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EVALUATION OF DIFFERENT ADJUVANT FORMULATIONS OF INACTIVATED TRIVALENT FOOT AND MOUTH DISEASE VACCINE IN CATTLE

MVSc. THESIS

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY, AND VETERINARY PUBLIC HEALTH

BY

GETU AYELE

AUGUST, 2021

BISHOFTU, ETHIOPIA

EVALUATION OF DIFFERENT ADJUVANT FORMULATIONS OF INACTIVATED TRIVALENT FOOT AND MOUTH DISEASE VACCINE IN CATTLE

MVSc THESIS

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology

By

Getu Ayele

August, 2021

Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by Getu Ayele entitled: **Evaluation of different adjuvant formulations of inactivated trivalent foot and mouth disease vaccine in cattle** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: **Masters of Veterinary Science in Veterinary Microbiology**.

_____	_____	_____
Chairman	Signature	Date
Professor Tesfaye Sisay (DVM, MVSc, PhD)	_____	_____
External examiner	Signature	Date
Professor Gezahegn Mamo (DVM, MVSc, PhD)	_____	_____
Internal examiner	Signature	Date
Dr. Bedaso Mammo (DVM, MVSc, PhD)	_____	_____
Major Advisor	Signature	Date
Dr. Belayneh Getachew (DVM, MVSc, PhD)	_____	_____
Co- Advisor	Signature	Date
Professor Gezahegn Mamo (DVM, MVSc, PhD)	_____	_____
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STATEMENT OF THE AUTHOR

First, I declare that this thesis is my authentic work and that all sources of material used for this thesis have been appropriately acknowledged. This thesis has been submitted in partial fulfilment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Name: Getu Ayele Signature: _____

College of Veterinary Medicine and Agriculture

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LIST OFABBREVATIONS

AAU-CVMA	Addis Ababa University College of Veterinary Medicine and Agriculture
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ARERC	Animal Research Ethics Review Committee
AS	Aluminum hydroxide gel-Saponin
BHK	Baby Hamster Kidney
CARD	Caspase Activation and Recruitment Domain
CD	Cluster Differentiation
CI	Confidence Interval
CMI	Cell Mediated Immunity
CPE	Cytopathic Effect
CTL	Cytotoxic T Lymphocytes
DC	Dendritic Cell
DPV	Day Post Vaccination
ELISA	Enzyme-Linked Immune Sorbent Assay
FAO	Food and Agriculture Organization
FeLV	Feline Leukemia Virus
FIA	Freunds Incomplete Adjuvant
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
HRPO	Horse Reddish Peroxidase
IFN	Interferon
Ig	Immunoglobulin
MAb	Monoclonal Antibody
MEM	Minimum Essential Medium
MHC	Major Histocompatibility Complex
NAHDIC	National Animal Health and Diagnostic Center
NLR	Nucleotide Binding Oligomerization Domain Like Receptor

NLRP	Nucleotide Binding Oligomerization Domain Like Receptor Protein
NOD	Nucleotide binding Oligomerization Domain
NSP	Non Structural Protein
NVI	National Veterinary Institute
O/W	Oil in Water
OD	Optical Density
OIE	Office International des Epizooties
ORF	Open Reading Frame
PANVAC	Pan African Veterinary Vaccine Center
PBS	Phosphate Buffer Saline
PI	Percent of Inhibition
PRR	Pattern Recognition Receptor
RNA	Ribonucleic acid
RPM	Revolution Per Minute
SAT	Southern African Territory
SC	Sub-Cutaneous
SE	Standard Error
SNT	Serum Neutralization Test
SPCE	Solid Phase Competitive Elisa
ssRNA	Single Stranded Ribonucleic acid
TCID	Tissue Culture Infectivity Dose
Th	T helper
TLR	Toll like receptor
TPB	Tryptose Phosphate broth
USD	United State Dollar
UTR	Untranslated Region
VNT	Virus neutralization test
VP	Viral protein
W/O	Water in oil
WRL	World reference laboratory

ABSTRACT

Foot-and-mouth disease is globally one of the most economically important viral diseases of cloven-hoofed animals that can primarily controlled by vaccination. Selection of the effective adjuvants formulated simultaneously with the antigen in the vaccine is crucial in ensuring the quality of vaccine and the protective effect of the vaccine against FMD. Aluminum hydroxide gel and saponin (AS) is the most used adjuvant, with its shortcoming in short duration and poor immune response in FMD vaccine. Therefore, the present experimental study was undertaken to evaluate different formulation of adjuvants for inactivated trivalent FMD vaccine containing A, O and SAT 2 serotypes in cattle. Additionally, this study was also performed to demonstrate the effect of booster dose administration on immune response. The vaccines were prepared by mixing 10 % of aluminum hydroxide gel, 0.3 % of saponin with the virus suspension. Oil based was prepared with equal volume of virus suspension (50:50). Twenty-nine cattle were classified into five groups, with four experimental groups consisting of six cattle in each group (n=6) and the fifth group is a control group with five cattle (n=5). The experimental cattle were grouped as: AS, AS boosted, oil based and AS + oil. The sera sample were collected on day 0, 7, 14, 21, 28 and 42, their immune response was measured using Solid Phase Competitive Enzyme Linked Immune Sorbent Assay (SPCE). There was a significant difference in the immune response between the adjuvant groups ($P<0.05$, ANOVA). The results showed that, the antibody level in cattle vaccinated with AS were significantly lower than AS boosted group for serotype A, O and SAT 2, indicating that the need for booster dose. Whereas the antibody response in the AS + oil group was higher followed by oil alone, AS boosted and AS. It can be concluded that oil based and AS with oil induce better antibody response relative to others and they could replace the aluminum hydroxide gel and saponin for FMD vaccine production to control the disease. On different note, challenge test was not successful in this study indicating the need for further research on the virus infectivity.

Keywords*: adjuvant, Foot and Mouth Disease, immune response, serotype, vaccine*

1. INTRODUCTION

Ethiopia is one of the country which has the largest livestock population in Africa (FAO, 2014). Despite this, the products and productivity of livestock are still very less due to the impacts of economically important diseases of animals (Sulayeman *et al.*, 2018). Among these, foot and mouth disease (FMD) is one of the most economically important, highly contagious viral disease of cloven-hooved animals affecting cattle, pigs, sheep, and goats (Çokçalışkan *et al.*, 2016). This disease is characterized by fever and the presence of vesicular lesion or sores on the tongue, lips, teats, in the mouth and between the hooves. It is known by causing high morbidity, low mortality, severe production and weight losses (Knight-Jones and Rushton, 2013)

The FMD virus (FMDV) is the member of the genus *Aphthovirus* belongs to the family *Picornaviridae.* The virus genome is a positive single stranded RNA, which is about 8.5 Kb, encodes structural proteins including viral proteins (VP)1, VP2, VP3, VP4 and nonstructural proteins (Doosti *et al.*, 2019). To date, seven serotypes that are responsible to cause FMD have been identified such as Southern African Territories (SAT) 1, SAT 2, SAT 3, A, O, C, and Asia 1 with different variants and many subtypes (Doosti *et al.*, 2019;Tadesse, 2018). In Africa, six of the seven serotypes of FMD viruses except Asia 1 have been reported in the last decade (Maree *et al.*, 2014; Ayelet *et al.*, 2009). Among these serotypes, SAT 1, SAT 2 and SAT 3 are endemic in sub-Saharan Africa (Bastos and Sangare, 2001; Brito *et al*., 2007).

