naïve and vaccinates as 99.1% and 96.4%, respectively.

In present study, seropositivity to 3AB3 NSP was evaluated by DIVA ELSA. The serum samples of sheep and goat collected on day zero were analyzed by r3AB3 I-ELISA to rule out the infected animals in the herd. Among all sheep, two sheep were found DIVA positive. All the goats were found DIVA negative.

DIVA positive animals were 12.5 % in case of sheep and in goat it was 0% in the present study. It was 4.65% in total sheep and goat taken together in the study.

In India, 35.20% DIVA positive animals were found in small ruminants from an organized sheep farm whereas, it was only 0.01% in Andaman and Nicobar Island. A serological investigation involving most of the states of India found 20.35% sheep and 13.60% goats to be positive for 3AB NSP antibodies thus, providing a serological evidence of extensive viral activity (Rout *et al.*, 2014 a and b).

In the present study, the animals which were found NSP positive had shown low SP titre when analyzed by LPB-ELISA. Since, the animals tested were not having the history of previous vaccination, 4.65% DIVA positive status indicates a mild viral activity suggesting its role in silent amplification and dissemination of virus in a mixed farming set up. This again strengthen the view of bringing small ruminants under the ambit of routine vaccination and surveillance programmes as practised for large ruminants under FMD control programme.

LPB- ELISA was used to measure the antibody titre against the different serotypes of FMDV in combined and monovac vaccines. This test is rapid, more sensitive, more specific, relatively simple to perform, economic and could be completed in shorter duration of time as compared to SNT (Hamblin *et al.*, 1986).

Serum antibody titre is an indication of immune response as well as disease status in animal (Hamblin *et al.*, 1987). Since the disease status was already ruled out by performing the 3AB DIVA ELSA test therefore, the serum antibody titre was indicating only the immune status of the animals.

No protective antibody titre ($\geq 1.8 \log_{10}$) was obtained at day zero in monovac as well as combined vaccine group both in sheep and goat. Confirming the history of no vaccination when the mean antibody titre against the different serotypes (A, O and Asia 1) of FMDV were compared at day zero, there was no significant difference between combined vaccine, monovac vaccine and control group ($p < 0.05$) in both sheep and goat.

On 30, 60 and 90 days post vaccination there was significant difference between the mean antibody titre against serotype O, A, Asia 1 of FMDV between control group and the vaccinated groups ($p < 0.05$) in goat. It indicates, that the animals in vaccinated groups have responded against the serotype specific FMDV antigen. It shows that seroconversion has taken place. The similar type of result was seen in sheep.

On day 30, 60 and 90 there was no significant difference between the mean antibody titre against serotype O, A and Asia 1 of FMDV in combined vaccine group and monovac group ($p < 0.05\%$) in goat as well as in sheep.

The results obtained were similar as Reddy *et al.* (1997) and Singh (2008) also could not found any significant difference between the mean antibody titre of FMDV in combined and monovac groups in cattle. They vaccinated one group of cattle with only FMD vaccine and FMD, HS vaccine was given simultaneously to another group. They did not found any significant difference between both the groups. In a similar type of study, Hedger *et al.* (1986) vaccinated two groups of cattle, one with the FMD vaccine alone and another group with the FMD, RP vaccine, simultaneously and observed that response were not different. The result is also supported by the study of the Joseph and Hedger (1984) in another case. They did not find any significant difference between immune response when vaccine was given alone and when it was given simultaneously with H. S. vaccine whereas, in a similar type of study by Chhabra *et al.* (2004), combined vaccine was found better than monovac vaccine in buffalo calves.

In goat, the peak immune response was obtained on day 30 against all three serotypes of FMDV in monovac vaccine. The similar result was obtained by Madamohan *et al.* (2008) against the serotype O, A and Asia 1 of FMDV in oil adjuvant FMD vaccine and also similar to Chhabra *et al.* (2004), where they found maximum immune response on day 30 in case of monovac vaccine in buffalo calves.

In goat, in case of combined vaccine, the maximum immune response was obtained on day 30 against serotype A and Asia 1 of FMDV whereas, the maximum immune response for serotype O was on day 60. On an average, it can be said that the maximum immune response against different serotypes of FMDV in combined vaccine in goat was observed on day 30 (except serotype O, where day 60 response was slightly higher). Chhabra *et al.* (2004)

also found maximum immune response on day 21 in case of different serotypes (O, A, and Asia 1) of FMDV in combined vaccine in buffalo calves. It seems there is no study on combined vaccines in case of goat which includes FMD antigen.

