SEROCONVERSION OF THREE DIFFERENT FOOT-AND-MOUTH DISEASE VACCINES IN CATTLE

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that this thesis entitled **"SEROCONVERSION OF THREE DIFFERENT FOOT-AND-MOUTH DISEASE VACCINES IN CATTLE"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy,
11.04.2001

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CERTIFICATE

Certified that the thesis, entitled **"SEROCONVERSION OF THREE DIFFERENT FOOT-AND-MOUTH DISEASE VACCINES IN CATTLE"** is a record of research work done independently by **Dr. Bipin, K.C.,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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BIPIN, K.C.

Dedicated to
My Parents and Teachers

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1. INTRODUCTION

Domestication of cattle for milk and draught power was a major milestone in man's progress from primitive existence to civilized life. Eighty per cent of India's population live in rural areas and depend on agricultural and animal husbandry activities for their livelihood. By adopting scientific practices in dairy cattle production, India emerged as the top milk producing country in the world during recent years. Our country has a huge cattle population of around 200 million including cows and bullocks. The contribution of dairy sector to nation's economy is estimated to be approximately 50,000 crore rupees annually (Ramaswamy, 2000). Any threat to the milk production, therefore will seriously affect the nation's economic scenario.

Among various conditions adversely affecting livestock health and productivity, Foot-and-Mouth disease (FMD) stands first which cripples the livestock industry and adversely affects export of livestock products. Though not a killer disease, FMD causes considerable reduction in the milk production and work potential of convalescent animals. A heavy calf mortality and secondary complications like mastitis adds to this. Being an FMD endemic country, India could not export many of our livestock products to FMD free countries. Because of all these reasons, Foot-and-Mouth disease attracted the attention of scientific community all over the country during last few decades.

Repeated epidemic episodes has also invited Government's interest for developing satisfactory control measures.

The strategy adopted for control and eradication of Foot-and-Mouth disease varies from country to country depending on geographical location, technological and economical development and prevailing political attitude besides its endemic nature. Stamping-out policy considered to be the most successful measure for eradication of FMD and this has been practiced by countries like United Kingdom with satisfactory results. But because of the compensation to be given to the farmers, stamping-out policy is economically not feasible in India. Strict control on cattle and other susceptible livestock movement across the state and national borders can be practiced, but having only limited results.

The economical, political and religious considerations prevailing in our country suggest that the systematic large scale vaccination is the most appropriate method to bring down the incidence of the disease. Although there is an increasing awareness of the importance of disease control through movement restriction and sanitary measures, immunization is still the most effective method of control for FMD which can be extensively applied in many parts of the world. Therefore, emphasis should be placed on improvements in techniques of vaccine production, vaccine quality and delivery system.

The most commonly used FMD vaccine in India has been an aqueous vaccine prepared from inactivated antigen adsorbed on aluminium hydroxide

gel and adjuvanted with saponin. This vaccine has been in use for last many years with good results. The vaccine however, still suffers certain disadvantages like need for repeated administrations to maintain protective levels of immunity in vaccinated animals. The immunity produced with aqueous FMD vaccine keeps the animal disease resistant only for a period of six months.

During last few years, there is an increasing interest in the use of oil-adjuvanted FMD vaccines all over the world. Unlike aqueous vaccines, oil emulsion vaccines reported to be producing a long lasting immunity in the vaccinated animals. Because of the need for only fewer musterings, oil-vaccines can considerably reduce the cost and labour involved in FMD control.

Many commercial biologicals pioneered in manufacturing of Foot-and-Mouth disease vaccine has now switched over to production of oil-adjuvant vaccines from the traditional algel vaccine. For the last one or two years, these oil-adjuvant FMD vaccine is being extensively used in the field. Whether these new vaccines produce effective immunity for a longer period with lesser number of administrations in vaccinated animals is a question of high importance not only to the professionals in this field, but also to the dairy farmers of our country who depend on cattle for their livelihood.

The present study was conducted with the following objectives:

1. To study the duration of immunity by routine FMD vaccination using oil-adjuvanted and gel vaccines in cattle.

2. To compare the immunopotency of oil-adjuvanted and gel FMD vaccines in producing effective seroconversion of neutralizing antibodies in cattle.

2. REVIEW OF LITERATURE

2.1 History

The earliest description of Foot-and-Mouth (FMD) disease was recorded by Hieronymus Fracastorius in northern Italy in 1514 (Anon, 1978).

In Britain, the disease first appeared in 1839 in dairies of Stratford, London (Anon, 1978; Henderson, 1978).

Although there is acceptable evidence that many of the great plagues of animals like anthrax, glanders, rabies, rinderpest and tuberculosis have existed for some thousands of years, Foot-and-Mouth disease appear to be of more recent origin (Henderson, 1978).

Foot-and-Mouth disease is endemic in South-America, Africa, Asia and parts of Europe (Kahrs, 1981).

The first systematic work of FMD in South-East Asia was initiated at the Indian Veterinary Research Institute, Mukteswar in 1943 for identification and typing of FMD virus using stock strains of type O, A and C from World Reference Laboratory, Pirbright, UK (Natarajan *et al.*, 1993).

FMD virus was the first animal virus reported by Loeffler and Frosch in 1897 responsible for causing Foot-and-Mouth disease (Dhanda and Gopalkrishan, 1998).

2.2 Etiology

Foot-and-Mouth disease virus (FMDV) is a member of the genus Aphthovirus belonging to the family Picorna viridae (Murphy *et al.*, 1999).

2.2.1 Virus properties

Galloway and Elford (1931) reported that size of Foot-and-Mouth disease virus is 8-12 µm using gradocol membranes.

According to Bachrach *et al.* (1964), the Foot-and-Mouth disease virus has a sedimentation co-efficient of 140s.

According to Sellers (1968) chemical substances like phosphoric, sulphuric, citric, acetic and formic acids and sodium carbonate, sodium metasilicate and sodium hydroxide inactivate FMD virus in short time.

Brown (1972) described the size of viral particle as 24 nm.

The antigenic property of FMD virus was discovered by Vallee and Carre in 1922 in France and Waldmann and Trautman in 1926 in Germany (Henderson, 1978).

The virus occurs as seven major serotypes, *viz.*, O, A, C, Southern African Territories (SAT) 1, SAT 2, SAT 3 and Asia-1. Within these major serotypes, many antigenically and serologically distinct subtypes have been identified (Kahrs, 1981; Murphy *et al.*, 1999; Radostits *et al.*, 1994).

Many commonly used disinfectants are ineffective in destroying the Foot-and-Mouth disease virus, but the virus is liable to acids and alkalies (Cottral and Callis, 1995).

The FMD virions are icosahedral in shape with no envelope, core consists of single stranded RNA and a small protein (3B VPg) co-valently linked to its 5'-end. The capsid is composed of 60 protein subunits, each consisting of four proteins (Murphy *et al.*, 1999).

Suryanarayanan *et al.* (1998) studied the antigenic relationship of type O outbreak virus isolated from Tamilnadu and Karnataka with vaccine strain and concluded that virus from outbreak in vaccinated cattle was a variant which could escape neutralization by antibodies against vaccine virus.

Kumar *et al.* (1999) reported that a close antigenic relationship (r = 0.9 to 1.0) existed between type 'O' FMD outbreak virus from Haryana during 1996-'97 and vaccine strain, thus ruled out the possibility of a variant virus for the outbreak.

2.3 Epidemiology

2.3.1 Prevalence

2.3.1.1 Global

Castro *et al.* (1967) conducted a five year study on epidemiology of FMD in Sao Polo State of Brazil during 1961- '66. Type O virus was detected in 60 per cent cases, type A in 22 per cent and C in 18 per cent cases.

During 1920's the number of outbreaks of FMD in Europe were 4,973, Germany 3,17,783, Denmark 1,59,279, Holland 1,92,050 and France 1,02,852 (Anon, 1978).

According to Mowat (1978), the number of FMD outbreaks in various European countries during 1952 were, France 3,20,016, Belgium 8,943, Holland 7,054, West Germany 54,572 and Italy 28,579 which drastically reduced to 2, 21, 2, 13 and 31 respectively during 1975 by adopting various control programmes.

United States had suffered nine outbreaks of FMD and the last outbreak occurred in 1929. The last occurrence of FMD in North America was in 1952 (Graves, 1979).

North America, Central America and Panama have been free of FMD for more than 25 years and Australia is the only other major country free of the disease (Graves, 1979).

Gleeson *et al.* (1995) investigated an outbreak of FMD in Thailand. A close antigenic relationship (r=0.61) was observed between type A outbreak virus and the vaccine strain.

During 1951-'52, over 9,00,000 outbreaks of Foot-and-Mouth disease were reported in Europe (Kumar, 1996).

Shieh (1997) reported that 6,147 pig farms of Thaiwan were affected with outbreak of FMD during March-July, 1997. The disease was controlled within two months by culling and blanket vaccination. A total of 1,300 farms were affected in March, 3,864 in April, 975 in May, five in June and three farms in July.

Foot-and-Mouth disease is endemic in South America, Africa, Asia and parts of Europe. Australia, New Zealand and South America have prevented its establishment by adopting drastic control measures (Dhanda and Gopalkrishan, 1998).

2.3.1.2 India

Datt *et al.* (1968) studied the incidence and distribution of different types of FMD virus in India, using complement fixation test on the vesicular materials collected from field cases of FMD. An incidence of 55 per cent for type 0, 23 per cent type A, 9 per cent type C and 13 per cent Asia-1 were detected.

Sharma and Asthana (1978) reported an outbreak of FMD in Regional Pig Breeding Station, Aligarh in Uttar Pradesh.

Mukhopadhyay (1992) stated that every year, approximately 5,000 outbreaks of FMD occurs in India affecting nearly 3,00,000 animals. Same report was also given by Kumar (1996).

Natarajan *et al.* (1993) reported that number of FMD outbreaks occurred in India were 1,940 during 1988, 790 during 1989, 4,186 during 1990, 524 during 1991 and 950 during 1992. Serotypes O, A, C and Asia-1 were recorded every year.

Saxena (1995) conducted a survey throughout India during January to March, 1991 to study the annual incidence rate of FMD in individual states proportionate to their livestock population. Incidence by region was 35 per cent in east, 24 per cent in west, 20 per cent in north and 11 per cent in south.

Maan *et al.* (1998) studied the distribution of FMD virus types recorded in north-west region of India during 1994-'96. Type O was the most predominant (79.67 per cent) followed by A_{22} (17.89 per cent) and Asia-1 (2.44 per cent).

2.3.1.3 Kerala

Anon. (1983) reported 9,122 cases of FMD among cattle, 636 cases among buffaloes and 1,463 cases among goats of Kerala during 1983.

During 1990-'91, out of 28 samples collected from FMD outbreaks in Kerala, 16 were found type O, and 2 as type Asia-1 (Anon, 1991a).

Anon (1994) reported an incidence of FMD in Kerala during 1994 as 800 among cattle, 84 among buffaloes and 90 among goats.

Vijayakumar (1999) reported that the FMD outbreak that occurred in almost all districts of Kerala during 1998 was the most severe outbreak which occurred in the state in last 12 years with a total of 14,905 cattle, 66 buffaloes, 910 goats and 22 pigs affected.

2.3.2 Hosts

Cases of Foot-and-Mouth disease in man has been reported in many literatures (Armstrong *et al.*, 1967; Eissner *et al.*, 1967; Howie and Weipers, 1967).

Hedger (1976) reported persistent infection of FMD in African buffalo (*Syncerus caffer*) without clinical lesions.

Confirmed cases of Foot-and-Mouth disease in elephants have been reported by Pyakural *et al.* (1976), Rahman *et al.* (1987) and Chakrabarti and Majumder (1990).

Sharma and Asthana (1978) in a report of FMD outbreak in pigs pointed out that 22 per cent of mortality occurred among unweaned pigs and 16.8 per cent among pigs aged two to six months. Yorkshire breed was least susceptible.

Graves (1979) reported cases of FMD in horses.

Foot-and-Mouth disease virus affects all cloven-footed animals, both domestic and wild. Among domestic animals, cattle, sheep, goats, pigs, buffaloes and camel and among wild animals, African buffaloes, deers, warthogs, bush pigs,

elephants, yak, neelgai and giraffe are affected by the virus (Mann and Sellers, 1990).

According to Mann and Sellers (1990), cattle are more susceptible to Foot-and-Mouth disease than sheep, goats and pigs, because cattle breaths more volume of air than other species and also the infectious dose required for the cattle is less.

Foot-and-Mouth disease was reported in deer, Nilgiri bison and mithun by Ahuja *et al.* (1991).

Verma and Sarma (1997) reported a series of FMD outbreaks in mithun (*Bos gaurus*) in Arunachal Pradesh during 1994-'95.

Farag *et al.* (1998) studied the susceptibility of camels to natural infection with FMD virus. None of the 645 camel sera samples tested were positive for virus infection associated antibodies against antigen types A Sau 41/91 and O_1 *Manisa/68*.

Barman *et al.* (1999) studied FMD in wild and semi-domesticated animals of North-Eastern states of India. Three outbreaks were reported in elephants, one in wild buffalo, two in sambar deer, one each in spotted deer and barking deer, four in yak and twelve in mithun.

2.3.3 Transmission

During incubation period, the virus from the pharynx of cattle can be recovered for several days and from milk and semen four days before apparent symptoms (Burrows, 1968; Sellers, 1969).

A study conducted by Polyakov and Naurysbaev (1968) showed that FMD virus remained virulent in dung for 29 to 33 days in summer, 156 to 168 days in winter, eight days in dung subjected to mesophilic fermentation at 32°C and for 39 days in liquid manure.

The disease spreads through contact and by other means like virus contaminated animal products such as meat, milk, semen, embryos and by direct mechanical transfer of virus on man, wild animals, vehicles, birds, fomites and by air-borne routes extending over short and long distances (Hyslop, 1970; Gloster *et al.*, 1982; Donaldson, 1986).

After slaughter, the virus present in muscles is inactivated by lactic acid produced, but no pH change occurs in offal and bone marrow and may lead to transmission of disease (Mann and Sellers, 1990).

Sharma *et al.* (1991) observed that seasonal peaks of FMD epidemics were related to more movement and congregation of animals caused by migration to new pastures and animals fairs.

According to Gleeson *et al.* (1995), a close contact is required between animals in hot climates for FMD virus to spread.

The incubation period of FMD virus varies from 24 hours to 15 days, but the usual period being from three to 10 days (Dhanda and Gopalkrishan, 1998).

2.4 Economic importance

An estimated loss of 10 crore pounds occurred in France during 1952 due to FMD mainly as a result of reduced milk production (Anon, 1978).

The annual estimated loss in India due to Foot-and-Mouth disease is approximately 4,300 crores of rupees (Anon, 1991b).

Kumar (1996) reported that economic impact of FMD also include export embargo on animal products and bye-products and repeat breeding.

The estimated direct economic loss per annum due to FMD in India is rupees 500 crores due to loss in milk yield and impairment of motive power (Dhanda and Gopalkrishan, 1998).

The economic effects of Foot-and-Mouth disease in a herd are very serious. The loss of milk yield, abortions and deaths of young ones contribute to major losses. Mastitis in convalescent milch animals contributes to more than 25 per cent loss in milk production (Dhanda and Gopalkrishan, 1998).

The economic losses to Indian dairy industry caused by FMD comes to more than Rs.5000 crores per annum (Manickam, 1998).

Vijayakumar (1999) reported that an FMD outbreak occurred in Kerala during 1998 which caused an economic loss of Rs.66.33 lakhs due to death of animals and Rs.121.50 lakhs due to reduced milk yield.

2.5 Control

Naurysbaev (1967) employed 0.8 per cent formaldehyde solution for the disinfection of experimentally infected fodder grain at the rate of 10 litres per 100 kg grain for six hours and concluded as a satisfactory method of destroying FMD virus.

Procedures most widely employed for the control of Foot-and-Mouth disease are by eradication and by vaccination or employing both methods together. For disinfection of barns, one to two per cent sodium hydroxide or formalin or four per cent sodium carbonate solution can be used (Radostits *et al.*, 1994).

Control of cattle movement is very important in preventing the spread of Foot-and-Mouth disease. Access of wild animals and birds to the premises of domestic animals must be prevented. Slaughter of infected animals is essential for proper control of FMD (Dhanda and Gopalkrishan, 1998).

Singh and Killari (1998) reported that by adopting solid vaccination coverage of all susceptible animals from 1957 onwards, European countries completely eradicated FMD and from 1992 onwards, they are following a non-vaccination slaughter policy.

Control on cattle movement is important during FMD outbreak. Door-to-door vaccination is to be practised and never allow congregation of animals (Azad, 1999).