In Ethiopia, the disease is endemic. According to the ministry of agriculture records, the outbreak incidence of FMD was occurred from 1997 to 2006 throughout the country, with the highest incidence in the central part of Ethiopia (Ayelet *et al*., 2008). A sero-epidemiological surveys conducted in different parts of the country have been reported up to ~26 % prevalence of FMD. Recently, among the seven serotypes of FMDV that are immunologically different, five of them (O, A, SAT 1, SAT 2 and C) were reported from FMD samples collected throughout the country (Yirgalem *et al.*, 2020 ; Megersa *et al.*, 2009 ; Yahya *et al.*, 2013). The FMDV serotype O is the most prevalent (73.3 %) followed

by serotype A (19.5 %), SAT 2 (4.1 %) and SAT 1 (1.8 %). After 1983 serotype C has not reported in Ethiopia (Ayelet *et al*., 2009; Jemberu *et al*., 2016 ; Yirgalem *et al.,* 2020 ; Ayelet *et al*., 2009).

The endemic presence of the FMDV is highly challenging due to its impacts on the trade market and productivity of the livestock. Thus, different measures are required to eradicate and control the disease, including vaccination strategies and control of animal movement (Arzt *et al.,* 2018). The use of inactivated FMD vaccine has been confirmed to be an effective tool to eradicate, control and prevent the disease. However, the vaccine requires continuous improvement to overcome the limitations of currently existing FMD vaccines and to increase its efficacy (Xiao *et al.,* 2007). Improvement of vaccine antigen and use of effective adjuvants are considered to be the corner stone to offer more efficient vaccine against FMD. The selection of vaccine adjuvants is one of the factors that impact vaccine performance in vaccination strategy against FMDV. As a result, the vaccine requires the selection of effective adjuvants to ensure sufficient protective level and provide a long-lasting immune response (Ibrahim *et al.,* 2015). An ideal adjuvant is the one which can stimulate specific components of humoral immune response early, that can promote production of high antibody titer with prolonged duration of immunity. It should also stimulate cell-based immune response (Park, 2013).

In Ethiopia, considering the wide prevalence of the disease, National Veterinary Institute (NVI) is producing inactivated trivalent (for serotype O, A and SAT 2) vaccine. The vaccine is produced only for the cattle and adjuvanted with aluminum hydroxide gel and saponin (Ashenafi, 2012). Aluminum hydroxide gel and saponin and oil adjuvant vaccines are the most commonly used adjuvant in inactivated FMD vaccine to control the disease. The aluminum hydroxide gel and saponin adjuvant has an advantage in that the antigen is released slowly as it is absorbed into the gel. However, vaccine containing aluminum hydroxide gel and saponin as an adjuvant has several deficiencies, including the induction of short-lived immune responses and the consequent need for frequent vaccination. In contrast, the oil-adjuvanted FMD vaccines are effective in providing longer lasting protection than a standard aluminum hydroxide gel and saponin based vaccines (Cloete *et*

al., 2008 ; Choi *et al.*, 2020). Since 1970, adjuvants used in FMD vaccine for pigs have been transformed from gel to oil, thereby overcome the problem of poor immunization in pigs (Park *et al*., 2014). Comparative studies of aluminum hydroxide gel and saponin and oil based adjuvant have been conducted and revealed that oil adjuvant showed better immune effects than aluminum hydroxide gel and saponin (Selim *et al*., 2010). Providing early protection against the disease is another advantage of oil-based adjuvant (Iyer *et al*., 2000). Furthermore, study conducted on combination of aluminum hydroxide gel and saponin and oil induces better immunity (Wael *et al*., 2019). Despite the advantage of oil based and combined adjuvants, in Ethiopia, NVI is producing the inactivated trivalent FMD vaccine formulated with aluminum hydroxide gel and saponin. This indicates that there is lack of scientific evidence on adjuvant evaluation and the selection of proper adjuvants in the country.

Therefore, the objectives of the present study were:

- to evaluate immune response induced by different formulation of adjuvants for inactivated trivalent FMD vaccines in cattle
- to assess immune response elicited with and without booster vaccine
- to test the potency of formulated vaccines through challenge with the live virus

2. LITERATURE REVIEW

2.1. General overview of Foot and mouth disease

FMD was first described since the 16th century and was the first animal pathogen recognized as a virus (Grubman and Baxt, 2004). The disease is a severe, highly infectious and contagious viral disease of cloven-hoofed species such as cattle, pigs, sheep and wildlife animals. FMD is characterized by the development of vesicular in the oral cavity, on the feet (Jamal and Belsham, 2013), on the teats, and between the toes that ruptured and cause painful sores and loss of the hooves (Morse and Meyer, 2017). According to World Organization for Animal Health (OIE) rank, FMD placed as economically one of the most important infectious diseases of animals that can significantly affect globally international trade of livestock products (Lubroth *et al.*, 2007; Maree *et al.*, 2014). It is transmitted from infected to health animals via, inhalation, ingestion of fomites and direct contact of infected animals with a susceptible host (Auty *et al.*, 2019).

A large number of cloven-hoofed animals can be affected by FMDV and causes significant economic losses in many countries of the world. The disease circulates in developing countries with high prevalence, especially widely distributed in sub-Saharan countries of Africa and Asia, while eradicated from developed countries (Lyons *et al.*, 2016). Morbidity of FMD is high, while mortality is low (<5%) and in different countries outbreaks are being reported from time to time throughout the year (Longjam *et al.*, 2011).

2.2. Etiology

FMDV is type of a single-stranded (ss) positive-sense RNA virus (Longjam *et al.*, 2011) that belongs to the genus *Aphthovirus* in the *Picornaviridae* family. The virus causing FMD was defined in 1963 by the International Committee of Taxonomy of viruses. The family name, *Picornaviridae* is derived from the Latin word 'pico' (small) and 'ma' (RNA) which refers to the size and genome type while the genus name '*aphthovirus*' refers to the

vesicular lesions produced in cloven hoofed animals. It has immunologically distinct seven serotypes named O, A, C, Asia1, SAT 1, SAT 2, and SAT 3 and subtypes within each serotype (Saeed *et al.*, 2015; Tesfaye, 2019). All the three types of Southern African Territories serotypes are maintained in African buffaloes, which can provide a possible source of infection to domestic livestock and other species (Ayebazibwe *et al*., 2010).

FMDV is a non-enveloped virus with icosahedral symmetry consists of genome ~8.5 Kb size and encodes a single long open reading frame (ORF) of about 7 kb. The viral capsid is composed of 60 copies of capsomer each consists of four (4) structural viral protein (VP-1, VP-2, VP-3, VP-4). The VP4 is located internally (buried within the capsid) whereas VP-1, VP-2, and VP-3 are exposed on the surface of the virus. The ssRNA genome of FMDV is enclosed by a protein coat. The RNA comprises, the 5′ UTR (5′ untranslated region), coding region and the 3′ UTR (3′ untranslated region) (Jamal and Belsham, 2013).