In both monovac and combined vaccine groups in goat, the immune response obtained against serotype Asia 1 of FMDV was always higher than that of serotype O and A throughout the whole course. Similar results were obtained by Chhabra *et al.* (2004) in buffalo calves and Madanmohan *et al.* (2008) in goats in case of monovac vaccines.

In sheep, maximum immune response against serotype O and serotype Asia 1 of FMDV in monovac vaccine was obtained on day 30. Immune response against serotype A was maximum on day 60. In a study of monovac vaccine in sheep, Patil *et al.* (2002) found similarly peak immune response against serotype A on day 60 and serotype O on day 30. Throughout whole course, immune response against serotype O of FMDV in monovac vaccine was higher than serotype A and Asia 1 in sheep.

In sheep, peak immune response against serotype A and Asia 1 of FMDV in combined vaccine, was obtained on day 60 while the peak response against serotype O was obtained on day 30. It seems there is no study of combined vaccine in sheep which includes FMDV antigen. But it can be compared with the response of combined vaccine in the buffalo calves by Chhabra *et al.* (2004). Chhabra *et al.* (2004) found serotype O, A and Asia 1 giving peak immune response on day 30 in buffalo calves. Throughout whole course, immune response against serotype O of FMDV in combined vaccine in sheep was higher than serotype A and Asia 1.

If we compare the mean antibody titre of serotype A, O and Asia 1 of FMDV of monovac vaccine in sheep and goat there was no significant difference ($p < 0.05$). There was no significant difference between the different serotypes (O, A, and Asia 1) of FMDV in combined vaccine in sheep and goat ($p < 0.05$).

CHAPTER-VI

SUMMARY AND CONCLUSIONS

FMD is a highly contagious disease of cloven footed animals including cattle, sheep and goat. FMD is caused by FMD virus which is member of genus *Aphovirus*, family *Picornaviridae*. FMDV has seven serotypes O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3. In India, FMD is caused by serotypes O, A, C and Asia 1. Last outbreak due to serotype C was reported in 1996. Although, FMD is subclinical in sheep and goat, the disease has epidemiological importance. In Indian conditions most of the time sheep, goat are generally reared with cattle. Since, the disease is subclinical or mild in sheep and goat, there may be silent transfer of the disease from these small ruminants to cattle and buffalo. In India, FMD vaccination is only followed in cattle and buffalo through an extensive biannual government programmes and there is no such programme for small ruminants which seems, must for the control of silent amplification and dissemination of the disease.

Furthermore, in countries like India where fund is always a problem, if the animals can be vaccinated with the vaccine that contains multiple antigens then it can decrease the stress from the farmers and will also be cost effective. The present study, was taken considering all these factors. A total 43 healthy sheep (n= 16) and goat (n= 27) were included in the study. The animals (sheep and goat, separately) were divided into the three groups. Group 1 was given combined vaccine (FMD, HS and BQ), Group 2 was given monovac vaccine (FMD only) while, group 3 was used as control and they were not vaccinated.

The blood samples were collected from all the animals at day 0 and animals of different groups were vaccinated by different vaccines. Post vaccination serum samples were collected at day 30, 60 and 90.

3AB3 DIVA ELISA test was performed to rule out the silent infection in herd. Only two sheep were found positive for R3AB3 DIVA test while, all the goats were found negative. Two sheep which were found DIVA positive were excluded from the study. All the animals seronegative for 3AB3 were finally included in the study. Serum samples collected on day 30, 60 and 90 post immunization were analyzed by the LPB- ELISA to know the humoral immune response in different groups in sheep and goat.

In case of monovac vaccine in goat, the peak immune response was observed on day 30 for all the three serotypes (O, A and Asia 1). In monovac vaccine in sheep, serotype O and Asia 1 showed peak immune response at day 30 whereas, it was on day 60 for serotype A.

On day zero (pre vaccination) in all the three groups, there was no significant difference against all the three serotypes (O, A and Asia) of FMDV in both monovac and combined vaccine in sheep as well as in goat ($p < 0.05$). The mean antibody titre at day 30, 60 and 90 was significantly different from that of day 0 in case of sheep as well as in goats ($p < 0.05$) indicating seroconversion has taken place. While comparing combined and monovac vaccine, no significant difference between mean antibody titre against serotype O, A and Asia 1 could be observed during whole trial period both, in sheep as well in goats. Thus, clearly indicating that combined vaccine is equally efficacious as monovac vaccine for protection against different serotypes of FMD virus.