Saseendranath (1999) suggested that livestock movement control, proper handling and use of vaccines, strict control over importation of livestock and animal products, increasing vaccine production, proper reporting of outbreak and proper disinfection of cattle premises are essential for control of FMD.

The most practiced method of control of FMD in countries where slaughter of affected animals are socially and economically not feasible is vaccination (Sulochana, 1999).

2.6 Vaccines

Formaldehyde inactivated Frenkel vaccine can be stored for as long as five years while aqueous vaccines formulated with azridine inactivated virus do not last long because formaldehyde chemically cross link the virus coat and thus improves the antigen stability (Wild and Brown, 1968).

The local side effects produced by Saponin due to haemolytic activity of some of its fractions can be overcome by its purification making use of dialysis (Dualsgard, 1974).

Mc Kercher *et al.* (1975) compared FMD vaccines prepared by virus inactivation with formaldehyde and adsorbed onto aluminium hydroxide gel with vaccine prepared by inactivation with AEI and adjuvanted with Freund's incomplete adjuvant. The results obtained with oil vaccines were less favourable than expected owing to less amount of antigen or use of poor antigen subtypes.

Wittman *et al.* (1975) demonstrated that the DEAE-Dextran exerts its optimal adjuvant effect when injected together with inactivated FMD virus as vaccine.

Gorard *et al.* (1977) reported that virus grown in IBRS-2 cells are inactivated with azridines like Acetyl ethyleneimine or binary ethyleneimine.

Maes *et al.* (1977) found out that protamine was more effective than DEAE-Dextran in immuno potentiation of FMD vaccines.

Adsorbing FMD virus extracted from tongue epithelium on to aluminium hydroxide gel followed by treatment with formaldehyde solution at a controlled temperature resulted in vaccines of greater potency (Mowat *et al.*, 1978).

The first steps in the development of inactivated FMD vaccines were taken 50 years ago when it was found that suspensions of the virus treated with a dilute solution of formaldehyde could give some degree of protection against infection (Mowat *et al.*, 1978).

Need for large amount of antigen as well as price of DEAE-Dextran made addition of this adjuvant unattractive and is more useful in those conditions when only aqueous vaccines are available for use in pigs (De Leeuw *et al.*, 1979a).

Solyom *et al.* (1980) observed that immunogenicity enhancing mechanism of saponin was more pronounced in young and growing animals. The immunity found to have increased significantly with the use of eight milligrams of saponin per dose.

Solyom and Bertok (1985) assessed the immunity enhancing adjuvant activity of radio detoxified endotoxin (RD-LPS) on the potency of C type FMD vaccine in different species. In cattle and sheep, the adjuvant effect of oil plus RD-LPS surpassed only slightly that of oil alone. The effect of RD-LPS was very pronounced in pigs.

Nair and Sen (1992) studied the effect of inactivant in the immunogenicity of FMD vaccines in sheep and found out that no significant difference exists in antibody response to vaccines inactivated with formaldehyde or binary ethyleneimine.

Brown (1993) reviewed the accidents caused by incomplete inactivation of viruses and described that Foot-and-Mouth disease vaccines prepared by inactivation with formaldehyde will carry residual infectivity.

Misra and Lal (1995) reported that monovalent type A FMD vaccine adjuvanted with bentonite did not show any deterioration on storage at 4°C for 12 to 24 months when compared to type O vaccine.

Portiansky *et al.* (1996) showed that injection of Cyclophosphamide (five mg/kg, Endoxan – Asta) intraperitoneally four days before FMD

vaccination produced higher neutralizing antibodies when compared to control animals which were given vaccine without pre-treatment with cyclophosphamide.

Volpina *et al.* (1996) synthesized the peptide palm 2, 135 to 159, a dipalmitoyl derivative of the 135 to 159 fragment of VP1 portion of A_{22} strain FMD virus. The trials conducted in sheep showed that synthetic polymethyl siloxane oil was found to be suitable adjuvant for this vaccine.

The earliest attempt to evaluate an FMD vaccine was carried out in Europe in 1938 by Waldmann who prepared a new vaccine by formalinizing the suspensions of tongue epithelium collected from experimentally infected animals (Dhanda and Gopalkrishan, 1998).

Filgueira *et al.* (1999) found out that immunomodulators like water soluble fraction of the cell wall of mycobacterium species and a synthetic lipoamide, avridine included in FMD oil vaccines can produce protective immunity even at low antigen concentration.

Sadir *et al.* (1999) reported that anti-FMDV antibody response was significantly ($P<0.05$) higher in animals immunized with the immunogen containing avridine.

2.6.1 Aluminium hydroxide gel vaccine

The effect of dose volume of vaccine and concentration of aluminium hydroxide on the immune response was studied by Hyslop and Morrow (1969) who found out that variation in dose volume from five to 15 ml had no effect provided that the quantity of antigen and amount of aluminium hydroxide were kept constant. They also found out that a reduction in quantity of aluminium hydroxide reduced the immune response to vaccine.

Auge-de-Mello and Gomes (1977) in a trial with FMD aqueous vaccines observed highest antibody titre at 30 days post vaccination and a gradual decline thereafter.

Rivenson *et al.* (1977) reported that the FMD vaccines prepared by inactivating the virus suspensions with formaldehyde or acetyl ethyleneimine (AEI) adsorbing the antigen on aluminium hydroxide gel and adding saponin produced immunity of shorter duration and repeated vaccinations at 4 months interval was needed.

Gel quality is usually evaluated by measurement of its adsorptive capacity for congo red or preferably, virus (Bekkum, 1978).

The method of adsorbing the virus extracted from tongue epithelium onto aluminium hydroxide gel followed by treatment with formaldehyde solution at a controlled temperature resulted in vaccines of greater potency (Mowat *et al.*, 1978).

Nair and Sen (1993a) observed no significant difference between aluminium hydroxide gel adsorbed saponified FMD vaccine type O and Asia-1 inactivated with formaldehyde or acetyl ethyleneimine in producing antibody response in sheep.

2.6.2 Oil-adjuvant vaccines

Freund *et al.* (1948) reported that combining paraffin oil and killed tubercle bacilli enhanced the immune response in various experimental animals. This later led to many studies with emulsion vaccines.

The use of oil-adjuvants with FMD vaccines first appeared in the reports of the investigators from the laboratory at Lindholm, Denmark, where a number of adjuvants were examined and an improved immune response was obtained with the oil-adjuvant mixture (Michelson, 1961).

Cunliffe and Graves (1963) compared the response of formalin inactivated vaccines combined with either aluminium hydroxide or oil-adjuvants and found the antibody response to be higher and of longer duration with the oil-adjuvant.

Most oil-adjuvants are a single water-in-oil emulsion, however the superiority of double emulsion FMD vaccines has been reported (Herbert, 1965; Anderson *et al.*, 1971).

Mowat (1974) observed no significant difference between aqueous and oil adjuvanted FMD vaccines while Mello *et al.* (1975) reported oil adjuvanted FMD vaccines as superior to aqueous vaccines.

The most widely used emulsifying agent in the water-in-oil emulsion is Arlacel A special (Mannide mono-oleate) which is combined with a light mineral oil: Bayol F, Drakeol 6 VR or Marcol 52 (Mc Kercher and Graves, 1977).

Solyom *et al.* (1977) found that oil-adjuvanted FMD vaccine failed to evoke an immune response in calves, but proved to be the preparation of choice for adult cattle.

Sutmoller (1977) reported that oil adjuvanted FMD vaccines have higher and long lasting protection levels when compared with aluminium hydroxide vaccines prepared from the same source of inactivated antigens.

Astudillo and Auge-de-Mello (1980) studied the relative cost-effectiveness of the use of FMD oil-adjuvant vaccine to immunize cattle against existing aluminium hydroxide vaccine and found oil-adjuvant vaccine as preferred one because of relatively fewer number of injections required and greater immunogenicity.

Rivenson *et al.* (1982) reported that oil-emulsion FMD vaccine containing 42.5 per cent marcol 52, 6.55 per cent Arlacel 83 and 0.95 per cent Tween 80 gave better results when compared to a hydroxy saponin FMD

vaccine prepared by adsorption on aluminium hydroxide and addition of 0.1 per cent saponin, five per cent glycerol and 1 in 30,000 thiomersal.

Roncha *et al.* (1983) conducted vaccination trials in young cattle with oil-adjuvant FMD vaccines and recommended that vaccination of young cattle should be performed atleast three times at intervals of six months followed by re-vaccination.

Sadir *et al.* (1988) observed that calves born to vaccinated dams did not respond to the aqueous FMD vaccine 30 or 90 days post partum. Calves which are 30 or more days old responded to oil-adjuvant FMD vaccine like adult cattle.

Nair and Sen (1993b) observed that immunogenicity of aluminium hydroxide gel and oil-adjuvanted FMD vaccines in sheep do not differ significantly over a period of eight weeks.

Rao *et al.* (1993) reported that marcol oil adjuvanted FMD vaccine produces better serological response than paraffin-emulsion and aluminium hydroxide-saponin FMD vaccines. Booster vaccination after 21 days with both oil-emulsion vaccines produced no significant anamnestic response.

Spath *et al.* (1995) studied the immune response of oil-adjuvanted FMD vaccines in calves. Results obtained indicated that calves aged three to four months with non-protective levels of colostral-derived antibodies responded with high antibody titres to vaccination.

Barnett *et al.* (1996b) compared two novel oil-adjuvants, montanide ISA 25 and 206 (Seppic, Paris). The results indicated that the vaccines adjuvanted with these oils retained potency for longer periods following storage at +4°C and elicited good immune response in both pigs and cattle regardless of injection route.

Hunter (1996) assessed the performance of selected oil-adjuvants containing SAT serotypes of FMD virus in cattle, sheep and goats. A commercial double oil emulsion vaccine elicited higher antibody titres and a more prolonged antibody response than conventional vaccines.

According to Doel (1999), potential variables in vaccination against FMD like use of oil-adjuvant for cattle are less critical when compared to elements like selection of appropriate strains and proper and timely administration.

2.6.3 Synthetic vaccines

Kleid *et al.* (1981) described the recombinant DNA technology to produce VP1 protein of FMD virus in *Escherichia coli*.

Experimental vaccine prepared with bio synthetic VP1 or synthetic peptide elicited neutralizing antibodies in cattle, but challenge experiments with cattle were often disappointing (Dimarchi *et al.*, 1986).

Morgan and Moore (1990) observed that a single dose of FMD virus protein, VP1 peptide expressed in *E. coli* as a fusion protein with 190 amino acids of the LE^1 protein of the tryptophan operon of *E. coli*, elicited an immune response in steers sufficient to withstand the challenge of exposure to animals with acute FMD.

Rieder *et al.* (1994) constructed a serotype A virus using an infectious cDNA of FMDV, in which the G-H loop has been substituted with the homologous sequences from serotypes O or C. These chimeric viruses replicated to high titre and chemically inactivated vaccines prepared from chimeric viruses induced antibodies in guinea pigs that neutralized both type A and either type O or type C viruses.

Barnett *et al.* (1996a) studied the immunogenicity of a synthetic peptide corresponding to VP1 sequence of FMDV strain A_{24} cruzeiro, which is found to elicit neutralizing and protective antibodies in guinea pig and cattle.

Taboga *et al.* (1997) studied four types of peptides representing sequences of Foot-and-Mouth disease type C. None of the tested peptides at several doses and vaccination schedules gave protection above 40 per cent. Protection showed limited correlation with serum neutralization activity and lymphoproliferation in response to whole virus.

The inactivated or live virus vaccines have certain limitations as there is risk of leakage from the manufacturing units and an inactivated vaccine may

contain some live virus. So the importance of synthetic peptide vaccines against FMD is increasing (Dhanda and Gopalkrishan, 1998).

2.7 Assessment of immune response

The tests generally used for the detection of antibodies to Foot-and-Mouth disease are virus neutralization test (VNT) and enzyme linked immunosorbent assay (ELISA) (OIE manual, 1992).

2.7.1 Virus neutralization test

Golding *et al.* (1976) described a standard procedure for the detection of antibodies against FMD virus using virus neutralization test.

The application of virus neutralization test for detection of antibodies to FMD virus had shown that the VN titres obtained have a direct correlation with protection against FMDV challenge in cattle (Sutmoller and Vieira, 1980).

According to Hamblin *et al.* (1987), the VN test measures those antibodies which neutralise the infectivity of the virions *in vitro*, whereas ELISA measures all classes of antibodies, including those generated against incomplete and non-infectious virus.

Westbury *et al.* (1988) conducted trials for measuring serum antibodies to FMD virus in immunised animals of Thailand. The virus neutralization and ELISA titres showed a positive correlation and a VN titre of 1:8 was found to be equivalent to an ELISA titre of 1:22.

The virus neutralization test is specific, sensitive and quantitative and takes two to three days to provide a result. Low titre - false positive reactions can be expected in a small proportion of sera (OIE Manual, 1992).

Kalanidhi *et al.* (1993) carried out microneutralization test (MNT) for serum antibody assay to compare the efficacy of FMD vaccines prepared from concentrated antigens stored at low temperatures and results obtained showed that vaccines formulated using antigens stored at +4°C and in liquid nitrogen for 18 to 30 months induced satisfactory titres for all the four valencies.

Rao *et al.* (1993) compared the efficacy of oil-adjuvanted and aqueous FMD vaccines by measuring the serum neutralizing antibody titres employing microneutralization test. The results obtained indicated that the antibody response pattern was similar in both MNT and ELISA, eventhough ELISA gave higher values.

Dekker and Terpstra (1996) employed virus neutralization test to detect Foot-and-Mouth disease antibodies in dairy herds of Netherlands four years after vaccination. Virus neutralization titres equal to or higher than the titre at which 95 per cent of the cattle would be expected to be protected against challenge were found in 57 to 73 per cent of the younger age groups and in 100 per cent of the older animals.

Bayri *et al.* (1999) assessed the protective immunity to a recombinant protein encoding C-terminal of the VP1 protein of type Asia-1 in guinea pigs

using virus neutralization test. The sera collected at intervals of 21, 42 and 63 days after booster showed high titres which could be protective.

2.7.2 Enzyme linked immunosorbent assay

Abuelzein and Crowther (1978) employed indirect ELISA technique for quantifying antibodies to FMD virus from cattle sera. On comparison of the results from ELISA and neutralization test, a low degree of correlation was obtained (r=0.693) between the two tests.

Hamblin *et al.* (1986a) described the development of a liquid phase blocking sandwich ELISA for the quantification of antibodies against Foot-and-Mouth disease virus, which can replace the virus neutralization test. The assay is rapid, relatively simple to perform, economic and results may be recorded within 24 hours.

Hamblin *et al.* (1986b) found out that a titre of one in 16 in virus neutralization was equivalent to one in 40 by ELISA as indicated by the overall regression between the ELISA and the VN data.

Hamblin *et al.* (1987) evaluated the antibody titre against Foot-and-Mouth disease after infection and vaccination using ELISA. The antibody titres recorded by ELISA were compared with virus neutralization test results and concluded that results were similar following primary vaccinations and until five days after secondary vaccination.

Many workers found out that a positive correlation exists between ELISA and MNT titres with ELISA gives higher titres for antibodies against Foot-and-Mouth disease (Westbury *et al.*, 1988; Kalanidhi *et al.*, 1993; Rao *et al.*, 1993).

Maanen *et al.* (1989) compared liquid phase blocking Enzyme linked immunosorbent assay (LPB-ELISA) and Serum neutralization test (SNT) for evaluating immunity in potency testing of FMD vaccines. The correlation co-efficient between LPB-ELISA and SNT were 0.91 for type O and A, and 0.82 for type C.

A complex trapping-blocking (CTB) ELISA was described by Maanen and Van Maanen (1990) for FMD antibody detection. Results obtained from CTB-ELISA showed a positive correlation with VNT results.

The ELISA is specific, sensitive and quantitative, and also has the advantage that it is more rapid to perform, is less variable, gives fewer false-positive results and is not dependent on tissue culture systems over microneutralization test (OIE manual, 1992).

Periolo *et al.* (1993) assessed specific serum activity levels against four reference strains of Foot-and-Mouth disease virus in animals vaccinated with quadrivalent oil-vaccines using LPB-ELISA. Serum LPB-ELISA titres were directly correlated with percentage of protected animals.

According to Gruia *et al.* (1995), the LPB-ELISA method eliminates the need for the maintenance of cell culture and live FMD viruses and the results of the test are obtained within 24 hours. The sensitivity and reproducibility of the test are comparable to those of virus neutralization test.

Saha and Sen (1995) described the application of liquid phase blocking ELISA for detection of FMD antibodies. The animals of an organised dairy farm were selected and screened for type O FMD antibody one month after vaccination with monovalent vaccine. A comparison was made between Liquid Phase Blocking-ELISA (LPB-ELISA) and sandwich competition ELISA (SWCOM-ELISA). Higher titres observed in LPB-ELISA (2 to 2.8) than SWCOM-ELISA (1.1 to 2.0).