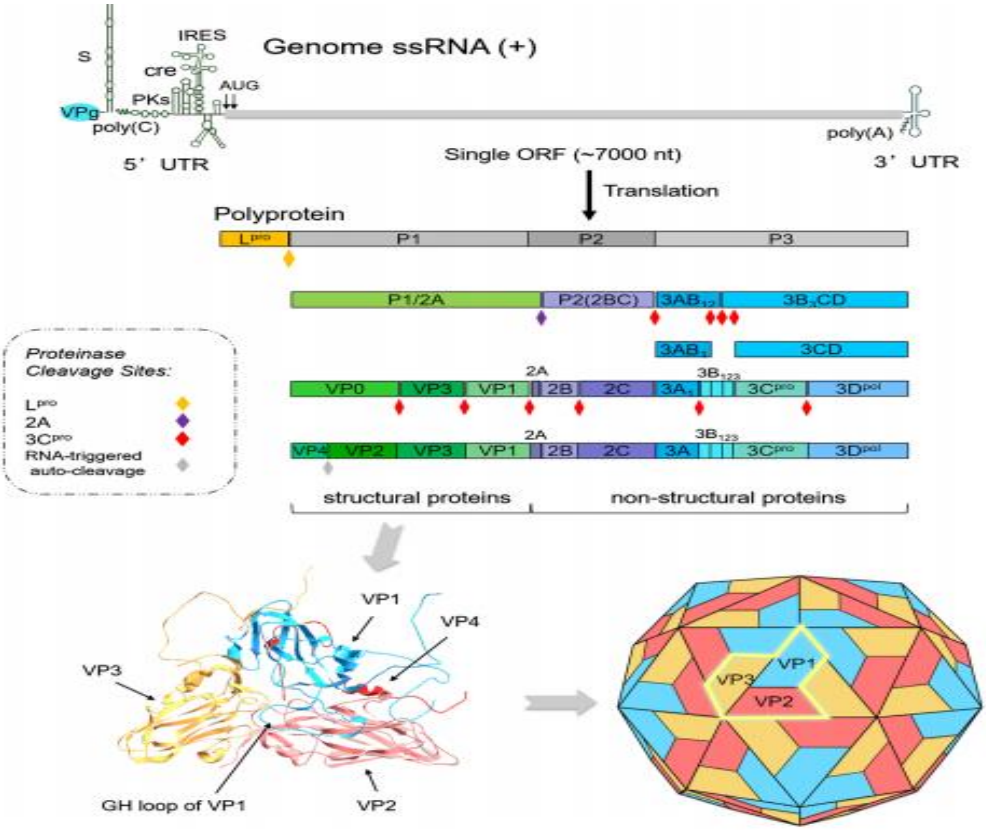

Figure 1: Schematic diagram showing the genome organization and structure of FMDV displaying structural and nonstructural proteins

Source *(*Gao *et al, 2016)*

2.3. Antigenic structure of FMDV

The main location of cell attachment site and the immunodominant region of FMDV are both found on a dissolvable exposed region at the surface of the virion, specifically in trypsin-sensitive zones of VP1, particularly the G-H loop in VP1. This site has been identified by monoclonal antibody (mAb) (Bari *et al.*, 2015; Grazioli *et al.*, 2006). It is the antigenic and immunogenic site found in all serotypes. Prior serological studies, revealed that diverse serotypes of FMDV shared a highly variable region of VP1, as one of the major antigenic sites of the virus. Within this region, a number of overlapping B-cell epitopes are located and are able to initiate both neutralizing and non-neutralizing antibody responses. The high sequence variability found in this region accounts for the low cross reactivity watched among distinctive serotypes (Carvallho and Paula, 2000).

2.4. Epidemiology of foot and mouth diseases

2.4.1. Host range and geographical distribution

The disease is described as an acute, highly contagious viral infection of cloven-hoofed animals involving domesticated livestock species and water buffalo (*Bubalus bubalis*) are susceptible to infection and can spread the disease. The disease is considerably less obvious or sub-clinical in species of cattle, sheep and goats indigenous to Africa and Asia where FMD is endemic and these animals are supposed to have been the source of infection for countries previously considered disease-free (Tesfaye, 2019).

Among the seven serotypes of FMDV, type O, A and Asia 1 are found to be continuously circulating as endemic in Asia, Europe and African countries. Whereas, SAT 1, SAT2, and SAT 3 are commonly distributed in African countries. The existence of serotype C has

been decreased in the last 30 years and recently its distribution becomes low. In Kenya, outbreak due to this serotype was reported in 2004 for the last time (Rodriguez and Grubman, 2009).

In African continent the epidemiology of the disease is influenced by two different cycles, including involving wildlife, especially the African buffalo (*Syncerus caffer*), and an independent cycle maintained within domestic animals. Another distinctive feature of FMD epidemiology in Africa is the presence of the three SAT serotypes, including SAT1, SAT2 and SAT3, with multiple genetics and antigenic variants in different geographical regions, defined as topotypes. All the three SAT serotypes are maintained within the African buffalo populations (Maree *et al*., 2015)

Recently, increased field surveillance, intensive investigation of the outbreak, and submission of virus samples to reference laboratories such as World Reference Laboratory (WRL) and FAO/OIE reference laboratory for further analysis resulted in improved disease reporting transparency. However, efforts are still not sufficient for comprehensive control and complete disease eradication (Saeed *et al.*, 2015).

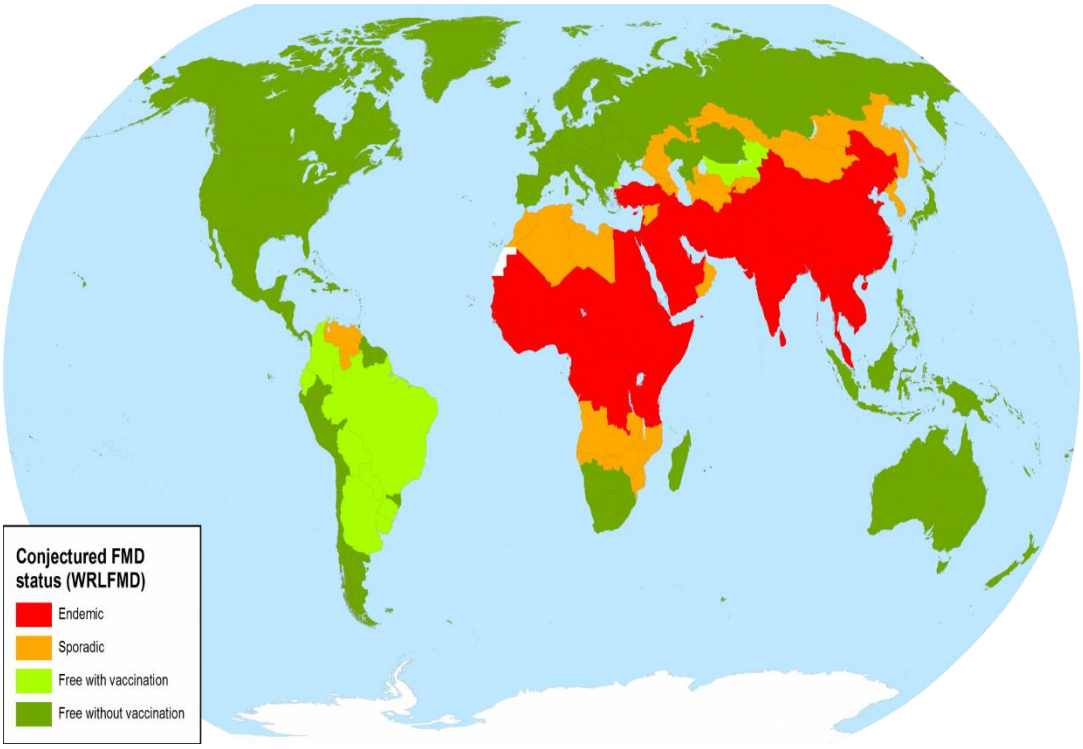

Figure 2: The global distribution of Foot and Mouth Disease
Source (https://www.wrlfmd.org/foot-and-mouth-disease/occurrence)

2.5. The status of FMD in Ethiopia

FMDV was first reported in Ethiopia from 1957 by FAO as the cause of FMD outbreak. (Aman *et al.*, 2018). Of the seven serotypes of FMDV five serotypes, O, A, C, SAT 1, and SAT 2 were responsible for the outbreak occurred from 1974 to 2007 with serotype O predominate followed by serotype A. After 1983 serotype C has not reported in Ethiopia (Ayelet *et al*., 2009; Jemberu *et al*., 2016). FMD is endemic in Ethiopia, causing several outbreaks every year (Ayelet *et al*., 2012). Different epidemiological survey carried out in different parts of the country reported up to 26 % and 48 % sero-prevalence of FMDV at the animal and herd level respectively (Megersa *et al.*, 2009 ; Mohamoud *et al*., 2011).