In case of combined vaccine in goat, the peak response was seen on day 30 by serotype A and Asia 1 while serotype O showed maximum immune response on day 60. In sheep, serotype A and Asia 1 showed maximum immune response on day 60.

The vaccinal response of monovac and combined vaccines were almost comparable and no significant difference was observed between sheep and goat for serotype O, A, and Asia 1 of FMD antigen ($p < 0.05$).

It can be concluded that

- 1) Both, combined and monovac vaccines are equally efficacious in sheep and goats while comparing immune response against FMDV serotypes O, A and Asia 1.
- 2) There was no species specific immune response and no significant difference between the immune response in sheep and goat for both the vaccines
- 3) The maximum immune response was seen at day 30 in most of the cases.
- 4) No ill affect was seen in the animals vaccinated with the combined triovac vaccine.
- 5) Further, study is needed to know the immune response against all the other antigens included in the combined vaccine and their comparison with their monovac counterparts.

ABSTRACT

FMD is a contagious disease of cloven footed animals including cattle, sheep, goat and others. FMD is caused by virus of family *Picornaviridae*, genus *Aphovirus*. FMDV has seven serotypes O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3. In India, most of the outbreaks is due to serotype O, A, C and Asia 1. Since last 20 years there is no report of FMD outbreak due to serotype C. The disease is very severe in cattle and buffalo while in sheep and goat it is subclinical in nature. But small ruminants have epidemiological importance in spreading the disease. In the present study, a total 43, sheep (n=16) and goat (n=27) were included. They were divided in three groups separately. Group 1 received combined triovac vaccine (FMD, HS and BQ), group 2 received monovac (FMD only) vaccine and group 3 were used as control. The serum samples were collected on day 0, 30, 60 and 90. NSP based 3 AB3 DIVA ELISA was performed for day 0 samples. A total 12.5% of sheep and 0% of goat were found positive suggesting a mild viral activity. Serum samples were also evaluated by LPB- ELISA to know the humoral immune response. Both monovac and combined vaccines showed peak immune response on day 30 (except for serotype O in combined vaccine) in goat. In sheep, serotype O and Asia 1 in monovac vaccine and serotype O in combined vaccine got peak immune response on day 30. While serotype O in monovac and serotype A and Asia 1 in combined vaccine showed peak immune response on day 60 in sheep. No significant difference was found between the monovac and combined vaccine in both sheep as well as in goat ($p<0.05$). There was also no significant difference between sheep and goat for combined as well as for monovac vaccine ($p<0.05$). It can be concluded that combined vaccines can replace the monovac vaccine but further study for antigens other than FMD, is needed in evaluation of immune response. It can be concluded that small ruminants should be brought under FMD surveillance and control programmes to prevent FMD dissemination from them.

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Washing Buffer (DIVA- ELISA), pH - 7.2- 7.4

NaH ₂ PO ₄ .2H ₂ O	0.39 g (0.0025 M)
NaHPO ₄ .2H ₂ O	1.336 g (0.0075 M)
NaCl	29.325 g (2.93% w/v)
Twen-20	500 µl (0.05% v/v)
Distill water to make	1 lit

Washing Buffer (LPB- ELISA), pH - 7.2- 7.4

NaH ₂ PO ₄ .2H ₂ O	0.39 g (0.0025 M)
NaHPO ₄ .2H ₂ O	1.336 g (0.0075 M)
NaCl	29.325 g (2.93% w/v)
Twen-20	1000 µl (0.1% v/v)
Distill water to make	1 lit

Phosphate Buffer Saline, pH -7.4 (0.15 M)

NaCl	8.0 g
KCl	0.20 g
Na ₂ HPO ₄	1.15 g
KH ₂ PO ₄	0.20 g
Distill water to make	1 lit

Sterilize at 15 lb pressure (autoclave) for 30 min and store at + 4 C

Diluent Buffer: 20 ml

Skimmed milk Powder	0.6 g (3 % w/v)
Horse serum	2 ml (10 % v/v)
E. coli lysate	2.5 µl (0.01 % v/v)
Washing Buffer	upto 20 ml

Stopper Solution (1M H₂SO₄): 100 ml

H ₂ SO ₄ : 95-97%	5.55 ml
Distilled Water	94.45 ml

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- 2) NCC B and C certificates holder.
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