Araujo *et al.* (1996) used liquid phase blocking sandwich ELISA for detection of antibodies against FMD virus in water buffalo sera. The antibody titres obtained in the blocking ELISA had a high positive correlation coefficient with VNT (r=0.9 for type O, and 0.82 for type A).

O' Donnel *et al.* (1996) applied liquid phase blocking sandwich ELISA for detection of antibodies against FMD virus with a bio-engineered 3D protein. The assay was able to detect antibodies as early as five days post inoculation.

Armstrong (1997) employed ELISA for detecting FMD antibodies in cattle milk. Sample from convalescent cattle showed a high correlation between antibody levels in milk and serum.

The enzyme-linked immunosorbent assay is marked by its rapidity, specificity and sensitivity for assessing the antibody titre against Foot-and-Mouth disease (Dhanda and Gopalkrishan, 1998).

Smitsaart *et al.* (1998) studied the herd immunity level induced in cattle by Foot-and-Mouth disease oil vaccines using ELISA. Ninety nine per cent of the native cattle serum samples had titres below $\log_{10} = 1.2$, and none had a titre above $\log_{10}=1.5$.

2.8 Vaccination failure/vaccine failure

2.8.1 The virus/antigen factor

De Leeuw *et al.* (1979b) showed that a level of immunity that is adequate to protect pigs against a moderately heavy challenge can be overwhelmed by the massive challenge that results from close contact with an animals with fully generalised infection.

Srinivasan *et al.* (1983) studied the serological relationship between six type O FMD virus isolates from different parts of India. The vaccine strain O IND 53/79 exhibited broadest serological spectrum.

Many research results have shown that the virus neutralizing antibody responses of cattle were a linear fraction of the 140s antigen dose after inoculation of FMD vaccines containing type O virus (Rweyemamu *et al.*, 1984).

The antigenic diversity among type A FMD viruses in India indicated that a proposed candidate vaccine strain need to be thoroughly compared with isolates from different parts of the country (Belwal *et al.*, 1986).

Black *et al.* (1986) reported that doubling the antigen dose increased the antibody titre against O_1 and A_{24} antigen by approximately 0.15 $\log_{10}$. They also found out that A_{24} antigen is about 30 times more immunogenic than O_1 with C_3 intermediate between the two.

Belwal *et al.* (1987) compared an FMD type A strain isolated from an outbreak in a vaccinated herd with vaccine virus using two-dimensional micro-neutralization test, and immunologically through cross-immunity test in cattle. The antigenic diversity of the field virus from vaccine virus revealed by serological analysis was substantiated by lack of cross protection in primo vaccinated animals.

Belwal *et al.* (1989) found out considerable antigenic variations among aphthovirus types Asia-1 isolates recovered from India. Two vaccine strains and three field strains were studied. Strain Asia-1 WBN 117/85 showed broad spectrum antigenecity which has been recommended for incorporation in quadrivalent vaccine.

In vaccination programmes, the paramount consideration is whether the available vaccine antigen is likely to induce sufficient antibody levels in

vaccinated animals to protect such animals against a strain of virus responsible for the disease situations in the field (Kitching *et al.*, 1989).

Serological study of type A Indian FMD isolates by Azad *et al.* (1995) indicated that type A IND 17/82 had a broad immunogenic spectrum and could be considered as a candidate vaccine strain for incorporation in FMD vaccines in India.

Gleeson *et al.* (1995) observed a close antigenic relationship between vaccine virus and outbreak virus (r=0.61). The investigation suggested the requirement of close contact between animals for FMD to spread in tropical environment.

Farag *et al.* (1999) studied the epizootiology of an FMD outbreak in Saudi Arabia during 1992-'98. All type O and Asia-1 isolates showed close antigenic relations with vaccine strain while three type A isolates showed antigenic variation.

Kumar *et al.* (1999) compared the FMD type O virus isolated from outbreaks in Haryana during 1996-'97 with vaccine strain. For all field strains tested, r value obtained was 0.9 to 1.0 indicating close relatedness of vaccine strain with outbreak strain.

2.8.2 The host factor

Immunity of Foot-and-Mouth disease in cattle appears to be mainly dependent on serum neutralizing antibody levels present at the time of exposure to infection. A linear correlation has been described between the log SNT produced in cattle following a primary vaccination and the log antigen dose (140s) (Pay and Parker, 1977; Rweyemamu *et al.*, 1982).

Pay and Parker (1977) reported that animal to animal variation in the serum neutralizing antibody response produced in cattle even of the same age and breed following primary vaccination with a fixed antigen dose is quite large with a standard deviation of the mean log SNT of 0.4.

According to Mc Kercher and Graves (1977), oil-adjuvanted FMD vaccines appear to be only vaccine which afford a reasonable degree of protection in swine.

A number of protozoan diseases are known to cause immunosuppression, and trypanosomiasis have been shown to suppress the response of cattle to FMD vaccination (Scott *et al.*, 1977; Sharpe *et al.*, 1982).

Wild ruminants also respond well to aqueous aluminium hydroxide/saponin FMD vaccines (Hedger *et al.*, 1980). However, pigs require atleast two inoculations to produce an IgG response (Pay, 1991).

Significant differences have been described for the responses obtained in three genetically distinct strains of Friesian cattle of the same ages and under identical systems of husbandry (Frenkel *et al.*, 1982).

Gomes (1984) observed the influence of colostral antibodies on the anamnestic response of calves re-vaccinated against FMD. Two doses, 105 days apart were given to 59 calves aged six to 169 days which were born to regularly vaccinated dams. A relationship was found between the presence of colostral antibodies and the secondary response in 28 per cent and 37 per cent of animals re-vaccinated with aluminium hydroxide - saponin and oil-vaccines.

Black *et al.* (1986) studied the influence of age on the immune response to FMD vaccines and observed that no difference could be demonstrated between the responses of 12 and 24 month old cattle.

Sadir *et al.* (1988) observed that calves born to vaccinated dams did not respond to the aqueous vaccine 30 or 90 days post partum. When oil-adjuvanted vaccines were given to same group of animals, no response was elicited upto 21 days, but calves above 30 days old responded like adult cattle. In colostral antibody free calves, good antibody response was observed.

Ahmed *et al.* (1991) studied the immune response to FMD vaccines (monovalent type A_{22}) in *Trypanosoma evansi* infected guinea pigs. The infected animals showed a significant suppressions of both humoral and CMI responses.

The presence of maternal antibody will depress the response of young animals to FMD vaccination in varying degrees, depending on the level of antibody present and the antigen mass present in the vaccine (Pay, 1991).

2.8.3 The human factor (Handling and storage)

FMD vaccines, to be effective must be applied on continuing basis in a locality to lower the disease incidence (Graves, 1979).

FMD antigens are relatively labile, and their decay rate will be proportional to temperature and time. Maintaining FMD vaccines at 2 to 8°C upto the moment they are injected is necessary (Pay, 1991).

The shelf life of FMD vaccines is one year at 4°C and the vaccine must not be frozen for evoking an immune response in vaccinated animals (OIE manual, 1992).

The use of chemicals to sterilize the syringes, excessive use of alcohol while swabbing skin, administration through unconventional routes or inadequate dose may result in failure of an effective vaccine to stimulate protective immunity (Tizard, 1994).

Kumar (1996) stated that shorter shelf life and absolute requirement of cold chain are major deficiencies of present Foot-and-Mouth disease vaccines.

A study on the effect of storage temperature on the shelf life of FMD vaccine revealed that the vaccines stored at 6 to 8°C and 35 to 37°C maintained the protective immunity upto 30 days, but those samples kept at 41 to 43°C were not efficacious even on 10 days of storage (Anon, 1998).

Materials and Methods

3. MATERIALS AND METHODS

The study was carried out at the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy during June 1999 to July 2000.

3.1 Materials

3.1.1 Glasswares and reagents

All glasswares used were of either Borosil or Vensil brand and chemicals were of analytic or guaranteed reagent grade. All materials were processed by standard procedures (Hoskins, 1967) and sterilized by either keeping in hot air oven at 160°C for 60 minutes or autoclaving at 121°C for 15 minutes at 15 lbs pressure, depending on the material sterilized.

3.1.2 Experimental animals

Calves which were above four months of age and not vaccinated against Foot and Mouth disease were selected for the study from different Kerala Agricultural University farms viz., University Livestock Farm, Mannuthy, Cattle Breeding Station, Thumburmuzhi and Livestock Research Station, Thiruvazhamkunnu.

A total of 43 calves were selected which were apparently healthy and free from clinical illness. They were grouped into three at random as follows:

Group I : 12 animals

Group II : 12 animals

Group III : 10 animals

3.1.3 Vaccines

Three different commercial Foot and Mouth disease vaccines were used for the study. They are:

Vaccine I : Raksha*

Vaccine II : Raksha-O Vac**

Vaccine III : Clovax***

3.1.4 Liquid phase blocking sandwich enzyme linked immunosorbent assay (LPB-ELISA)

3.1.4.1 ELISA plates

Flat bottom 96 well ELISA plates (TARSON) were used as the test plates and 'U' bottom 96 well plates (TARSON) were used as the carrier plates for the LPB-ELISA.

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* Raksha – Inactivated quadrivalent aluminium hydroxide gel vaccine against O, A, C and Asia-1 strains of Foot-and-Mouth disease, manufactured by Indian Immunologicals Ltd.

** Raksha-O Vac – Inactivated quadrivalent oil-adjuvanted vaccine against O, A, C and Asia-1 strains of Foot-and-Mouth disease, manufactured by Indian Immunologicals Ltd.

*** Clovax – Inactivated quadrivalent oil-adjuvanted vaccine against O, A, C and Asia-1 strains of Foot-and-Mouth disease, manufactured by Hoechst Roussel Vet. (P). Ltd.

3.1.4.2 Reagents

a. Coating Buffer (0.5 M carbonate – Bicarbonate Buffer) pH 9.6

Sodium carbonate	3.18 g
Sodium bicarbonate	5.86 g
Distilled water to make	2000 ml

(First dissolved the reagents in 500 ml distilled water and made upto 2000 ml).

b. Dulbecco's Phosphate Buffered Saline (DPBS) pH 7.2

(i) Stock solution (5x)

Sodium chloride	40.0 g
Potassium chloride	1.0 g
Magnesium chloride ($MgCl_2.6H_2O$)	0.5 g
Potassium dihydrogen orthophosphate	1.0 g
Disodium hydrogen ortho phosphate	7.15 g
Calcium chloride ($CaCl_2.2H_2O$)	0.5 g
Distilled water to make	1000 ml

(Dissolved $CaCl_2$:$2H_2O$ separately in distilled water and added)

(ii) Working solution (1x)

DPBS stock solution (5x)	1000 ml
Distilled water	4000 ml

c. Dulbecco's Phosphate Buffered Saline – Tween-20 (DPBS-T)

Tween-20	0.5 ml
DPBS(1x)	1000 ml

d. Citrate Buffer (Substrate buffer) pH 5.0

Citric acid	5.11 g
Disodium hydrogen ortho phosphate	7.3 g
Distilled water to make	2000 ml

e.(i) Substrate solution

Orthophenylene diamine dihydrochloride (Sigma)	30 mg
Citrate buffer	75 ml

(iii) Activated substrate solution

30% Hydrogen peroxide	0.001 ml
Substrate solution	2 ml

f. Reaction stopper solution (1M H_2SO_4)

Conc. Sulphuric acid	60 ml
Distilled water	2000 ml

g. Blocking buffer

Normal bovine serum	10 ml
Normal rabbit serum	5 ml
DPBS-T	85 ml

3.1.4.3 Biologicals

a. Antigen

Inactivated O, A, C and Asia-1 Foot and Mouth disease virus antigens were used.

b. Anti '146s' immune rabbit serum (IRS)

Type specific rabbit antisera against O, A, C and Asia-1 FMDV antigens were prepared by two subcutaneous inoculation of inactivated 146s FMDV in Freund's complete adjuvant (FCA) (Have and Jensen, 1983). Animals were exsanguinated after the second inoculation. Sera were dispensed into aliquots and stored at –20°C.

c. Anti '146s' immune guinea pig serum (IGPS)

Type specific guinea pig antisera against O, A, C and Asia-1 FMD antigens were prepared by single inoculation of inactivated 146s FMDV in FCA as described by Ferris and Donaldson (1984). Guinea pigs were exsanguinated after 28 days. Collected sera were pooled, dispensed into aliquots and stored at –20°C.

d. Anti guinea pig - Horse radish peroxidase conjugated IgG

Anti guinea pig horse radish peroxidase conjugated IgG (Sigma) was used at a working dilution of 1 in 2000 in blocking buffer.

3.2 Methods

3.2.1 Vaccination of animals

Group I

All the 12 calves of this group were vaccinated with vaccine I as follows.

Primary vaccination : at 4 months of age

First booster dose : at 5 months of age

Second booster dose : at 11 months of age

Dose : 3 ml

Route of vaccination : Subcutaneous

Group II

All the 12 calves of this group were vaccinated with vaccine II as follows.

Primary vaccination : at 4 months of age

First booster dose : at 13 months of age

Dose : 2 ml

Route of vaccination : Deep intramuscular

Group III

All the 10 calves of this group were vaccinated with vaccine III as follows.

Primary vaccination : at 4 months of age

First booster dose : at 7 months of age

Second booster dose : at 13 months of age

Dose : 3 ml

Route of vaccination : Deep intramuscular

3.2.2 Collection of serum samples

All the calves were exsanguinated before vaccination. Separated the serum and inactivated at 56°C for 30 minutes in water bath. The samples were stored at –20°C which formed the pre-vaccinated, 0^{th} month samples.

All the calves were bled at monthly interval for a period of one year from the date of primary vaccination. Serum samples were separated and inactivated at 56°C for 30 minutes in water bath. All the serum samples were stored at –20°C.

3.2.3 Liquid phase blocking sandwich enzyme linked immunosorbent assay (LPB-ELISA)

LPB-ELISA was employed for the assessment of serum neutralizing antibody titre against O, A, C and Asia-1 foot and mouth disease virus antigens. The procedure was carried out as per Hamblin *et al.* (1986a).

3.2.3.1 Antigen

BHK-21 cell adapted, azridine inactivated O, A, C and Asia-1 FMD virus antigens were used for the LPB-ELISA.

3.2.3.2 Standardization of reagents

The working dilution of antigens, immune rabbit serum, immune guinea pig serum and anti guinea pig HRPO conjugated IgG were assessed by checker board titration procedure.

The working dilution of different reagents are as follows:

a. Antigen

O	:	1 in 4
A	:	1 in 4
C	:	1 in 4
Asia-1	:	1 in 4

Antigen dilutions were made in DPBS-T.

b. Immune rabbit serum (IRS)

O	:	1 in 2000
A	:	1 in 2000
C	:	1 in 2000
Asia-1	:	1 in 1500

IRS dilutions were made in coating buffer.

c. Immune guinea pig serum (IGPS)

O	:	1 in 2000
A	:	1 in 2000
C	:	1 in 2000
Asia-1	:	1 in 2000

IGPS dilutions were made in blocking buffer.

c. Anti guinea pig HRPO conjugated IgG (Sigma)

Working dilution 1 in 2000 (in blocking buffer)

3.2.3.3 Test procedure

3.2.3.3.1 Coating of test plates

All the four types of IRS were made into corresponding working dilutions with coating buffer (0.5 M carbonate bicarbonate buffer).

Flat bottom 96 well ELISA plates (TARSON) were used for coating with IRS. Added 50 µl of IRS at working dilution to all the 96 wells. Sealed the plates and kept at room temperature in a moist chamber overnight for coating.

3.2.3.3.2 Preparation of carrier plates

All the four types of antigens were made into corresponding working dilution with DPBS-T.

'U' bottom 96 well microtitre plates (TARSON) were used. Added 50 µl of DPBS-T to all the 96 wells. Added 50 µl of test serum samples in 1 to 10 wells of first row, i.e. sample I in 1A, Sample II in 2A etc. (ten samples on a single plate). Two-fold dilutions were made column-wise (A to H wells of 1 to 10 columns).

Added 50 µl of corresponding antigen at working dilution to all the wells except in 12th column of the carrier plate. Sealed the plates and kept at +4°C overnight for neutralization.

3.2.3.3.3 Transfer of serum-antigen mixture to test plates

Washed the IRS coated plates five times with DPBS-T and tapped to dry. Transferred the contents of carrier plates to the corresponding wells of test plates. Only 50 µl of serum antigen mixture was transferred from each carrier plate well. Sealed the plates and incubated at 37°C for one hour with intermittent shaking.