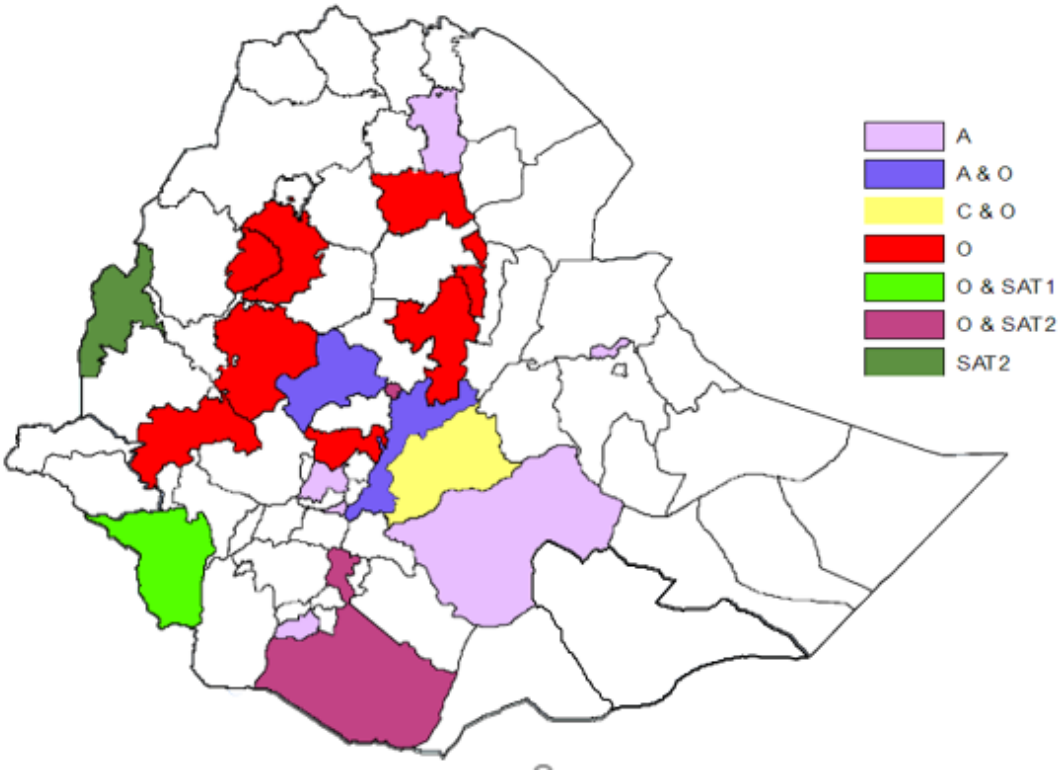

Figure 3: FMDV serotypes identified in Ethiopi

Source (Ayelet *et al*., 2008)

The disease has a significant economic impact in Ethiopia, economic losses were significantly higher particularly for the pastoral system. Reduction in milk yield, losses of draft power are the major impacts caused due to FMDV, nonetheless mortality is low (Jemberu *et al.*, 2014). The targeted vaccination strategy is shown to provide the largest economic return with a relatively low risk of loss (Jemberu *et al.*, 2016). Considering its socio-economic impact, to control the disease around the infected area and to protect susceptible animals inactivated trivalent vaccine that comprise serotype O, A and SAT 2 of FMDV is produced at National veterinary institute. However, vaccination strategy has a limitation because of insufficient dose produced at NVI and diversity of serotypes that are not included in the vaccine production (Aman *et al*., 2018).

Table 1: The overall seroprevalence study of FMDV conducted in different parts of Ethiopia

Study area	Sample size	Prevalence (%)	References
Southern Ethiopia	1020	9.5	*Megersa et al.,2009*
Benchi maji zone	273	12.08	*Gelaye et al.,2009*
Borena and Guji	460	24.56	*Mekonen et al.,2011*
South omo zone	770	8.18	*Molla et al.,2010*
Tigray region	390	15.38	*Zerabruk et al.,2014*
Borena	768	23.04	*Bayissa et al.,2011*
Jijjiga	384	14.58	*Mohamoud et al.,2011*
Central Ethiopia	38,187	14.49	*Alemayehu et al.,2014*
In and around Adama	4321	12.86	*Birhanu, 2014*
Amhara region	1672	11.48	*Mesfine et al.,2019*

2.6. Economic importance of Foot and mouth disease

Foot and mouth disease is considered as severe, highly contagious viral disease of livestock in the world in terms of economic impact that leads to severe economic losses. This impact can be divided into two components: direct impacts due to high morbidity resulted in production losses and change in herd structure. The other is indirect impacts due to the incentive to invest on control strategies, limit on trade market on countries where the disease is endemic (Alemayehu *et al*., 2014). The disease impairs the trade market potential of the country for animals, milk, meat and other agricultural products. The impacts of FMD in terms of visible animal and animal products losses and vaccination cost in affected countries can cause losses of >USD 1.5 billion per year (Knight-Jones and Rushton, 2013).

Foot and mouth disease, deeply affect the production of causes the highest economic impact on the developing countries like Ethiopia, where the livelihood of most of the people depends dominantly on livestock. It reduces productivity of herd, leading to less efficient herd structure, high control costs (Tesfaye, 2019). Study conducted by Bayissa *et*

al. (2011) on economic impact of FMD shows that, acute phase of the infection resulted in reduction of milk about 0.5-1.5 L per day per lactating cow for 25.5 days. This was accounted about 73.3 % of milk which was supposed to be produced within those days, but only 7.7 % of total milk production of a lactating cow per lactation period. According to Alemayehu *et al*. (2014), the total economic loss resulted due to FMD influences from international market during the study period was estimated to be 3,322, 269 USD (56,345,682.24 ETB).

2.7. FMD Vaccines and adjuvants

Vaccines play an important role in controlling and preventing both animals and humans from infectious diseases (Knight-Jones *et al*., 2014). Vaccination is used in maintaining animal health and to improve overall production, controlling and eradicate both veterinary important and zoonotic diseases such as FMD, anthrax, brucellosis, rabies, influenza, and also control of emerging as well as exotic diseases of animals and humans. Furthermore, vaccination strategy plays an essential role to reduce the cost of treatment (Zhou, 2019).

Currently, the most effective vaccines in use are attenuated and inactivated forms of infectious pathogens or their products. However, both types of vaccines have their advantage and disadvantage related to safety, stability, and efficacy in inducing an immune response (Francis, 2018). The aim of these vaccines is to provide long-lasting protection against the diseases caused by specific pathogens through generating effective immune response. A live-attenuated vaccine that consists of live organisms can induce strong humoral and cell-mediated immunity (CMI). Whereas, inactivated vaccine types are relatively safer than live-attenuated and principally induce humoral immunity, but little or no cell mediated immunity. Therefore, additions of adjuvant to a vaccine is essential to confer strong protective humoral and CMI (Lee and Nguyen, 2015).

The control of FMD can be achieved by four integrated activities which includes separation of infected from susceptible animals, movement restriction, vaccination strategy and surveillance (Kasem *et al.*, 2017). Vaccination strategy is a major tool for FMD control to

mitigate the impact of the disease, or to eliminate the virus circulation from the endemic area. The effect of vaccination is not only depending on the quality and suitability of the chosen vaccine, but also depends on the selection of adjuvant formulated with the vaccines to protect the susceptible animals against FMD (Ibrahim *et al.*, 2015).

The ideal adjuvant is one that encourage in early stimulation of the humoral immunological response, and to induce production of higher antibody. It could also prolong the immune response as well as activate specific components of either cell-mediated or humoral immune response (E. I. *et al.*, 2020). In addition, Combinations of multiple compounds have a synergistic effect when used as an adjuvant. New adjuvant advancement should recognize novel combinations of adjuvants and formulations capable of inducing stronger and longer-lasting humoral and cellular immune responses (Cao, 2014). Currently, different adjuvants are in development and in use for inactivated FMD vaccine which includes mainly aluminum hydroxide gel and saponin, oil-based, different particles (virus-like particles, liposomes) and derivative of bacteria (Vogel, 2000).

An inactivated vaccine formulated with either aluminum hydroxide gel and saponin or oil is an effective means to limit the spread of the disease in endemic areas (Kasem *et al.*, 2017). However, the vaccine containing aluminum hydroxide gel and saponin adjuvant has several deficiencies in relative to oil based, including the induction of short-lived immune responses and the consequent need for frequent vaccination. In contrast, the oil adjuvanted inactivated FMD vaccines are effective in the induction of high titer and rapid humoral immune response as well as providing longer lasting protection than a standard aluminum hydroxide gel and saponin gel-saponin adjuvanted vaccines (Cloete *et al*., 2008).