3.2.3.3.4 Addition of detecting antibodies

All the four types of IGPS were made into corresponding working dilution with blocking buffer.

Test plates were washed five times with DPBS-T and tapped to dry. Added 50 µl of corresponding IGPS at working dilution to all the wells. Sealed the plates and incubated at 37°C for one hour with intermittent shaking.

3.2.3.3.5 Addition of conjugate

Anti guinea pig HRPO conjugated IgG (Sigma) was made into a working dilution of 1 in 2000 with blocking buffer.

Test plates were washed five times with DPBS-T and tapped to dry. Added 50 µl of conjugate at working dilution to all the wells. Sealed the plates and incubated at 37°C for one hour with intermittent shaking.

3.2.3.3.6 Addition of substrate

Washed the test plates five times with DPBS-T and tapped to dry. Added 50 µl of activated substrate solution to all the wells. The plates were kept in darkness for 15 minutes.

3.2.3.3.7 Addition of stopper solution

After 15 minutes, plates were taken out and added 50 µl of 1M H_2SO_4 to all the wells.

3.2.3.3.8 Reading of the plates

The optical density (O.D.) values were assessed using a multi-scan spectrophotometer at a wavelength of 492 nm after setting the 12^{th} column as the column blank.

3.2.3.4 Controls

The 12^{th} column of each plate was taken as the blank where antigen was not added. The 11^{th} column of each plate was taken as the antigen control for the corresponding antigen where test serum is not added.

3.2.3.5 Interpretation of readings

The serum neutralizing antibody titre against the corresponding Foot and Mouth disease antigen was estimated as the 50 per cent optical density end point of each serum dilution obtaining from the mean O.D value of antigen control and expressed as $\log_{10}$ of the serum dilution.

3.2.3.6 Statistical analysis

Statistical analysis of the results obtained were done as per Snedecor and Cochran (1994).

Results

4. RESULTS

All the serum samples collected from the test animals were subjected to Liquid-phase blocking ELISA (Plate 1) for estimation of serum neutralizing antibody titres against FMDV types O, A, C and Asia-1. From the optical density values obtained, SN_{50} log_{10} values were calculated and were taken as the antibody titres. The antibody titres obtained are presented in Tables 1 to 12.

Comparison of results between groups for all the four serotypes were done by analysis of variance and presented in Tables 13 to 16. Comparison of results between adjacent months were done by paired students t-test and the table of t-values obtained are presented in Tables 17 to 20.

4.1 Seroconversion produced following vaccination in three different groups

4.1.1 Seroconversion of type O antibodies

4.1.1.1 Group I

Table 1 shows the type O antibody titres of all the animals belonging to Group I from zero to 12^{th} month. The mean type O antibody titres of group I before vaccination was 1.209 ± 0.150 and became 1.507 ± 0.136 in twelfth month of study. The highest mean type O antibody titre of 2.202 ± 0.248 was obtained during the second month and a lowest mean titre of 1.147 ± 0.115 was obtained in the fourth month of study.

There was a significant rise in antibody titre ($P<0.05$) from 1.681 ± 0.214 to 2.202 ± 0.248 during first to second month and a significant fall in antibody titre ($P<0.05$) from 1.662 ± 0.092 to 1.394 ± 0.121 during ninth to tenth month. The mean type O antibody titre of 1.813 ± 0.139 during third month was reduced to 1.147 ± 0.115 during fourth month which was significant at $P<0.01$ (Table 17).

4.1.1.2 Group II

Type O antibody titres of Group II animals are presented in Table 2. The mean type O antibody titres of group II before primary vaccination was 0.985 ± 0.107 and the mean titre during twelfth month was 1.741 ± 0.162. The highest mean antibody titre after primary vaccination was 2.066 ± 0.133 during second month and the lowest mean type O antibody titre during the study period following primary vaccination was 1.458 ± 0.101 during ninth month.

There was a highly significant increase in mean type O antibody titres ($P<0.01$) from 0.985 ± 0.107 to 1.932 ± 0.231 following primary vaccination from zero to first month (Table 17).

4.1.1.3 Group III

Table 3 shows the type O antibody titres of all the animals in Group III. The mean type O antibody titre before vaccination was 0.988 ± 0.139 before primary vaccination in group III. The mean titre recorded during twelfth month

was 1.286 ± 0.142. Following primary vaccination during the zero month, the highest mean antibody titre obtained was 1.832 ± 0.126 during the sixth month and the lowest mean antibody titre obtained was 0.895 ± 0.085 during fourth month.

There was a significant increase ($P<0.05$) in mean antibody titres from 0.895 ± 0.085 to 1.242 ± 0.139 during fourth to fifth month. A highly significant rise in mean type O antibody titres during fifth to sixth month (1.242 ± 0.139 to 1.832 ± 0.126) and a highly significant fall ($P<0.01$) in mean type O antibody titres during sixth to seventh month (1.832 ± 0.126 to 1.457 ± 0.120) were also recorded (Table 17).

4.1.1.4 Comparison of type O FMD antibody titres between groups

Table 13 and Figure 1 shows the comparison of type O antibody titres between three of groups in each month. A significant difference in antibody titres between groups I and III, and groups II and III were observed during second and third month of study. In fourth month, a significant difference in antibody titres between groups I and II, and Groups II and III were present. In eighth month, antibody titres between groups I and II, and I and III were significantly different.

4.1.2 Seroconversion of Type A antibodies

4.1.2.1 Group I

Table 4 shows the type A antibody titres of Group I animals. The pre-vaccination mean type A antibody titre of group I was 1.504 ± 0.153. The mean type A antibody titre was recorded as 1.507 ± 0.136 during twelfth month. Following the primary vaccination, highest mean titre was recorded during eighth month (2.032 ± 0.165) and lowest mean titre during fifth month (1.010 ± 0.098).

There was a highly significant ($P<0.01$) rise in mean antibody titre from zero to first month (1.054 ± 0.153 to 1.929 ± 0.202) and fall in mean titres during fourth to fifth month (1.580 ± 0.064 to 1.010 ± 0.098) - Table 18.

4.1.2.2. Group II

The antibody titres against type A FMD antigen in group II animals are presented in Table 5. The mean type A antibody titres of this group was 1.210 ± 0.202 before vaccination and was 1.913 ± 0.121 during twelfth month. Following primary vaccination, the highest mean titre of 2.319 ± 0.139 was obtained during tenth month and the lowest mean titre of 1.299 ± 0.115 was obtained during fifth month.

A significant rise in mean antibody titres ($P<0.05$) was noted during zero to first month (1.210 ± 0.202 to 1.989 ± 0.240) and a reduction from third to fourth month (2.109 ± 0.159 to 1.738 ± 0.121). The increase in mean titres

from 1.562 ± 0.133 to 2.319 ± 0.139 during ninth to tenth month was highly significant (P<0.01). A highly significant (P<0.01) reduction in mean type A antibody titres were observed during fourth to fifth month (1.738 ± 0.121 to 1.299 ± 0.115) and during tenth to eleventh month (2.319 ± 0.139 to 2.010 ± 0.136) -Table 18.

4.1.2.3. Group III

Table 6 shows the antibody titres of animals in Group III against type A FMD antigen. Group III showed a mean type A antibody titre of 0.729 ± 0.095 before primary vaccination and it became 1.160 ± 0.123 during twelfth month, at the end of the study. Following the primary vaccination in zero month, the highest mean antibody titre was noted during sixth month (2.033 ± 0.253) and the lowest mean titre during fifth month (0.713 ± 0.047).

A highly significant rise (P<0.01) in mean serum antibody titres was noticed from zero to first month (0.729 ± 0.095 to 1.734 ± 0.183) and from fifth to sixth month (0.713 ± 0.047 to 2.033 ± 0.253). A highly significant fall in mean titres from 1.169 ± 0.108 to 0.713 ± 0.047 was observed during fourth to fifth month (P<0.01). A significant (P<0.05) reduction in mean titres from 2.033 ± 0.253 to 1.577 ± 0.193 during sixth to seventh month was observed (Table 18).

4.1.2.4 Comparison of type A FMD antibody titres between groups

Table 14 and Figure 2 presents the comparison of type A antibody titres of the animals of all the three groups. A significant difference was observed between groups I and III, and groups II and III during third and fourth month. During fifth month, all the three groups showed significantly different antibody titres. During tenth and eleventh month, significant difference between groups I and II and groups II and III were noted. A significant antibody titre difference was observed between groups I and III and groups II and III was observed in the twelfth month.

4.1.3 Seroconversion of Type C antibodies

4.1.3.1 Group I

Table 7 shows the antibody titres of serum samples collected from group I animals against type C antigen. The mean type C antibody titres before vaccination in group I was 0.853 ± 0.121. A mean antibody titre of 1.760 ± 0.089 was observed during twelfth month. Following primary vaccination highest mean antibody titre was recorded during eighth month (2.232 ± 0.072) and lowest mean titre during fourth month (1.508 ± 0.104).

On statistical analysis, a highly significant ($P<0.01$) increase in mean titres from zero to first month (0.853 ± 0.121 to 1.803 ± 0.286) was recorded. A significant fall in mean type C antibody titres from 1.898 ± 0.124 to 1.508 ±

0.104 during third to fourth month (P<0.05) and from 2.066 ± 0.107 to 1.813 ± 0.133 during tenth to eleventh month (P<0.01) were also observed (Table 19).

4.1.3.2 Group II

The type C antibody titres of group II animals are presented in Table 8. The mean type C antibody titres of group II at the beginning of study was 1.211 ± 0.208 before vaccination and was 1.924 ± 0.136 at the end of study, during twelfth month. Highest mean antibody titre following primary vaccination was noted during tenth month (2.394 ± 0.095) and lowest mean titre following vaccination during ninth month (1.556 ± 0.104).

A highly significant rise in mean antibody titres (P<0.01) was recorded during zero to first month (1.211 ± 0.208 to 2.294 ± 0.196) and during ninth to tenth month (1.556 ± 0.104 to 2.394 ± 0.095). A significant fall in antibody titres from 1.958 ± 0.179 to 1.583 ± 0.176 during third to fourth month (P<0.05) and from 2.394 ± 0.095 to 2.003 ± 0.144 during tenth to eleventh month (P<0.01) were also observed (Table 19).

4.1.3.3 Group III

Type C antibody titres of group III animals are presented in Table 9. The mean type C antibody titres of group III animals were 1.062 ± 0.164 before vaccination and 1.374 ± 0.136 during twelfth month, at the end of study.

Highest mean titre of 2.016 ± 0.130 during sixth month and lowest mean titre of 1.204 ± 0.108 during fourth month were recorded following vaccination.

On statistical analysis, a highly significant (P<0.01) rise in mean antibody titres from 1.400 ± 0.187 to 2.016 ± 0.130 was observed during fifth to sixth month. Significant reduction in mean titres from 1.789 ± 0.218 to 1.204 ± 0.108 during third to fourth month (P<0.05) and from 2.016 ± 0.130 to 1.326 ± 0.126 during sixth to seventh month (P<0.01) were also recorded (Table 19).

4.1.3.4 Comparison of type C FMD antibody titres between groups

Table 15 and Figure 3 shows the comparison of type C antibody titres of all the three groups in different months. In seventh month, groups I and III were found as heterogenous. In eight and ninth months, significant difference was recorded between groups I and II and groups I and III. In tenth month, all the three groups were heterogenous with regards to their type C antibody titres. Significant difference observed between groups I and III and groups II and III were observed during eleventh and twelfth month.

4.1.4 Seroconversion of Type Asia-1 antibodies

4.1.4.1 Group I

The type Asia-1 antibodies of group I animals are presented in Table 10. The group I animals showed mean type Asia-1 antibody titre of 1.023 ± 0.110 before vaccination and it reached 1.485 ± 0.078 during twelfth month. Highest

mean antibody titre following vaccination was 1.904 ± 0.193 during second month and lowest mean titre following vaccination was 1.163 ± 0.089 during fifth month.

On statistical analysis, significant rise in antibody titres observed ($P<0.05$) during zero to first month (1.023 ± 0.110 to 1.696 ± 0.162), fifth to sixth month (1.163 ± 0.089 to 1.747 ± 0.176) and during seventh to eighth month (1.499 ± 0.144 to 1.864 ± 0.101). Highly significant reduction in mean type Asia-1 antibody titres recorded ($P<0.01$) during fourth to fifth month (1.671 ± 0.064 to 1.163 ± 0.089), eighth to ninth month (1.864 ± 0.101 to 1.583 ± 0.127) and during tenth to eleventh month (1.580 ± 0.107 to 1.389 ± 0.095) - Table 20.

4.1.4.2 Group II

Table 11 shows the type Asia-1 antibody titres of group I animals during the study period. The mean antibody titre of this group was 0.823 ± 0.118 before vaccination. A mean type Asia-1 antibody titre of 1.823 ± 0.162 was observed during twelfth month. Following vaccination, highest mean antibody titre observed during tenth month (2.375 ± 0.104) and lowest mean titre during fifth month (1.250 ± 0.115).

Statistical analysis revealed highly significant rise in mean titres ($P<0.01$) during zero to first month (0.823 ± 0.118 to 1.813 ± 0.196) and during ninth to tenth month (1.376 ± 0.136 to 2.375 ± 0.104). The fall in mean titres

during fourth to fifth month (1.927 ± 0.115 to 1.250 ± 0.115) and during tenth to eleventh month (2.375 ± 0.104 to 1.937 ± 0.153) were significant at P<0.01 (Table 20).

4.1.4.3 Group III

Table 12 shows the type Asia-1 antibody titres of group III animals. The mean type Asia-1 antibody titre of this group before vaccination was 0.627 ± 0.016 which became 1.364 ± 0.139 after twelve months, following vaccination. The highest mean titre following vaccination was noted during sixth month (1.859 ± 0.108) and lowest mean titre following vaccination was observed during second month (1.139 ± 0.206).

Statistical analysis of seroconversion revealed highly significant (P<0.01) rise in mean antibody titres during zero to first month (0.627 ± 0.016 to 1.174 ± 0.171), fifth to sixth month (1.289 ± 0.101 to 1.859 ± 0.108) and during ninth to tenth month (1.209 to 0.044 to 1.777 ± 0.092) -Table 20.

4.1.4.4 Comparison of type Asia-1 FMD antibody titres between groups

Table 16 and Figure 4 shows the comparison of type Asia-1 antibodies between three groups from zero to twelfth month. A significant difference in antibody titres were observed between group I and III before vaccination and on second month. During first month, group II and group III were found significantly different in type Asia-1 antibody titres. Significant difference

between groups I and III and groups II and III were observed in third and fourth months. During eighth month, significant difference was observed between groups I and II. Significant difference was observed between groups I and II. Significant difference was evident between groups I and II and groups II and III during tenth and eleventh months.

4.2 Protection attained by vaccination

According to Bengelsdorff (1989), the antibody titre required for protection of cattle against FMD virus was taken as 1:20 (SN_{50} $\log_{10}$ = 1.3) regardless of the virus type.

4.2.1 Group I

The mean antibody titres of group I animals against O, A, C and Asia-1 FMDV virus types and protective level required are shown in Fig 5.

4.2.1.1 Type 0

Mean type 'O' antibody titres of group I animals were above protective level during the entire study period except before vaccination and during fourth and fifth months. (Table 13, Fig 5).

4.2.1.2 Type A

Mean type 'A' antibody titres of group I animals were above protective level during the entire study period except before vaccination and during fifth month (Table 14, Fig 5).

4.2.1.3 Type C

The group I animas showed mean type 'C' antibody titres above protective level during the entire study period except before vaccination (Table 15, Fig. 5).

4.2.1.4 Type Asia-1

The mean type Asia-1 antibody titres of group I animals were above protective level during the entire study period except before vaccination and during fifth month (Table 16, Fig. 5).

4.2.2 Group II

The mean antibody titres of group II animals against FMDV types O,A,C and Asia I are presented in Fig.6 along with the antibody titre required for protection in cattle.

4.2.2.1 Type O

Group II animals showed protective mean type O antibody titres during the entire study period except before vaccination (Table 13, Fig. 6).

4.2.2.2 Type A

The mean type A antibody titres of group II animals were above protective level during the entire study period after vaccination except on fifth month (Table 14, Fig.6).

4.2.2.3 Type C

Group II animals had mean type C antibody titres above protective level during the entire study period after vaccination (Table 15, Fig.6).

4.2.2.4 Type Asia-1

The mean type Asia-1 antibody titres of group II animals were above protective level during the entire study period, except before vaccination and during fifth month (Table 16, Fig.6).

4.2.3 Group III

The mean antibody titres of group III animals against O,A,C and Asia-1 FMDV types along with level of protection required are plotted in Fig. 7.

4.2.3.1 Type O

The mean type O antibody titres of group III animals were above protective level except before vaccination and during first, second, third, fourth, fifth and twelfth months (Table 13, Fig. 7).

4.2.3.2 Type A

The group III animals showed mean type A antibody titres above protective level except before vaccination and during third, fourth, fifth, eleventh and twelfth months (Table 14, Fig. 7).

4.2.3.3 Type C

The mean type C antibody titres of group III animals were above protective level during the entire study period except before vaccination and during fourth month (Table 15, Fig.7).

4.2.3.4 Asia 1

The mean type Asia-1 antibody titres of group III animals were above protective level except before vaccination and during first, second, third, fifth, eighth and ninth months (Table 16, Fig. 7).

4.3 Economic assessment between groups

The cost factor involved in the vaccinations of three groups for the first year are shown in Table 21. The cost required for vaccinating a single animal of group I was Rs. 29.70 while that for group II and group III were Rs. 26.40 and Rs. 66.00 respectively for the first year (Fig. 8).

Table 1. The Type O FMD antibody titres of group I animals

Anim. No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
567	1.599	2.151	2.860	1.476	1.496	1.637	1.082	1.741	1.582	1.280	1.260	1.671	1.186
582	1.120	2.458	1.500	1.237	1.020	0.600	2.800	2.800	1.111	1.380	0.600	0.600	2.800
605	1.660	0.610	0.610	2.860	1.747	1.442	1.500	2.135	2.231	1.922	1.803	1.593	1.500
610	0.610	1.870	2.193	1.678	0.610	0.600	1.500	1.512	1.851	1.432	1.447	1.217	1.500
622	1.792	2.860	2.860	1.904	1.276	0.600	1.995	1.567	1.645	1.805	1.284	2.653	1.475
627	0.970	1.601	2.323	1.636	1.235	1.160	0.926	0.736	1.539	1.547	0.983	1.046	1.045
635	0.770	1.812	2.860	2.683	0.830	0.629	1.910	2.707	1.951	1.855	1.115	1.862	1.043
648	2.080	1.507	2.026	1.620	0.610	0.600	1.960	1.570	1.605	1.662	1.420	1.407	1.226
1262	0.610	2.077	2.860	1.730	1.446	1.522	1.861	1.229	2.260	1.156	1.351	1.686	1.268
1265	0.970	0.610	2.860	1.721	1.437	1.669	1.209	1.180	2.168	2.095	2.001	1.013	1.785
1266	1.665	2.003	2.860	1.483	0.610	1.306	1.292	1.415	1.783	1.643	1.381	1.339	1.603
1268	0.610	0.610	0.610	1.726	1.449	2.020	2.054	1.999	2.593	2.169	2.081	2.172	1.657
Mean±SE	1.209± 0.150	1.681± 0.214	2.202± 0.248	1.813± 0.139	1.147± 0.115	1.149± 0.150	1.674± 0.150	1.716± 0.176	1.860± 0.115	1.662± 0.092	1.394± 0.121	1.522± 0.159	1.507± 0.136

Table 2. The Type O antibody titres of group II animals

Anim. No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
568	0.702	2.723	3.359	1.597	1.275	1.321	1.095	1.543	1.835	1.749	1.890	1.975	2.021
586	1.088	2.860	2.860	2.198	2.036	1.540	0.600	1.250	1.871	1.729	1.830	1.955	1.866
569*	1.088	2.932	2.860	1.933	1.865	1.955	1.650	1.750	1.404	1.432	1.837	1.661	1.380
602	0.610	2.635	2.100	0.610	0.977	1.564	1.539	1.449	1.337	1.597	1.732	1.500	1.799
607	1.110	1.883	1.728	1.782	0.610	0.946	1.848	1.500	1.396	1.280	1.738	1.411	1.184
625	1.470	2.329	2.229	1.981	1.587	2.267	2.252	2.042	1.662	1.837	2.800	2.168	2.383
631	1.437	1.442	1.992	1.704	0.925	0.600	1.758	0.979	0.600	1.215	1.486	1.084	0.759
637	0.900	1.621	1.638	1.728	1.858	2.017	1.657	1.567	1.257	1.595	1.154	1.018	1.500
652	1.622	0.966	1.633	2.122	1.534	1.056	1.210	2.323	1.666	1.217	1.526	1.717	1.790
1269	0.610	0.610	1.648	1.383	1.586	1.246	1.923	0.907	1.368	1.810	1.629	1.767	2.800
1272	0.610	1.044	1.556	2.028	1.887	2.394	2.231	2.007	2.044	0.600	2.483	2.800	2.093
1276	0.610	2.143	2.194	1.656	1.791	1.328	1.273	1.287	1.479	1.431	1.616	1.001	1.315
Mean±SE	0.985± 0.107	1.932± 0.231	2.066± 0.133	1.727± 0.121	1.494± 0.130	1.519± 0.159	1.586± 0.139	1.551± 0.124	1.493± 0.107	1.458± 0.101	1.810± 0.127	1.671± 0.150	1.741± 0.162

Table 3. The Type O antibody titres of group III animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
578	0.610	0.592	1.557	0.610	1.270	0.978	1.245	0.600	0.978	0.992	1.515	1.217	1.074
604	0.720	1.746	1.342	1.423	1.138	1.740	2.003	1.500	1.230	1.500	1.983	2.032	1.981
609	0.610	1.445	1.087	1.266	0.610	0.950	2.145	2.051	1.662	1.384	1.789	0.600	0.600
619	1.640	1.130	0.783	0.760	0.610	1.071	1.665	1.756	1.500	1.262	1.588	1.206	1.500
626	1.210	0.610	1.455	1.488	0.861	0.871	1.634	1.354	1.337	1.268	1.600	1.777	1.500
632	0.830	1.456	1.278	1.620	0.833	1.252	1.662	1.350	1.048	1.232	1.500	1.673	1.283
646	1.670	1.269	1.638	1.533	0.610	1.302	2.704	1.657	1.820	1.497	2.797	1.692	1.731
653	1.366	1.376	0.610	2.237	0.695	0.600	1.741	1.537	1.078	1.105	0.600	0.800	0.600
1279	0.610	1.613	1.595	0.610	1.233	2.001	1.984	1.483	1.943	2.786	1.944	1.490	1.500
1281	0.610	1.292	1.534	1.118	1.098	1.660	1.540	1.277	1.505	1.267	2.543	1.431	1.093
Mean±SE	0.988± 0.139	1.253± 0.120	1.288± 0.114	1.267± 0.161	0.895± 0.085	1.242± 0.139	1.832± 0.126	1.457± 0.120	1.410± 0.104	1.429± 0.158	1.786± 0.193	1.392± 0.139	1.286± 0.142

Table 4. The Type A antibody titres of group I animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
567	1.081	1.864	2.093	1.818	1.765	1.637	0.914	1.925	1.500	1.289	1.090	1.314	1.029
582	1.081	2.039	0.610	0.859	1.309	0.600	2.800	0.600	0.945	0.600	0.681	0.600	1.500
605	2.020	0.610	0.610	2.381	1.882	1.038	1.174	2.289	2.800	2.053	2.195	1.966	1.914
610	0.610	1.316	2.166	1.633	1.204	0.600	1.500	2.800	2.800	2.800	2.415	1.333	1.993
622	1.620	2.780	2.180	1.880	1.524	1.119	2.800	2.800	2.800	2.051	2.800	1.750	2.799
627	0.610	2.370	2.360	1.808	1.480	0.837	2.186	1.706	1.924	0.930	1.314	1.299	1.107
635	0.610	1.097	2.055	2.121	1.477	0.725	2.800	2.365	2.134	1.891	1.359	2.739	2.400
648	1.683	1.632	2.609	1.907	1.422	0.770	2.800	2.559	2.162	2.800	1.982	2.118	1.820
1262	0.610	2.496	2.860	1.686	1.593	1.098	0.618	0.700	1.897	1.252	1.346	1.355	1.511
1265	0.610	0.610	1.621	1.785	1.684	1.500	0.715	2.369	1.753	1.755	1.968	1.053	1.249
1266	1.500	2.200	1.929	1.655	1.951	0.915	1.160	1.415	1.598	1.931	1.447	0.999	1.078
1268	0.610	2.860	1.611	1.565	1.671	1.281	1.185	1.090	2.059	1.981	1.767	1.606	1.344
Mean±SE	1.054± 0.153	1.929± 0.202	1.893± 0.202	1.758± 0.104	1.580± 0.064	1.010± 0.098	1.721± 0.257	1.885± 0.225	2.032± 0.165	1.778± 0.193	1.697± 0.173	1.436± 0.199	1.646± 0.162

Table 5. The Type A antibody titres of group II animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
568	1.691	2.343	1.829	1.788	1.656	1.025	0.920	1.263	1.132	1.500	1.716	1.465	1.359
586	1.862	2.860	2.860	2.860	2.045	1.511	0.600	1.473	1.966	1.748	2.857	2.800	2.133
569*	1.053	3.024	2.640	2.417	2.564	1.278	1.090	1.432	1.051	1.289	1.667	1.500	2.256
602	2.260	1.910	2.136	1.422	1.047	1.055	2.226	2.124	1.882	1.858	2.800	1.826	2.056
607	0.610	1.797	2.051	1.615	1.320	0.672	2.800	2.800	2.089	2.087	2.466	2.116	1.887
625	2.350	2.498	2.564	2.070	1.799	1.931	2.800	2.336	2.014	1.905	2.800	2.800	2.605
631	0.610	1.200	1.598	2.420	1.552	1.100	2.800	2.286	1.102	2.261	2.800	2.190	2.146
637	0.610	0.917	2.458	1.665	1.930	1.968	1.920	1.831	1.185	1.278	1.884	1.641	1.085
652	1.650	0.710	1.727	2.825	1.274	1.081	2.800	2.126	2.040	1.440	2.800	2.342	2.102
1269	0.610	2.860	2.097	1.510	1.573	0.997	1.863	0.689	1.032	1.675	1.828	1.510	1.461
1272	0.610	1.100	0.610	2.860	2.064	1.687	1.818	1.548	2.172	0.600	2.138	2.050	1.915
1276	0.610	2.646	2.253	1.858	2.032	1.279	1.153	1.279	0.717	1.100	2.077	1.881	1.950
Mean±SE	1.210± 0.202	1.989± 0.240	2.069± 0.173	2.109± 0.159	1.738± 0.121	1.299± 0.115	1.899± 0.234	1.766± 0.170	1.532± 0.153	1.562± 0.133	2.319± 0.139	2.010± 0.136	1.913± 0.121

Table 6. The Type A antibody titres of group III animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
580	0.610	0.532	1.419	1.484	1.126	0.600	1.042	0.600	0.600	0.600	0.600	2.800	2.800
604	0.610	1.396	1.175	0.610	1.555	0.858	2.207	1.500	1.609	1.955	1.500	1.373	1.417
609	0.610	2.280	1.396	0.855	1.245	0.600	2.800	1.500	2.028	1.753	1.976	0.600	0.886
619	0.610	1.579	1.960	0.610	0.870	0.600	2.930	2.443	2.094	1.973	1.952	1.201	1.719
626	0.640	1.630	1.623	0.730	0.691	0.673	2.110	1.976	1.863	1.754	1.568	1.701	1.475
632	0.610	1.050	1.537	1.473	1.270	0.600	2.095	1.752	1.810	2.268	1.380	1.891	1.021
646	0.610	1.503	1.779	1.903	0.672	0.788	2.800	1.883	2.314	1.697	2.108	1.921	1.635
653	1.552	2.860	0.610	2.480	1.071	0.600	2.420	2.152	1.970	1.914	1.775	1.675	1.216
1279	0.610	2.344	1.496	1.253	1.511	1.042	1.339	0.600	0.600	2.261	1.095	0.843	0.603
1281	0.610	1.588	1.441	1.588	1.216	0.769	1.028	1.365	0.600	0.672	2.800	0.864	0.910
Mean±SE	0.729± 0.095	1.734± 0.183	1.451± 0.114	1.211± 0.202	1.169± 0.108	0.713± 0.047	2.033± 0.253	1.577± 0.193	1.549± 0.215	1.685± 0.187	1.725± 0.164	1.281± 0.158	1.160± 0.123

Table 7. The type C antibody titres of group I animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
567	0.847	2.760	1.687	1.867	1.677	1.064	1.495	1.275	1.917	2.185	1.978	1.688	1.797
582	0.847	3.876	1.823	1.423	1.219	2.800	2.108	2.570	1.865	1.633	1.521	1.010	1.790
605	0.610	0.610	0.610	2.559	1.955	1.643	1.500	2.511	2.022	2.243	2.237	1.985	2.143
610	0.610	1.546	2.505	1.890	0.610	2.800	1.500	2.136	2.214	2.358	2.166	1.236	1.798
622	0.920	2.445	2.094	1.703	1.489	1.523	2.800	2.147	2.145	1.218	2.088	1.789	1.954
627	0.552	1.874	2.752	1.370	1.669	1.286	2.397	1.945	2.603	1.747	2.031	2.078	1.897
635	0.610	1.218	2.860	2.092	1.563	2.800	2.634	1.971	2.182	1.756	1.649	1.598	1.205
648	1.710	1.663	1.599	2.860	1.181	2.800	2.057	1.936	2.230	1.756	1.863	1.677	1.457
1262	0.610	2.323	1.594	1.833	1.757	1.429	1.740	1.691	2.367	2.566	2.386	2.332	2.260
1265	0.610	0.610	1.652	1.684	1.600	1.532	1.901	1.722	2.631	2.238	2.978	2.765	1.462
1266	1.700	2.106	1.803	1.712	1.710	2.800	1.430	1.676	2.473	1.534	1.863	1.752	1.492
1268	0.610	0.610	0.990	1.782	1.660	1.801	1.926	1.907	2.136	2.467	2.028	1.847	1.865
Mean±SE	0.853± 0.121	1.803± 0.286	1.831± 0.191	1.898± 0.124	1.508± 0.104	2.023± 0.205	1.957± 0.133	1.957± 0.104	2.232± 0.072	1.975± 0.121	2.066± 0.107	1.813± 0.133	1.760± 0.089

Table 8. The Type C antibody titres of group II animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
568	1.610	2.740	2.322	1.800	1.684	2.125	1.775	1.250	1.537	1.537	2.304	2.062	1.974
586	1.128	2.860	2.860	2.860	2.346	2.800	0.600	1.508	2.043	1.909	2.467	2.220	1.875
569*	1.128	3.267	2.860	2.182	2.107	2.800	1.996	1.689	0.870	1.500	2.237	1.906	1.462
602	2.270	1.883	1.060	1.826	1.035	2.800	1.987	1.958	1.540	1.732	2.175	1.497	1.980
607	0.610	2.860	2.471	1.950	1.367	1.147	2.098	1.496	1.604	1.980	2.479	1.985	1.755
625	2.670	2.860	2.710	2.860	1.923	2.126	2.800	2.397	2.379	2.175	2.800	2.886	2.957
631	0.610	1.512	1.556	1.498	1.072	0.908	1.572	0.997	1.653	1.150	2.083	1.539	1.721
637	0.881	1.152	2.351	1.757	2.052	2.318	2.065	1.844	1.926	1.852	2.190	1.192	1.352
652	1.800	1.890	1.397	2.393	0.610	1.547	1.967	1.878	1.249	1.511	2.907	2.097	2.198
1269	0.610	2.640	0.610	1.591	1.530	1.226	1.569	1.047	0.923	1.351	2.709	2.191	2.175
1272	0.610	1.473	0.610	2.235	2.122	2.800	1.843	1.923	2.412	0.850	2.698	2.800	2.116
1276	0.610	2.391	2.391	1.890	1.910	1.068	1.658	0.844	1.320	1.305	2.333	1.857	1.712
Mean±SE	1.211± 0.208	2.294± 0.196	1.929± 0.228	1.958± 0.179	1.583± 0.176	1.962± 0.222	1.832± 0.147	1.613± 0.139	1.651± 0.147	1.556± 0.104	2.394± 0.095	2.003± 0.144	1.924± 0.136