Study conducted in sheep vaccinated with inactivated bivalent FMD vaccine formulated with aluminum hydroxide gel and oil adjuvant revealed that, sheep vaccinated with oil based adjuvant were better in inducing higher long-lasting immunity than aluminum hydroxide gel vaccinated sheep by ELISA and serum neutralization test (SNT). Thus, oil based adjuvant has a potential to replace commercial aluminum hydroxide gel adjuvant (Selim *et al*., 2010). Furthermore, immunological study was conducted to reveal the

combination effect of aluminum hydroxide gel and oil based adjuvants on the immune response of trivalent FMD vaccine in cattle shows better immune response than oil alone (Mosad *et al*., 2019). Similarly, Park *et al.* (2016) reported aluminum hydroxide gel combined with various oil-based adjuvants showed increase in the level of antibody response in mice, pigs and target animals. On the other hands, combined oil and saponin adjuvant demonstrated in mice and co-administered with FMDV antigen induces significantly higher humoral immune response than FMDV administered alone (Song *et al.*, 2009)

2.8. Mechanisms of action of adjuvants

A better understanding of the adjuvant's mode of action or immunological mechanisms is critical in designing new vaccine adjuvants that enhance effective immune responses towards pathogen-specific and appropriate memory. It also assists in the evaluation of adjuvant safety at the developmental stage (Mastelic *et al.*, 2010). Based on their mechanism of action they have been divided into the delivery system and immuno-stimulatory adjuvants. In the early time, the delivery system was considered only to act as a depot adjuvant while immune-stimulatory induce innate immune response, but nowadays there is confirmation that some delivery systems can also activate cells of the innate immune systems (Awate *et al*., 2013).

Vaccine adjuvants are commonly used in billions of doses of humans and animals' vaccines. However, their mode of action are not understood well (Awate *et al*., 2013). But, some mechanisms of adjuvant action have been revealed. They can act by slowing the release of antigen or trap at the site of injection present a sustained supply to local APCs (Depot effect). This effect helps to reduce the removal or degradation of the antigen by immune cells (i.e. liver). Recruitment of cells at the site of injection, regulation of cytokines and chemokines, enhancement of expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules, and induction of inflammatory cascades are mechanisms employed by adjuvants to invoke immune response (Spickler and Roth, 2003).

Generally, all the mechanisms include stimulation of APCs directly or indirectly, mainly dendritic cells (DC). DC activates the innate and adaptive immune system by processing the antigens and presenting to specific T-cells. The trapped antigen is taken up through the process called phagocytosis or pinocytosis by DC and then detected by pattern recognition receptors (PRR). During stimulation of PRR, several soluble inflammatory mediators such as cytokines and type 1 interferon (IFN-1) are released by naive DC as part of innate immunity (Spickler and Roth, 2003). Additionally, the adaptive immune response is also stimulated by activated DC, via processing and presenting the antigens to specific T-cells (CD4+). Increased CD4+ is stimulated as a result of the interactions between DC and CD4+, but this cascade is not adequate for CD8+ T cell stimulation, which is important for the efficacy of a vaccine against intracellular pathogens and cancer. Therefore, novel adjuvants are targeted to receptors of APCs expressed on DCs to activate the innate immune system. PRRs include the TLRs, CLRs, and NLRs. Presenting antigens to APCs with adjuvant permits induction of immunity against tumors or antivirals (Agrawal *et al*., 2013; Azmi *et al*., 2014).

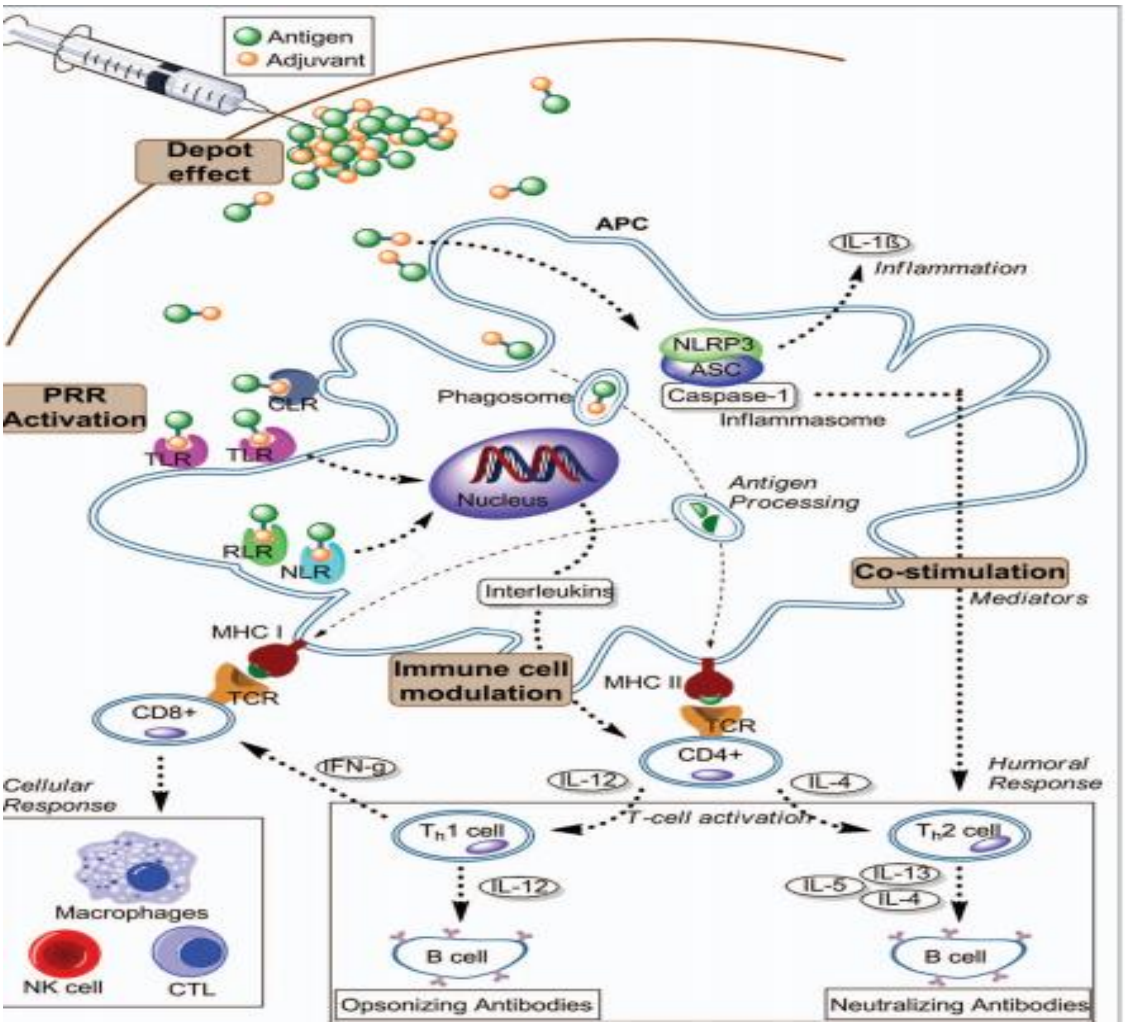

Figure 4: Schematic overview of the immunological cascade induced by adjuvants Source (Reed *et al*., 2013)

2.9. Common adjuvants used in FMD vaccine

2.9.1. Aluminum based adjuvant

In the past few years, number of materials have been tried as adjuvant. However, only aluminum-based adjuvants continue to be used widely in the world as compared to others. Aluminum hydroxide ($Al(OH)_{3)}$ and aluminum phosphate ($AlPO_4$), often mentioned as an alum are principally the foremost used adjuvants within the vaccine for human and animals

for over 80 years (Mohan *et al*., 2013). Glenny and his colleagues first reported the use of aluminum-based as an adjuvant in 1926. They reported that aluminum with toxoid vaccine injected into laboratory animals induces higher antibody response than the toxoid alone (Agrawal *et al*., 2013). Of aluminum-based adjuvants, aluminum hydroxide is the most widely used chemical as adjuvant in the vaccines. According to HogenEsch *et al*. (2018), this chemical is considered as the gold standard against all the new proposed adjuvants often compared for new candidate vaccines. In spite of its wide spread use of this adjuvant its mechanism of action remains fully unclear. Aluminum hydroxide adjuvant are preferentially known by inducing Th2-type immune responses, but little or no Th1 immune reaction. However, recent studies demonstrated that it can provoke both Th1 also as Th2 immune responses (He *et al*., 2015).