Table 9. The Type C antibody titres of group III animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
578	1.074	0.610	2.860	0.910	1.443	1.500	1.369	0.696	1.906	0.913	1.933	1.289	0.904
604	1.250	0.976	1.787	2.109	1.431	1.374	1.927	1.170	1.978	1.894	1.715	1.836	2.160
609	0.610	1.504	1.989	1.860	1.298	2.800	2.800	1.500	1.371	2.519	1.746	0.850	1.111
619	0.610	1.681	1.715	0.680	1.148	1.782	2.193	1.272	1.585	0.997	1.253	1.041	1.634
626	1.739	1.411	1.698	1.795	0.663	1.063	2.133	1.417	1.146	1.341	1.185	1.776	1.500
632	0.610	2.860	1.222	2.630	1.489	0.600	2.227	1.436	0.994	1.524	1.840	1.630	1.250
646	1.800	1.476	1.956	2.186	0.610	1.515	2.057	2.129	1.754	1.301	2.350	1.375	1.980
653	1.710	2.860	0.610	2.800	0.993	1.018	2.079	1.615	0.770	1.492	1.248	0.744	0.941
1279	0.610	1.825	1.665	1.364	1.490	1.144	1.985	0.844	1.843	2.088	2.283	1.145	1.212
1281	0.610	1.600	1.467	1.559	1.480	1.205	1.386	1.178	1.304	1.764	1.478	1.194	1.048
Mean±SE	1.062± 0.164	1.680± 0.225	1.697± 0.180	1.789± 0.218	1.204± 0.108	1.400± 0.187	2.016± 0.130	1.326± 0.126	1.465± 0.130	1.583± 0.155	1.703± 0.133	1.388± 0.155	1.374± 0.136

Table 10. The Type Asia-1 antibody titres of group I animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
567	0.883	1.710	2.113	2.080	1.849	1.173	1.062	1.761	1.585	1.139	1.264	1.236	1.125
582	1.000	2.117	2.860	1.633	1.403	0.600	2.800	0.600	0.975	0.787	0.654	0.669	2.117
605	1.007	0.610	0.610	1.664	2.116	1.571	1.500	2.036	2.136	1.803	1.633	1.662	1.483
610	1.008	1.308	1.724	1.256	1.385	1.012	2.543	1.652	2.029	1.929	2.011	1.349	1.247
622	0.610	2.590	2.860	2.370	1.716	0.976	2.396	1.752	1.672	1.800	1.350	1.024	1.421
627	1.660	1.242	2.240	2.117	1.681	0.890	1.472	0.800	1.781	1.499	1.622	1.586	1.242
635	0.675	1.127	1.952	1.620	1.718	0.983	1.968	1.438	2.003	1.437	1.663	1.500	1.663
648	1.490	1.474	1.781	1.430	1.543	1.000	2.127	2.246	1.953	2.149	1.854	1.798	1.699
1262	0.610	2.110	2.251	1.197	1.739	1.324	1.221	0.953	1.974	1.114	1.770	1.500	1.394
1265	1.510	1.900	0.892	1.911	1.500	1.242	1.157	1.544	2.351	2.066	1.870	1.404	1.509
1266	1.210	2.030	1.908	1.860	1.501	1.552	1.026	1.392	1.753	1.293	1.419	1.157	1.289
1268	0.610	2.130	1.654	2.013	1.900	1.637	1.689	1.809	2.150	1.975	1.855	1.778	1.627
Mean±SE	1.023± 0.110	1.696± 0.162	1.904± 0.193	1.763± 0.104	1.671± 0.064	1.163± 0.089	1.747± 0.176	1.499± 0.144	1.864± 0.101	1.583± 0.127	1.580± 0.107	1.389± 0.095	1.485± 0.078

Table 11. The Type Asia-1 antibody titres of group II animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
568	0.823	1.849	1.830	2.015	1.776	1.132	0.971	1.228	1.349	1.589	2.880	1.925	1.997
586	0.610	2.860	2.860	2.860	2.493	1.486	0.744	1.337	1.806	1.882	2.742	2.800	2.292
569*	0.823	2.550	1.712	2.690	2.548	1.290	1.707	1.646	1.500	1.129	2.393	1.862	1.048
602	0.610	2.151	0.673	1.880	1.192	0.954	1.789	1.809	1.506	1.598	2.367	1.037	1.811
607	0.610	1.540	2.361	1.544	1.557	0.951	2.041	1.338	1.770	1.315	2.036	1.807	1.312
625	0.884	2.445	1.763	1.847	2.051	2.141	2.036	2.726	2.279	2.130	2.800	2.800	2.800
631	0.823	1.200	1.803	2.103	1.738	0.709	1.985	1.147	0.827	0.600	2.037	1.257	2.569
637	2.070	0.898	1.513	1.470	2.322	1.808	1.371	2.264	1.689	1.500	1.986	2.224	1.663
652	0.610	1.600	1.923	0.941	1.597	1.014	1.638	1.584	1.239	1.146	2.470	1.627	1.425
1269	0.610	2.050	2.260	2.025	1.895	1.272	1.497	0.688	1.067	1.836	2.032	1.980	1.215
1272	0.790	2.000	0.610	2.860	1.880	1.026	1.671	1.966	1.365	0.668	2.800	2.259	2.241
1276	0.610	0.610	1.540	2.056	2.076	1.220	1.307	1.375	1.005	1.117	1.952	1.663	1.500
Mean±SE	0.823± 0.118	1.813± 0.196	1.654± 0.208	2.024± 0.165	1.927± 0.115	1.250± 0.115	1.563± 0.118	1.592± 0.156	1.450± 0.115	1.376± 0.136	2.375± 0.104	1.937± 0.153	1.823± 0.162

Table 12. The Type Asia-1 antibody titres of group III animals

Anim.No.	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
578	0.610	0.610	2.620	0.610	1.667	1.016	1.092	0.979	1.027	1.125	1.465	1.513	1.199
604	0.610	1.262	1.096	1.349	1.634	1.611	2.027	2.776	1.165	1.241	1.931	1.999	1.935
609	0.610	0.610	0.863	0.833	1.420	1.016	2.109	1.551	1.500	1.432	2.063	0.668	1.053
619	0.780	1.740	0.610	1.083	1.229	1.277	2.295	1.614	0.877	1.379	1.522	1.500	1.175
626	0.610	1.172	0.683	2.295	0.865	1.100	1.671	1.821	0.600	1.182	2.004	1.487	1.638
632	0.610	1.990	0.610	1.449	1.529	1.230	1.935	1.364	1.019	1.074	1.581	1.500	0.669
646	0.610	0.610	1.267	0.610	0.634	1.166	2.127	1.393	0.757	1.224	2.227	1.778	1.771
653	0.610	2.061	0.610	0.610	1.201	0.924	1.935	1.133	1.332	0.972	1.500	1.333	0.796
1279	0.610	0.766	1.818	1.484	1.518	1.830	1.729	1.389	1.920	1.276	1.988	1.500	1.645
1281	0.610	1.192	1.210	1.550	1.749	1.719	1.675	1.318	1.299	1.187	1.488	1.444	1.755
Mean±SE	0.627± 0.016	1.174± 0.171	1.139± 0.206	1.187± 0.174	1.318± 0.114	1.289± 0.101	1.859± 0.108	1.534± 0.158	1.150± 0.123	1.209± 0.044	1.777± 0.092	1.472± 0.108	1.364± 0.139

Table 13. Comparison of the mean Type O (mean ± SE) antibody titres of three groups

Groups	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Group I	1.209± 0.150	1.681± 0.214	2.202± 0.248[a]	1.813± 0.139[a]	1.147± 0.115[a]	1.149± 0.150	1.674± 0.150	1.716± 0.176	1.860± 0.115[a]	1.662± 0.092	1.394± 0.121	1.522± 0.159	1.507± 0.136
Group II	0.985± 0.107	1.932± 0.231	2.066± 0.133[a]	1.727± 0.121[a]	1.494± 0.130[b]	1.519± 0.159	1.586± 0.139	1.551± 0.124	1.493± 0.107[b]	1.458± 0.101	1.810± 0.127	1.671± 0.150	1.741± 0.162
Group III	0.988± 0.139	1.253± 0.120	1.288± 0.114[b]	1.267± 0.161[b]	0.895± 0.085[a]	1.242± 0.139	1.832± 0.126	1.457± 0.120	1.410± 0.104[b]	1.429± 0.158	1.786± 0.193	1.392± 0.139	1.286± 0.142
Cd (1,2)	NS	NS	0.511	0.392	0.323	NS	NS	NS	0.312	NS	NS	NS	NS
Cd (1,3) and (2,3)			0.536	0.411	0.338				0.327				

Values in same column bearing same superscript do not differ significantly (P<0.05)
NS – No significant difference between the groups

Table 14. Comparison of the mean Type A (mean ± SE) antibody titres of three groups

Groups	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Group I	1.054± 0.153	1.929± 0.202	1.893± 0.202	1.758± 0.104[a]	1.580± 0.064[a]	1.010± 0.098[a]	1.721± 0.257	1.885± 0.225	2.032± 0.165	1.778± 0.193	1.697± 0.173[a]	1.436± 0.199[a]	1.646± 0.162[a]
Group II	1.210± 0.202	1.989± 0.240	2.069± 0.173	2.109± 0.159[a]	1.738± 0.121[a]	1.299± 0.115[b]	1.899± 0.234	1.766± 0.170	1.532± 0.153	1.562± 0.133	2.319± 0.139[b]	2.010± 0.136[b]	1.913± 0.121[a]
Group III	0.729± 0.095	1.734± 0.183	1.451± 0.114	1.211± 0.202[b]	1.169± 0.108[b]	0.713± 0.047[c]	2.033± 0.253	1.577± 0.193	1.549± 0.215	1.685± 0.187	1.725± 0.164[a]	1.281± 0.158[a]	1.160± 0.123[b]
Cd (1,2)	NS	NS	NS	0.435	0.281	0.267	NS	NS	NS	NS	0.447	0.471	0.388
Cd (1,3) and (2,3)				0.456	0.295	0.280					0.469	0.494	0.407

Values in same column bearing same superscript do not differ significantly (p<0.05)
NS – No significant difference between the groups

Table 15. Comparison of the mean Type C (mean ± SE) antibody titres of three groups

Groups	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Group I	0.853± 0.121	1.803± 0.286	1.831± 0.191	1.898± 0.124	1.508± 0.104	2.023± 0.205	1.957± 0.133	1.957± 0.104 [a]	2.232± 0.072 [a]	1.975± 0.121 [a]	2.066± 0.107 [a]	1.813± 0.133 [a]	1.760± 0.089 [a]
Group II	1.211± 0.208	2.294± 0.196	1.929± 0.228	1.958± 0.179	1.583± 0.176	1.962± 0.222	1.832± 0.147	1.613± 0.139 [ab]	1.651± 0.147 [b]	1.556± 0.104 [b]	2.394± 0.095 [b]	2.003± 0.144 [a]	1.924± 0.136 [a]
Group III	1.062± 0.164	1.680± 0.225	1.697± 0.180	1.789± 0.218	1.204± 0.108	1.400± 0.187	2.016± 0.130	1.326± 0.126 [b]	1.465± 0.130 [b]	1.583± 0.155 [b]	1.703± 0.133 [c]	1.388± 0.155 [b]	1.374± 0.136 [b]
Cd (1,2)	NS	NS	NS	NS	NS	NS	NS	0.349	0.337	0.354	0.309	0.402	0.339
Cd (1,3) and (2,3)								0.366	0.354	0.372	0.324	0.422	0.356

Values in same column bearing same superscript do not differ significantly (P<0.05)
NS – No significant difference between the groups

Table 16. Comparison of the mean Type Asia-1 (mean ± SE) antibody titres of three groups

	Months												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Group I	1.023± 0.110	1.696± 0.162 [a]	1.904± 0.193 [ab]	1.763± 0.104 [a]	1.671± 0.064 [a]	1.163± 0.089	1.747± 0.176	1.499± 0.144	1.864± 0.101 [a]	1.583± 0.127	1.580± 0.107 [a]	1.389± 0.095 [a]	1.485± 0.078
Group II	0.823± 0.118	1.813± 0.196 [ab]	1.654± 0.208 [a]	2.024± 0.165 [a]	1.927± 0.115 [a]	1.250± 0.115	1.563± 0.118	1.592± 0.156	1.450± 0.115 [b]	1.376± 0.136	2.375± 0.104 [b]	1.937± 0.153 [b]	1.823± 0.162
Group III	0.627± 0.016	1.174± 0.171 [b]	1.139± 0.206 [b]	1.187± 0.174 [b]	1.318± 0.114 [b]	1.289± 0.101	1.859± 0.108	1.534± 0.158	1.150± 0.123 [ab]	1.209± 0.044	1.777± 0.092 [a]	1.472± 0.108 [a]	1.364± 0.139
Cd (1,2)	0.276	0.498	0.570	0.416	0.276	NS	NS	NS	0.317	NS	0.288	0.346	NS
Cd (1,3) and (2,3)	0.290	0.523	0.598	0.436	0.290				0.333		0.303	0.363	

Values in same column bearing same superscript do not differ significantly (P<0.05)
NS – No significant difference between the groups

Table 17. Table of t- values between months- Type O

	0&1	1&2	2&3	3&4	4&5	5&6	6&7	7&8	8&9	9&10	10&11	11&12
Group I	2.002	2.337*	1.250	4.331**	0.014	2.074	0.321	0.649	1.902	2.969*	0.773	0.057
Group II	3.647**	1.009	2.031	1.803	0.200	0.395	0.213	0.514	0.232	2.068	1.521	0.534
Group III	1.310	0.196	0.092	1.719	3.074*	3.557**	3.742**	0.439	0.173	1.698	2.210	1.620

Table values of t_{11}: at 5% level: 2.201 at 1% level: 3.106 * significant at 5% level
Table values of t_9 : at 5% level: 2.228 at 1% level: 3.169 ** significant at 1% level

Table 18. Table of t- values between months- Type A

	0&1	1&2	2&3	3&4	4&5	5&6	6&7	7&8	8&9	9&10	10&11	11&12
Group I	3.697**	0.133	0.647	1.822	7.477**	2.174	0.549	0.926	1.841	0.636	1.207	1.340
Group II	2.777*	0.410	0.155	2.366*	3.824**	2.200	0.858	1.534	0.154	5.851**	3.960**	0.928
Group III	6.805**	1.059	0.857	0.166	4.373**	4.918**	3.015*	0.243	0.700	0.142	1.934	0.976

Table values of t_{11}: at 5% level: 2.201 at 1% level: 3.106 * significant at 5% level
Table values of t_9 : at 5% level: 2.228 at 1% level: 3.169 ** significant at 1% level

Table 19. Table of t- values between months- Type C

	0&1	1&2	2&3	3&4	4&5	5&6	6&7	7&8	8&9	9&10	10&11	11&12
Group I	3.384**	0.092	0.254	2.393*	1.855	0.269	0.001	1.893	1.836	0.790	3.396**	0.354
Group II	4.153**	1.662	0.122	2.283*	2.002	0.479	1.718	0.272	0.593	5.698**	3.900**	0.711
Group III	2.145	0.042	0.255	2.229*	0.902	3.585**	5.331**	0.647	0.573	0.657	1.735	0.076

Table values of t_{11}: at 5% level: 2.201 at 1% level: 3.106 * significant at 5% level
Table values of t_9 : at 5% level: 2.228 at 1% level: 3.169 ** significant at 1% level

Table 20. Table of t- values between months- Type Asia-1

	0&1	1&2	2&3	3&4	4&5	5&6	6&7	7&8	8&9	9&10	10&11	11&12
Group I	3.049*	1.280	0.700	0.868	6.667**	2.368*	1.062	2.936*	3.354**	0.025	3.118**	0.710
Group II	3.782**	0.713	1.349	0.658	6.437**	1.763	0.176	1.258	0.684	6.705**	3.199**	0.631
Group III	3.223**	0.104	0.160	0.629	0.260	3.892**	2.152	1.822	0.486	6.938**	2.174	0.884

Table values of t_{11}: at 5% level: 2.201 at 1% level: 3.106 * significant at 5% level
Table values of t_9 : at 5% level: 2.228 at 1% level: 3.169 ** significant at 1% level

Table 21. Cost of three vaccines required for first one year in cattle

Vaccine	Cost of vaccine per dose (Rs.)*	Number of doses required for first one year	Total cost (Rs.)
Vaccine I	9.90	3	29.70
Vaccine II	13.20	2	26.40
Vaccine III	22.00	3	66.00

* Maximum retail price as per Anon. (2000)