Mechanism of action of aluminum hydroxide adjuvants involves the depot (repository) effect, pro-phagocytic effect and activation of the nucleotide-binding oligomerization like receptor protein 3 (NLRP3) activation. These all mechanism are stimulate innate, adaptive immune responses and complement system (Azmi *et al.*, 2014). The depot effect is the process of slowing the speed of release of antigen to the system by being adsorbed or attached to the surface of adjuvants containing aluminum eventually results in retention of antigens at the injection site. However, recent studies have confirmed that the depot effect does not play an important part for alum adjuvanticity. Several studies suggest that, excision of the injection site didn't inhibit the event of immune response (Lee and Nguyen, 2015). Even though, the depot effect is vital in long-lasting and effective activation of the immune response (He *et al*., 2015).

The other mechanism of aluminum hydroxide is that the activation of nucleotide-binding oligomerization domain (NOD) like receptor protein 3 (NLRP3) inflammasome pathway. Aluminum hydroxide-based adjuvants activate cellular immune responses mediated by NLRP3 like macrophages, to promote the secretion of pro-inflammatory factors such as IL-1β and IL-18. Activation of (NLRP3) inflammasome pathway has been demonstrated by using ovalbumin antigen with alum in NLRP3 deficient mice and wild-type mice (with NLRP3), the result of this study revealed that antibody production in NLRP3 deficient mice

is significantly less than wild-type mice. This study tells the role of aluminum hydroxide in innate and the adaptive immune response via the activation of the NLRP3 pathway (Agrawal *et al*., 2013; Kool *et al*., 2012; Maughan *et al*., 2015).

Generally, experimental studies have tried to investigate cells targeted by aluminum hydroxide to trigger the immune response. Aluminum hydroxide acts on macrophages and facilitates their differentiation into dendritic cells and increase the delivery of antigens by macrophages instead of activating DCs (Rimaniol *et al*., 2004). It can also facilitate the recruitment of inflammatory monocytes to the injection site and their differentiation into DCs. Recruited inflammatory monocytes express higher levels of major histocompatibility complex class II (MHC II) due to an improved capacity to adsorb antigens. Therefore, DCs (antigen carrying) differentiated from inflammatory monocytes migrate to lymph nodes then encourage the proliferation of T cells (De Gregorio *et al*., 2013).

2.9.2. Saponins

Saponins are natural complex compounds used as an adjuvant and produced from plants, mostly from bark of *Quillaja Saponaria Molina* (Fernández-Tejada *et al*., 2016). Saponin-based adjuvants have on numerous occasions been used commonly in veterinary vaccines to increase both antibody and cell-mediated immune response. Despite, the wide use and immune-stimulating ability of saponin, the mechanism of action remains not well understood like the other adjuvants (Ahlberg *et al.*, 2017). Quil-A, isolated from *Quillaja Saponaria Molina* is the most commonly used saponin based adjuvants. Saponins can provoke strong Th1 immune response and cytotoxic T-lymphocytes (CTLs) which makes them effective to use in vaccines desired for intracellular pathogens and subunit vaccines (Sun *et al*., 2009). Moreover, it can induce the production of cytokines like interleukins and interferons which may mediate their immunestimulant effects (Rajput *et al*., 2007).

Quil A has been used broadly in veterinary medicine in vaccines of cattle, pigs, horses, dogs, and vaccines which contains equine influenza virus, canine parvovirus, and FeLV vaccines (Spickler and Roth, 2003). Combination of Quil-A and oil can synergistically

enhance IgG responses in mice and pigs when used as an adjuvant for FMD vaccines (Cao, 2014). The utilization of Quil-A adjuvant in humans is restricted due to its toxicity and reactogenic properties (Garçon *et al*., 2011).

2.9.3. Oil emulsion adjuvants

The major emulsions used in a vaccine, with one substance dispersed into the others are oil-in-water (O/W) and water-in-oil (W/O) emulsions. In 1930, W/O emulsion such as Freund's incomplete adjuvants (FIA) was introduced by Jules Freund, and it is a widely used adjuvant type in vaccine formulation. Like aluminum, the water-in-oil emulsions adjuvant function as delivery systems by the formation of depot effect that traps antigen at the injection site and by slowing the discharge of antigen to elicit a long-lasting immune response (Garçon *et al*., 2011). Besides, water-in-oil adjuvants can protect antigens from degradation by the peptidase enzyme (Azmi *et al*., 2014). Additionally, oil emulsions are act by activating the production of immune-stimulatory cytokines and chemokines. Thus, trigger the recruitment of dendritic cells (DCs), monocytes, which differentiate the latter to DCs (Haensler, 2011).

W/O adjuvant contains microdroplets of an aqueous phase in an oil, stabilized by a surfactant. Releasing the antigen slowly from the injection site to the system is the vital role in providing long-lasting immune response. However, reactions at the site of injection and viscosity are common with water in oil emulsion, due to these side effects using in the vaccine of human and companion animals are limited. While, it can be used in some vaccine for ruminant, poultry, and in research animals. Relative to W/O emulsions O/W emulsion is less viscous and less activate inflammation (Spickler and Roth, 2003).

Montanide is an oil-based adjuvant available commercially in various forms, like ISA 206 and ISA 201 are currently used for the FMD vaccine in different countries. Montanide ISA 201 is considered induce rapid, higher antibody level, long-lasting and CMI. Inactivated FMD vaccine formulated with aluminum hydroxide gel and saponin has been provided less protection in the pig. However, a recently developed double oil emulsion adjuvant is

proven to guard all susceptible species against FMD. Moreover, the oil-based adjuvant has able to induce long-lasting immunity than aluminum hydroxide gel and saponin adjuvant (Cao, 2014 ; Cloete *et al.*, 2008; EI. *et al.*, 2020).

The development of an improved Foot and mouth disease vaccine requires the selection of appropriate adjuvants, that improves sufficient protection level of the vaccines when used for susceptible target animals. In this study effects of experimental vaccines of FMDV formulated with different types of adjuvants were and immune response elicited with and without booster dose were also compared in cattle.

3. MATERIALS AND METHODS

3.1. Description of the Study area

The study was conducted from November 2020 to June 2021 in the National Veterinary Institute found at Bishoftu, Ethiopia. All experimental works were done at NVI and ELISA assay to measure antibody response against FMDV was carried out at National Animal Health Diagnostic and Investigation center (NAHDIC), Sebeta, Ethiopia. National Veterinary Institute was established in 1964 under Ministry of Agriculture and supporting the livestock industry in producing veterinary vaccine to combat devastating livestock disease. Today it is a well-known institution in Africa with producing several million doses of different vaccines to protect millions of animals for several infectious diseases. The institute has been producing a total of 22 veterinary vaccines including 9 for bacterial, 7 for viral and 6 for avian disease. Moreover, it is also undertaken vaccine related research different diagnostic tests for various disease.

The infrastructure of NVI is well developed and playing an important role in the attraction of international institution like Pan African Veterinary Vaccine Control Center (PANVAC), which is under the African union. Moreover, it has been given the responsibility to produce and supply enough vaccines for countries of Eastern, Western and Southern Africa. The institute has reached the level of using state of the art equipment and technologies for biological production and vaccine related research.