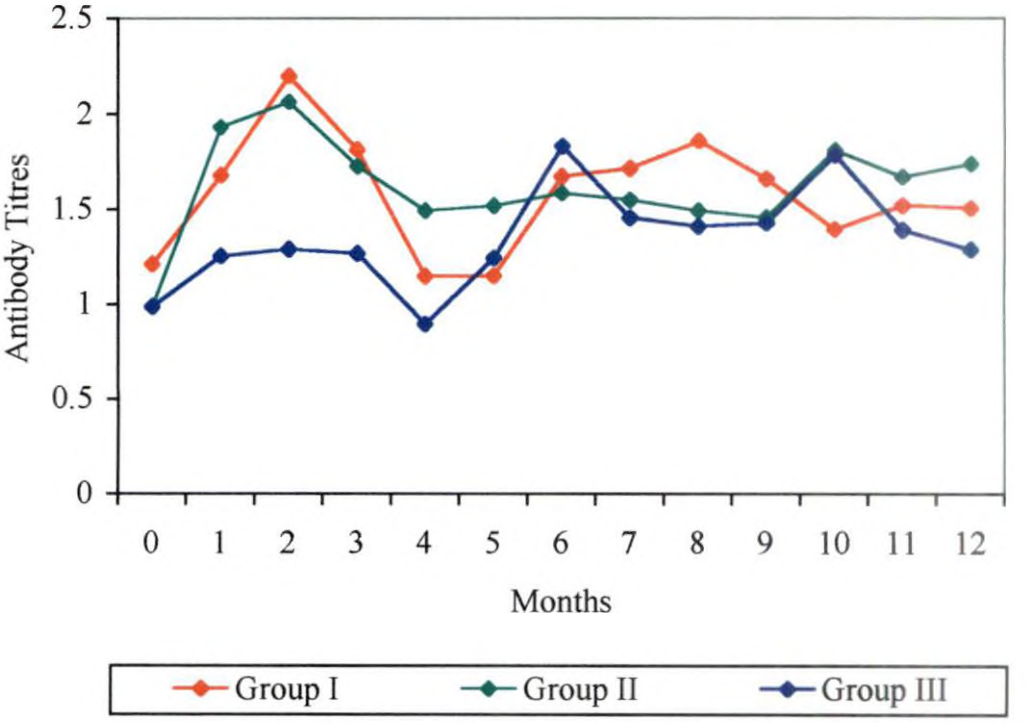

Fig.1. Seroconversion of Type O antibodies of three groups

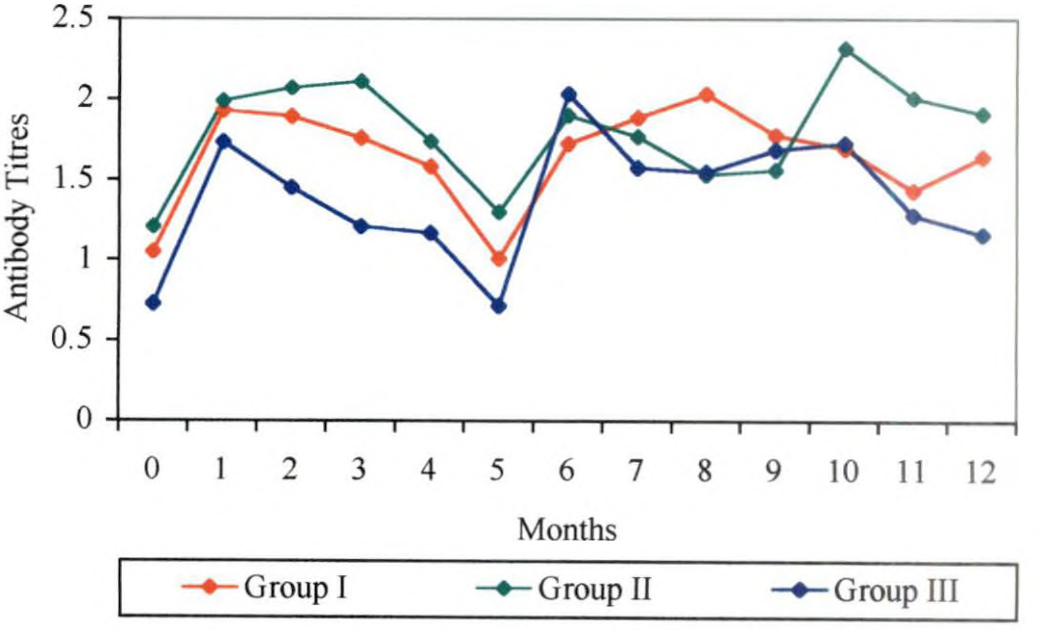

Fig.2. Seroconversion of Type A antibodies of three groups

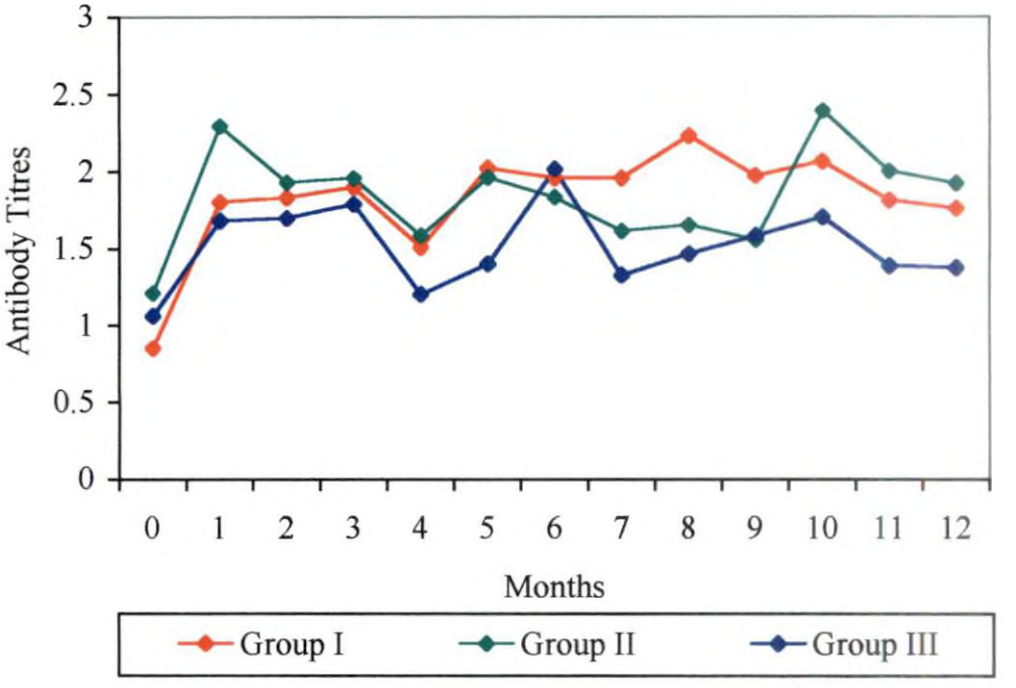

Fig.3. Seroconversion of Type C antibodies of three groups

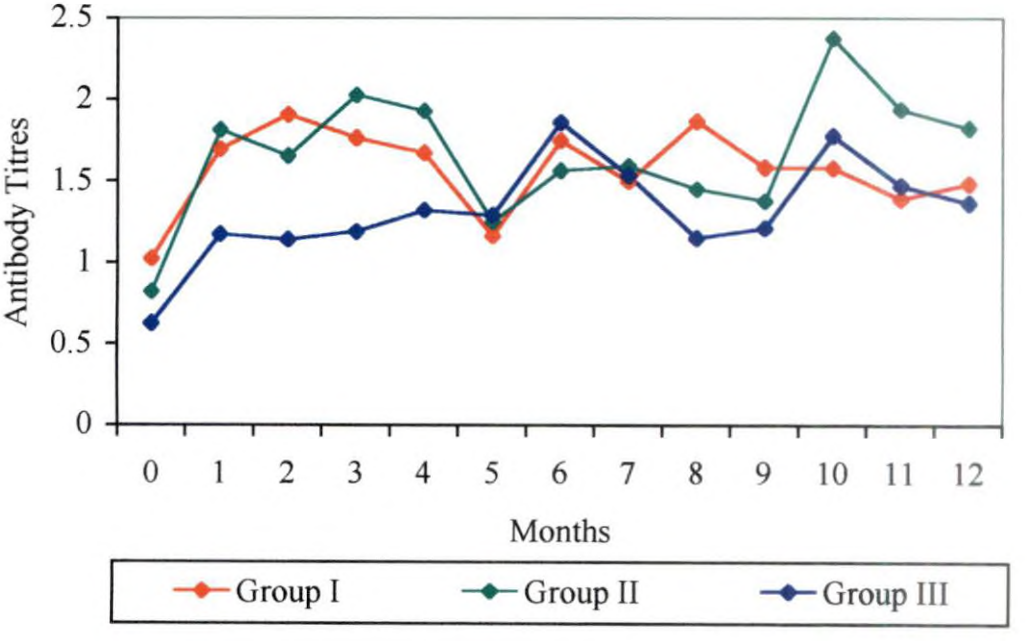

Fig.4. Seroconversion of Type Asia-1 antibodies of three groups

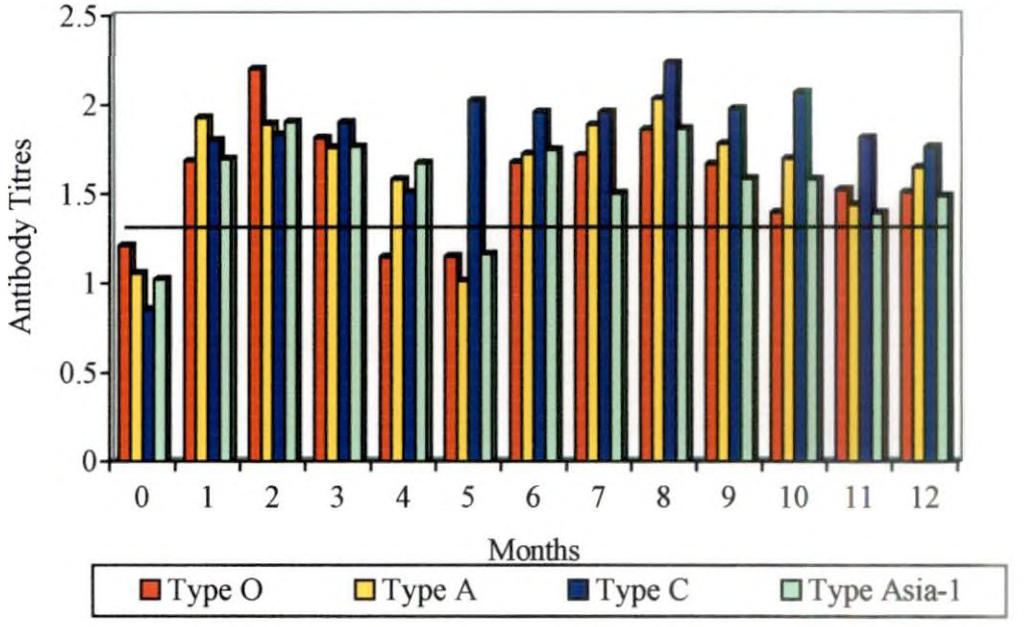

Fig.5. The antibody titres of Group I animals against the four FMDV types
(The black line indicates the level of antibody titre required for protection)

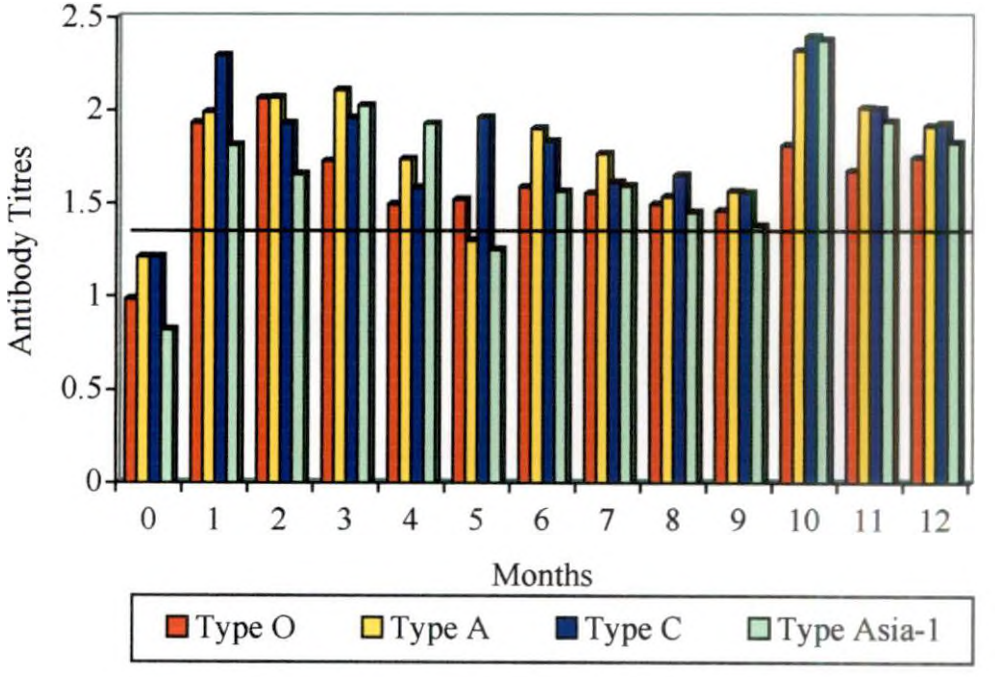

Fig. 6. The antibody titres of Group II animals against the four antibody types
(The black line indicates the level of antibody titre required for protection)

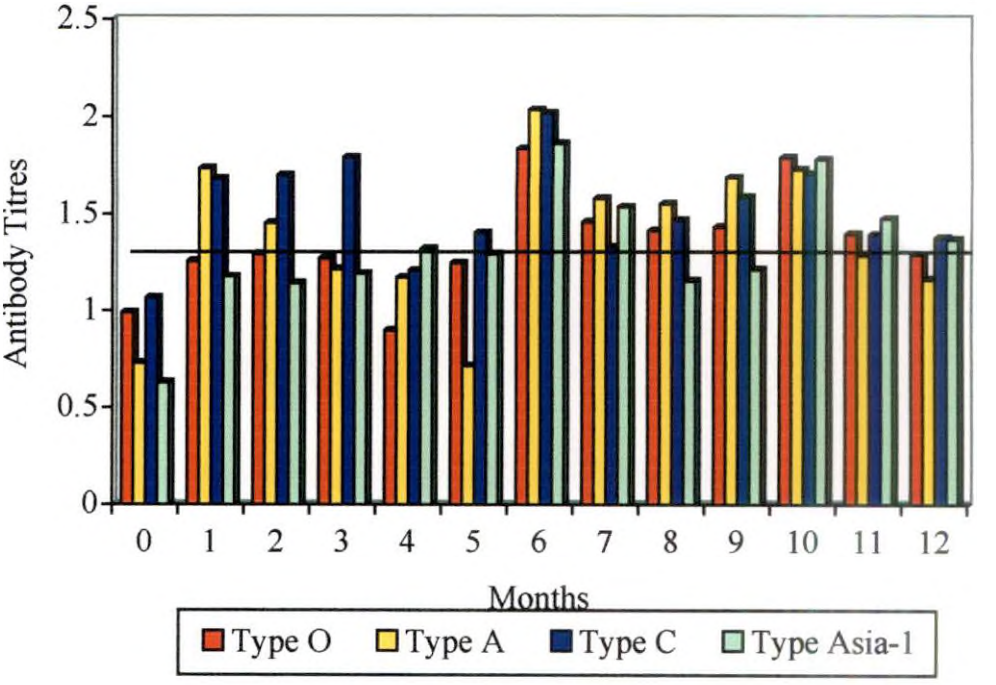

Fig.7. The antibody titres of Group III animals against the four FMDV types

(The black line indicates the level of antibody titre required for protection)