NAHDIC is another center found at Sebeta, Ethiopia located 35 km from Addis Ababa. This center provides huge economically significance support through testing export animals, investigating outbreaks and surveillance of economically important disease of animals. Currently NAHDIC is accredited for animal health related serological and molecular biology tests by National accreditation system.

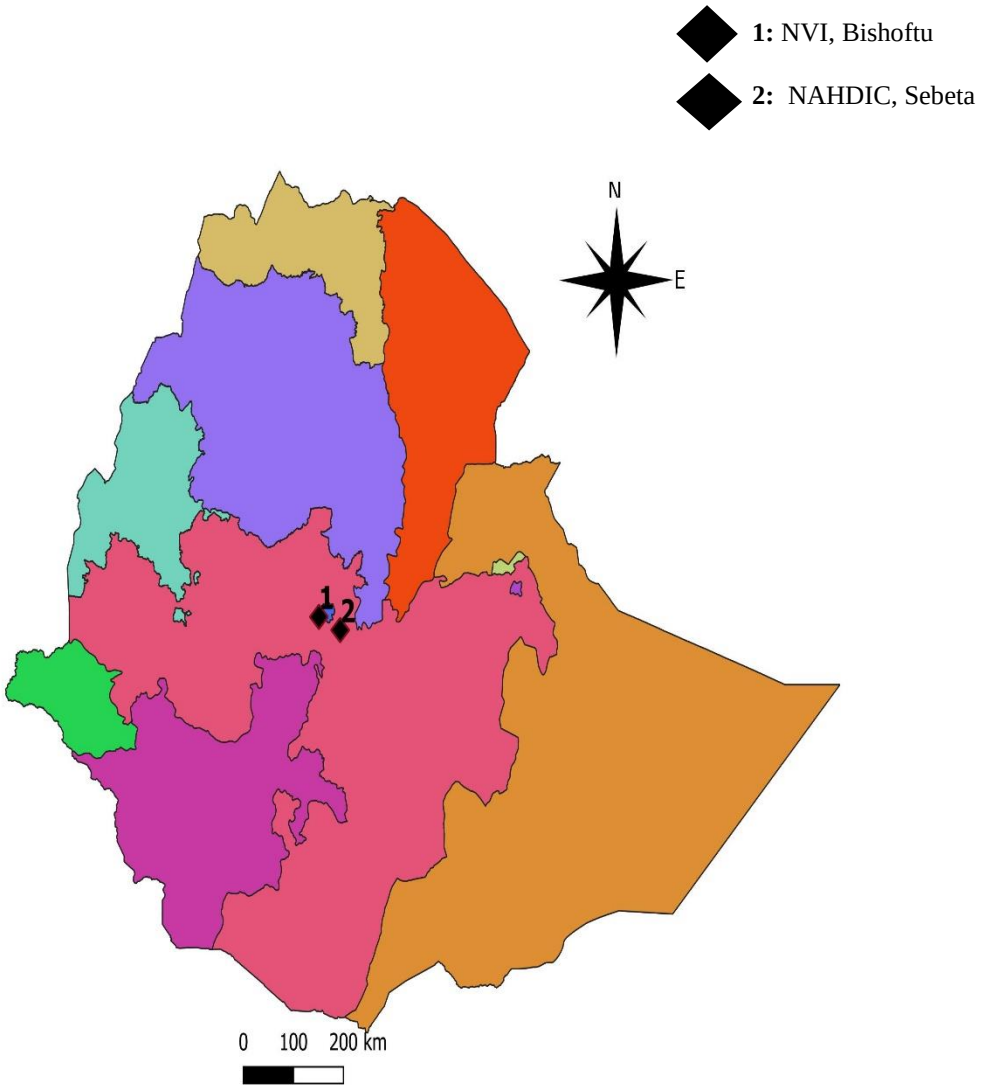

Figure 5: Geographical location of study area

3.2. Study animals

3.2.1. Cattle

The animals used in this experimental study were supplied by National Veterinary Institute. They were local breed male cattle aged three to four years old, apparently healthy or free from any clinical signs. Prior to vaccination, all cattle were ear tagged and identity number

was given. All the study animals used for this experimental study were bled and tested for the presence of anti-FMDV nonstructural protein (NSP) antibodies using the FMD nonstructural enzyme-linked immunosorbent assay (NS-ELISA). Only cattle with a negative result with the FMD NS-ELISA were used for the study. They were randomly allocated into five groups with six cattle in four groups (6 cattle/group) and five in control /non-vaccinated groups and a total of twenty-nine (29) cattle were used. Throughout the study period, they were kept separately and managed properly in animal experimental room of NVI with adequate provision of feed and water. The cattle in each vaccine group were vaccinated subcutaneously (SC) with inactivated trivalent (serotype A,O and SAT 2) FMD vaccine formulated with different adjuvants in the middle of the cervical area and blood samples were collected at 0, 7, 14, 21, 28 and 42 days after vaccination for serology testing. Generally, these cattle were used in the evaluation of humoral immune response induced because of vaccine formulated with different adjuvants and booster dose.

3.3. Study design

Longitudinal study was applied to evaluate immune response of the experimental animals. Twenty-nine (29) male cattle were grouped into five groups as follows to evaluate the immune response elicited by different formulation of adjuvants as well as to evaluate effect of booster dose. The first group was vaccinated with inactivated trivalent FMD vaccine adjuvanted with the conventional aluminum hydroxide gel and saponin (AS) (without boosting). The second group was vaccinated similarly as first group with AS adjuvanted vaccine and booster dose was given for this group at 14 days post vaccination. The third group was vaccinated with mineral oil adjuvanted inactivated trivalent FMD vaccine. The fourth group was vaccinated with vaccine formulated with AS and oil and the last group was left as control/non-vaccinated. In this experimental study, every vaccine formulates with a different adjuvant were equally likely applied to each group and every experimental calf within the group. Allocation of adjuvants to each calf was on a random basis to distribute experimental error equally for all groups. At the end of the experiment humoral immune response of each group were measured by using solid-phase competitive ELISA (SPCE) and the result of antibody response of each calf within each group was recorded.

3.4. Cell culture

Cryopreserved suspension of Baby hamster kidney cell line (BHK-21) was provided by Pan African Veterinary Vaccine Center (PANVAC) used for vaccine preparation and virus titration according to method described by (Kallel *et al.*, 2002). The cell preserved in liquid nitrogen (-196 ^{O}C) were thawed in water bath at 37 ^{O}C. The thawed cells were transferred into falcon tube and centrifuged at the speed of 1500 rpm for 10 minutes and the supernatant was discarded. Then about 5 ml of complete Minimum Essential Media (MEM) containing 10 % calf serum and Tryptose phosphate broth (TPB) was added to the pellet and transferred to flask of 25 cm^2. The culture medium and cells were mixed well by pipetting. The flask was allowed to incubate at 37 ^{O}C and 5 % CO_2 in a humidified incubator for 48 hours and the cell growth was followed up to show confluence. After 48 hour of incubation, good cell growth was seen under inverted microscope which was indicated by confluent cell. Once confluent cell was obtained, the medium was decanted, and the flask was washed 3 times with sterile phosphate buffer solution (PBS) to remove the residual of culture medium. Washing the flask with PBS was followed by addition of trypsin and EDTA to detach or dissociate adherent cells from the flask and observed for 3 minutes. After the cell confirmed to be detached, the cell was re-suspended in complete MEM media following by trypsinization process to get a single cell. Then the cell was passaged in flasks of 75 cm^2 and sub-cultured into another flask of 75 cm^2 accordingly.