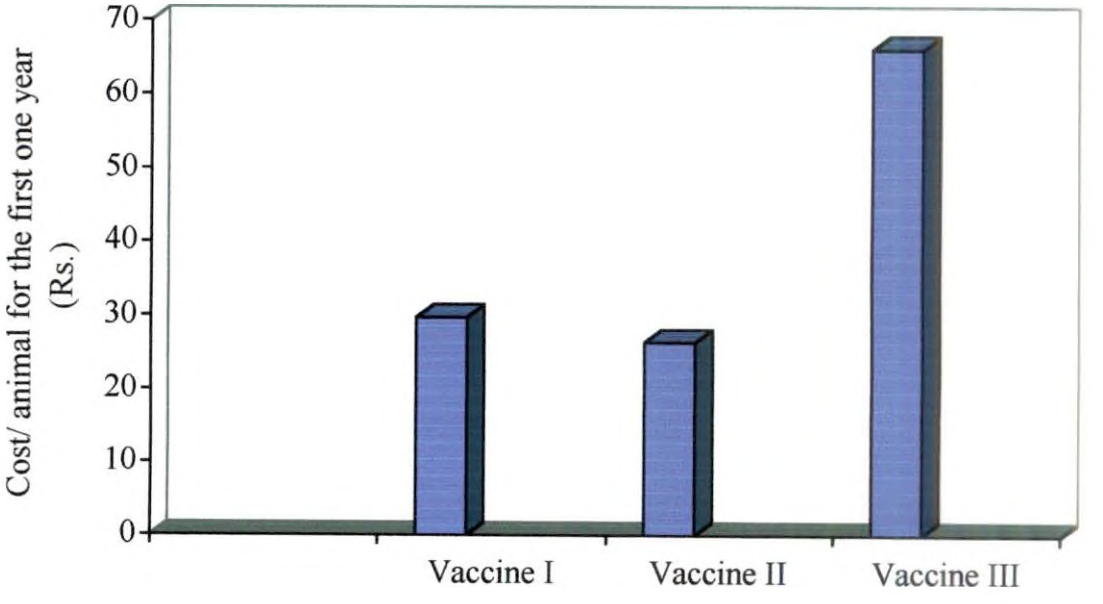

Fig.8. Cost of three vaccines required per cattle for the first one year

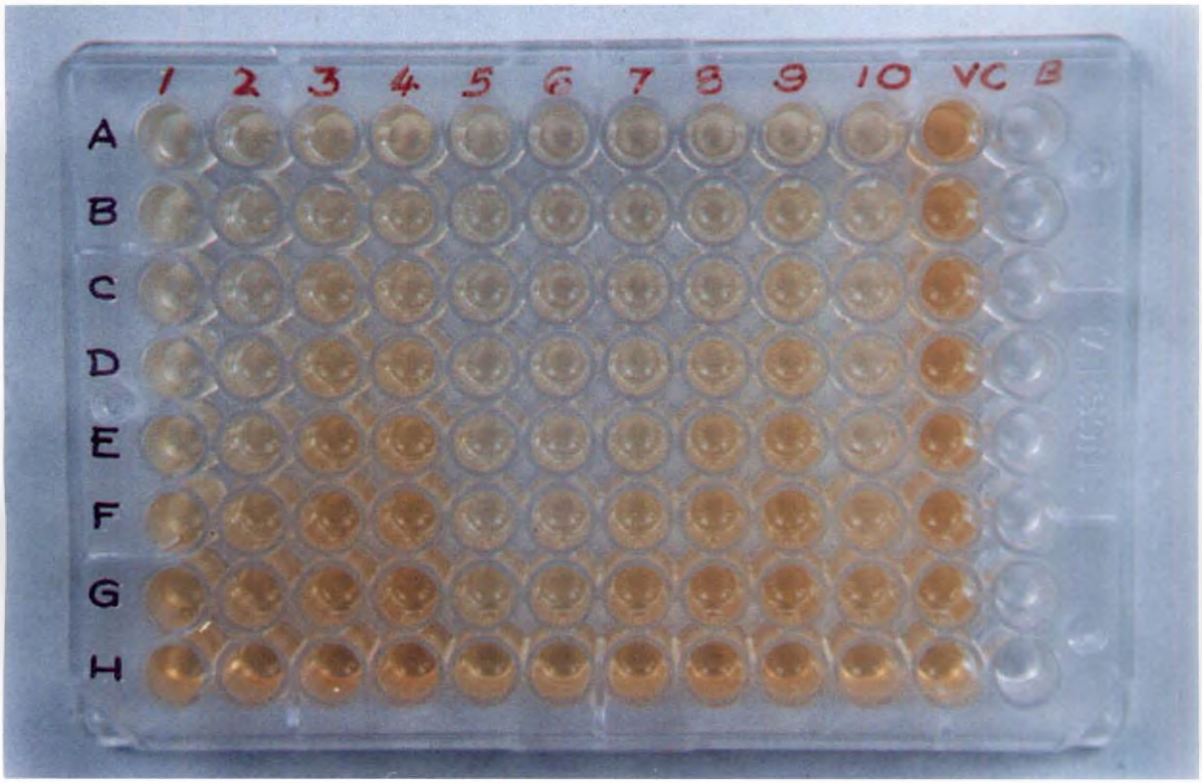

Plate 1. Liquid Phase Blocking Enzyme Linked Immunosorbent Assay (LPB - ELISA) - Test Plate

Columns 1 to 10
Samples 1 to 10 in two - fold dilutions from A to H

Column VC
Virus control

Column B
Column blank

5. DISCUSSION

In the present study, the seroconversion of three different Foot-and-Mouth disease vaccines were assessed in cattle, of which one vaccine was aluminium hydroxide gel adsorbed, saponin adjuvanted and two were oil adjuvanted vaccines. All the three vaccines used were inactivated quadrivalent FMD vaccines against types O,A,C, and Asia-1. Serum neutralizing antibodies against virus types O, A, C and Asia-1 were assessed monthly over a period of one year employing liquid phase blocking Enzyme linked immunosorbent assay (LPB-ELISA).

5.1 Seroconversion of type O FMD antibodies

Group I and group II animals attained a protective level of mean antibody titre in the first month itself while group III animals showed only a slight increase in antibody titre from a pre-vaccination titre of 0.988 ± 0.139 to 1.253 ± 0.120 within first thirty days. During second month, group III animals showed a significantly lower mean type O antibody titre compared to group I and group II.

Rao *et al.* (1993) studied the immune response of aqueous and oil adjuvanted FMD vaccines in cattle and observed that both vaccines produced satisfactory immune response in first 21 days. The results obtained in group I and II agrees with this findings, while group III animals, eventhough vaccinated with oil adjuvanted vaccine showed lower titre compared to group II animals. Group I and group II animals did not show any significant difference in mean type O FMD antibody titre throughout the study period, except during eighth month. Mc Kercher and Graves (1977) conducted a trial in South America and obtained a significantly higher immune response with oil-adjuvant vaccines, where as Mowat (1974) observed no significant difference between oil-adjuvant and aqueous FMD vaccine in eliciting immune response.

The significantly higher antibody titre in group I animals during eighth month is because of the anamnestic response produced by the booster vaccination given during seventh month.

Effect of booster vaccinations were clearly evident in all the three groups. Group I animals showed highest mean antibody titre during second month as a result of first booster vaccination. Rao *et al.* (1993) observed no significant difference between antibody titres of two groups,

one with a booster dose on 21 days post vaccination and another group without a booster vaccination in a trial with an aqueous FMD vaccine.

Animals of all the three groups showed a reduction in mean type O FMD antibody titre during fourth month. An outbreak of Foot-and-Mouth disease occurred all over the state during the same month. The virus type associated with the outbreak was detected as type O (Saseendranath, 2000). The possible reason for a reduced mean type O antibody titre during fourth month is the direct neutralization of antibodies with the virus. Eventhough, none of the test animals suffered with the disease, this neutralization might have reduced mean type O antibody titre. The immunosuppression occurred in the susceptible population due to the FMD outbreak is another reason for reduced antibody titre. Tizard (1994) described viral infections as one of the reasons for immunosuppression.

5.2 Seroconversion of Type A FMD antibodies

Animals of all the three groups showed increased mean type A FMD antibody titre as a result of primary vaccination. Mean antibody titres of all the three groups reached protective titre within first thirty days.

Group III showed a reduced level of mean type A antibody titre after first month, throughout the study period as compared to group I and group II. No significant difference was observed between group I and group II animals in terms of mean type A FMD antibody titres throughout the study period, except during fifth, tenth and eleventh months. The increased mean antibody titres of group II during tenth and eleventh months were because of the booster dose of vaccine II during ninth month. Nair and Sen (1993b) observed that immunogenicity of aluminium-hydroxide gel and oil adjuvanted FMD vaccines in sheep do not differ significantly over a period of eight weeks. But a higher response with oil adjuvant vaccine was observed by Mello *et al.* (1975), Mc Kercher and Graves (1977) and Rivenson *et al.* (1982).

Mean pre-vaccination titre of all the groups were below the protective level. All the three groups responded well to the primary vaccination. This result was in accordance with the observation made by Spath *et al.* (1995). First booster vaccination in group III during third month did not produce an anamnestic response while second booster dose during ninth month produced a slight increase in mean antibody titre.

In group I, a gradual decline in antibody titre was observed after the initial rise. Auge-de-Mello and Gomes (1977) in a trial with aqueous

FMD vaccines observed highest antibody titre at 30 days post vaccinations and a gradual decline thereafter.

All the three groups showed drastic reduction in mean type A FMD antibody titres during fourth and fifth months. The lowering in mean antibody titres in all the three groups occurred as a result of the FMD outbreak during these months can be the most probable reason for this poor response. None of the test animals were affected during this FMD outbreak, eventhough they showed a reduction in mean type A antibody titres.

5.3 Seroconversion of type C FMD antibodies

All the three groups did not show any significant difference in mean type C FMD antibody titres upto seventh month of study. Group I and group II showed close values for mean antibody titres throughout the study period, except during eighth, ninth and tenth months. During eighth and ninth months, group I animals showed an increased mean titre when compared to group II as a result of the booster dose of vaccine I given to group I animals during seventh month. A booster dose of vaccine II given to the animals of group II during ninth month resulted in an increased antibody titre during tenth month. Rivenson *et al.* (1982) observed higher immune response with oil-adjuvanted vaccine while the result obtained

did not agree with this. Mowat (1974) observed no significant difference between aqueous and oil adjuvanted vaccines in eliciting immune response.

Group III showed a lower mean type C antibody titre throughout the study period when compared to other two groups.

All the three groups responded positively to the booster vaccinations. Increase in mean antibody titres were recorded in all the three groups after respective booster vaccinations. Tizard (1994) describes that repeated injections of antigen produce immune response with shorter lag period and for a longer period of time than single inoculation.

The mean type C antibody titres of all the three groups showed a reduction during the fourth month and thereafter, antibody titres raised. The most possible reason for this reduction is the outbreak of FMD during that month among cattle all over Kerala.

The mean type C antibody titres of all the three groups were above the level required for protection throughout the study period, except for group III in the fourth month. The results obtained for all the

three groups over a period of one year was satisfactory, but the oil-adjuvanted vaccines did not produced significantly higher titres compared to aqueous vaccines which is not in accordance with the observations of Rivenson *et al.* (1982) and Rao *et al.* (1993).

5.4 Seroconversion of Type Asia-1 FMD antibodies

Mean type Asia-1 antibody titres of group I and group II were not significantly varying throughout the study period, except during eighth, tenth and eleventh months. A higher titre of groups I during eighth month recorded as a result of the booster vaccination with vaccine I during the seventh month. As a result of booster dose administration of vaccine II to the animals of group II during ninth month, the mean type Asia-1 antibody titres of group II found significantly higher than that of group I during tenth and eleventh months. Similarity between results obtained from group I and II are in agreement with Mello *et al.* (1975) and Nair and Sen (1993b).

Group III showed lower antibody titres compared to other two groups during most of the study period. Same was the condition for group III in case of mean antibody titres against FMD antigen types O, A and C also.

All the three groups responded to the booster vaccination by an increase in mean antibody titre. The response of group I and II were satisfactory while the primary vaccination in group III resulted only in a mild increase in mean antibody titre. Group III reached the protective titre only after the first booster dose with vaccine III during third month. Response of group I and group II animals to the booster vaccination agrees with Tizard (1994).

The mean type Asia-1 antibody titres of all the three groups came below the protective level during fifth month. The possible reasons for this have already discussed with other antibody types.

Group I and group II animals maintained protective level of type Asia-1 mean antibody titres throughout the study period, except during fifth month. A satisfactory level of antibody response with both aqueous and oil-adjuvanted vaccines upto 270 days was obtained by Rao *et al.* (1993). Group III animals, eventhough vaccinated with oil-adjuvanted FMD vaccine, reached protective antibody titre against FMDV type Asia-1 only during fourth month, then came below the protective level by eighth month and after the booster vaccination during ninth month, again reached above the protective level.

5.5 Economic assessment of the three different FMD vaccines used

The three different vaccines *viz.*, Vaccine I, II and III were compared on the basis of cost required for immunising a single calf for the first one year. Three doses each of vaccine I and vaccine III were required for the first year while only two doses of vaccine II was needed to be administered as per respective manufacturers recommendations. Total cost required for the first year was lowest for vaccine II and was highest for vaccine III. But the high cost required for vaccine III in the first year will reduce in the subsequent years, as vaccine III has to be administered at an interval of nine to twelve months subsequently as against Vaccine I, which is being an aluminium hydroxide gel vaccine which is to be administered in every six months for maintaining the protective level of antibodies. Mc Kercher and Graves (1977) opined that aqueous vaccines provides immunity only for a shorter period compared to oil-adjuvanted FMD vaccines. Astudillo and Auge-de-Mello (1980) reported that oil adjuvated FMD vaccines can be preferred over aluminium hydroxide gel vaccines in terms of cost factor. The oil-adjuvanted vaccines can reduce the cost of protecting animals, both in

price of vaccines and reduced labour requirement since only annual vaccination is recommended.

Summary

6. SUMMARY

The seroconversion of three different Foot-and-Mouth disease vaccines were studied in cattle of Kerala Agricultural University farms. For this study, two different oil-adjuvanted, and one aluminium hydroxide gel adsorbed-saponin adjuvanted inactivated quadrivalent FMD vaccines were used. Vaccinations were done in cattle above four months of age without previous vaccination, as per respective manufacturer's regime. The antibody titres against Foot-and-Mouth disease virus types O, A, C and Asia-1 were assessed in every month employing liquid phase blocking Enzyme-linked immunosorbent assay (LPB-ELISA).

Mean type O antibody titres in all the three groups found below protective level before the first vaccination. Group I and group II animals showed protective level of type O antibody titres throughout the study period except during fourth and fifth months, during which, group I showed a mean antibody titre below protective level. Group III animals showed a lower antibody titre throughout the study period. All the three groups responded to respective booster vaccinations.

Primary vaccination of all the three groups resulted in an increase in mean type A antibody titres above protective level. Group I and group II animals maintained the protective antibody titres throughout the study period

except during fifth month. Response to booster vaccinations were evident except in group I and group III for their first booster vaccinations.

All the three groups maintained protective mean type C antibody titres throughout the study period, except for group III during fourth month. Group III animals, eventhough maintained a protective level of immune response, showed lower mean antibody titres when compared to group I and group II during the study period. All the three groups showed a reduction in mean type C antibody titres during fourth month. Booster vaccinations produced an increased antibody titres in all the three groups, but a reduction in mean type C antibody titre was observed in group III following first booster vaccination during third month.

Group I and group II animals maintained protective mean type Asia-1 antibody titre during the entire study period, except during fifth month. Group III animals reached protective titre only during sixth month. All the vaccinations including primary and booster vaccinations produced increase in antibody titre than the previous month in all the three groups.

Comparison between the three vaccines in terms of cost required for immunising a single animal for the first year was made. Vaccine III required the highest cost and vaccine II required the lowest cost for the first year. Comparison between vaccine I and vaccine II showed that oil vaccines can reduce the vaccination cost compared to aqueous vaccines since the oil-

vaccines produce longer immunity. Vaccine III, eventhough an oil-adjuvanted vaccine required the highest cost for the first year as the vaccine has to be administered three times in the first year as per the schedule given by its manufacturers, but the subsequent vaccinations are required only at an interval of nine to twelve months.

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SEROCONVERSION OF THREE DIFFERENT FOOT-AND-MOUTH DISEASE VACCINES IN CATTLE

By

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ABSTRACT OF A THESIS

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ABSTRACT

Seroconversion of three different commercial inactivated quadrivalent Foot-and-Mouth disease vaccines were studied. One aluminium hydroxide gel vaccine and two oil-adjuvanted vaccines were used for the study in three groups of cattle. Monthly assessment of antibody titres against O, A, C and Asia-1 antigens were made by liquid phase blocking-ELISA (LPB-ELISA). Unvaccinated calves of four months and above age were grouped into three and vaccinations were made as per manufacturer's regime.

Group I and group II animals did not showed significant difference in type O antibody titres throughout the study period except following booster vaccination of group I in seventh month. Both groups maintained protective titres except in the fourth and fifth months of study. Group III showed a lower level of antibody titres throughout the study period.

Protective type A antibody titres were maintained by group I and group II animals during the entire study period except in the fifth month. No significant difference between these two groups observed except following the second booster vaccination in group II. Group III showed comparatively lower antibody titres against type A FMD antigen.

All the three groups showed protective mean type C antibody titres throughout the study period. But group III showed an antibody titre below protective level during fourth month. Group I and group II did not show significant variation in antibody titres except following respective booster vaccinations.

Group I and group II maintained a protective level of type Asia-1 antibody titres except during fifth month. Group III showed significantly low Asia-1 antibody titres throughout the study period.

The booster vaccinations produced anamnestic response in group I and group II in almost all cases. Group III animals showed lower antibody titres against all the four virus types when compared to group I and group II and response to booster vaccinations were poor in group III.

Comparison between the three vaccines in terms of cost required for immunising a single animal for the first one year revealed that oil-adjuvanted vaccine II required the lowest cost while oil-adjuvanted vaccine III required the highest cost for the first one year.