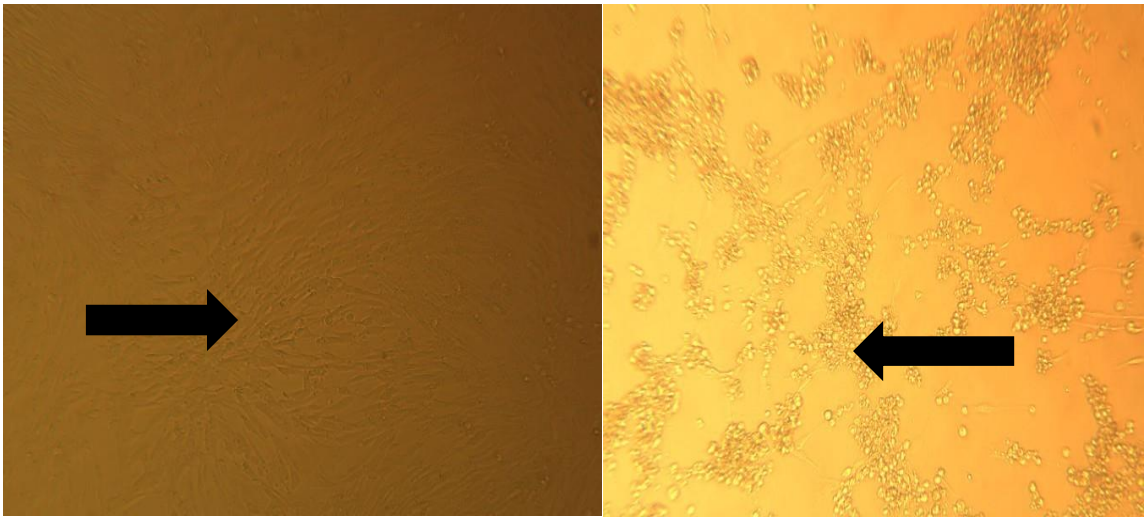

Figure 6: Normal confluent BHK-21 cell (left) and BHK-21 cell showing CPE after infection of the virus (right) observed under inverted microscope

3.5. Vaccine preparation

The FMDV serotype A/21/ETH/2008, O/ETH/38/2005, SAT 2/ETH/2009 locally isolated antigen were used for vaccine preparation using BHK-21 cell line. Maintenance media (Hanks media) was also used for cultivation and inoculum preparation as described by (Hassan, 2016). Before inoculation of the virus into the grown cell, the inoculum was prepared by diluting FMDV in1:100 dilutions in Hanks media and antibiotic was added in the inoculum. For sterility test, little amount of inoculum was inoculated into bacteriological media to identify the presence of any type of bacteria. The old media was removed. Consequently, the confluent BHK-21 monolayer cell was allowed to be infected with the diluted viruses on each flask for the three serotypes aseptically. The flasks were incubated at 37 ^{O}C and followed for 17-24 hours for development of cytopathic effect (CPE) by the virus. Within 24 hours of post infection, complete development of CPE in BHK-21 cell culture were observed under inverted microscope**.** CPE was indicated by the rounding and flattening of the cells and finally death of the cell that indicates the presence of the virus. The culture supernatants of virus adapted and grown on BHK-21 cell monolayers were separately harvested after 24 hours post-infection and poured into sterile container. The sterility test was conducted on bacteriological media. Then, the virus supernatant was preserved aseptically at -20 ^{O}C until further work.

3.6. Purification and inactivation of the virus

The harvested FMDV serotype A, O and SAT 2 from infected BHK-21 cell culture were initially concentrated by addition of 50 % of ammonium sulfate and stirred at low speed overnight (Kim *et al.,* 2019). Therefore, the viruses were further purified by using high-speed centrifugation at 3000 rpm for 30 minutes to remove unwanted substances or cell debris according to described by (Nazzaro *et al.,* 1997). The concentrated FMDV serotype

A, O and SAT 2 were re-suspended in PBS with pH of 7.2. Therefore, they were pooled in 1:1:1 ratio and inactivation of the viruses were achieved by using a final concentration of 0.06 % of formaldehyde according to method described by (Barteling and Cassim, 2005). Complete inactivation of the virus was confirmed by using BHK-21 cell culture. Finally, purified and inactivated FMDV serotypes were stored at +4^{o} C until used.

3.7. Virus titration

The three FMDV serotypes used as the vaccine strains were titrated by using 2 % incomplete MEM added to 96-microtiter plate wells. Tenfold serial dilution starting with 10^{-1} by mixing 0.5 ml of virus in 4.5 ml of MEM. Similarly, consequent transfer of 0.5 ml from the first virus dilution to the next using aseptic and sterile pipette for each dilution and finally 0.5 ml was discarded without changing the pipette. Thus, from each virus dilution in the universal bottles (10^{-1} to10^{-8}), 100 µl /wells were dispensed into the wells of their respective rows on 96-wells micro titer plates filled with 100 µl/wells of BHK-21 cells which was prepared 48 hours prior to infection. The plates were sealed and incubated at 37 °C in an atmosphere of 5 % Co_2 incubator for 48 hours. The cytopathic effect (CPE) was observed under a microscope after 48 hours and the infectivity titer of each virus were determined according to Spearman–Karber method (Kärber, 1931). The titer of the virus was expressed as $\log_{10}$ $TCID_{50}$ /ml.

3.8. Adjuvant preparation

3.8.1. Mineral oil adjuvant

Water in oil (W/O) emulsion or incomplete Freund's adjuvant (IFA) adjuvant used in this experimental study was prepared according to NVI protocol by mixing the following mineral oils: 3.6 % Montanide 888, 5 % Tween 80, and 48 % PBS and 43.3 % Marcol 52. The mixture was homogenized and sterilized by autoclaving at 121^{o} C for 15 minutes. The prepared adjuvant was stored at room temperature in dark place until used.

3.8.2. Saponin

The saponin adjuvant was prepared in 10 %, filtered by 0.22 µm pore filter sheet for sterilization. Final concentration of 0.3 % v/v was used to formulate with the vaccine (NVI protocol).

3.8.3. Aluminum hydroxide gel

10 % of aluminum hydroxide gel was prepared according to method described by (E. I. *et al.*, 2020) and autoclaved at 121 ^{o}C for 15 minutes and stored at 4 ºC until used.

3.9.Vaccine formulation with adjuvants

Formulation of the vaccine with adjuvants were performed as follows: Formulation 1: inactivated trivalent FMD vaccine with aluminum hydroxide gel and saponin (AS), Formulation 2: inactivated trivalent FMD vaccine mixed with mineral oil adjuvant, Formulation 3: inactivated trivalent FMD vaccine with combination of AS and mineral oil adjuvant. In these formulations, 0.3 % of saponin and 10 % of aluminum hydroxide gel were used to formulate with the vaccine and oil-based adjuvant was formulated with aqueous vaccine in ratio of 50:50 according to (E. I. *et al.*, 2020).

3.10. Sterility and safety testing of prepared vaccine

The prepared vaccine was tested for the presence of aerobic and anaerobic bacteria, fungal and mycoplasma contaminants by culturing of vaccine sample in thioglycolate broth, Sabouraud agar and mycoplasma medium. Complete inactivation of the virus was also tested for safety in vitro on BHK-21 cell line (NVI protocol).

3.11. Immunization of the animals

A total of 29 local zebu breed male cattle, aged 3 to 4 years' old, held at an experimental room of animal facility and quarantine department if NVI were selected for this study. The cattle were randomly grouped into five groups and the cattle in each group were vaccinated subcutaneously (SC) using individual syringes of 10 ml and 21G needle for each group in the middle of the cervical area with 4 ml of one of the following vaccine. Group 1 were vaccinated with trivalent FMD vaccine adjuvanted with conventional AS and group 2 were similarly vaccinated as group 1, but they took booster dose on 14^{th} day post vaccination. Group 3 were vaccinated with vaccine formulated with oil-based adjuvant. Group 4 were vaccinated with vaccine containing combination of standard AS and oil. The fifth group were left as control. After the cattle given first dose with 4 ml SC (at 0 day), all the group except group 2 were immunized twice at the interval of 14 days post vaccination with the same protocol and dose used for primary vaccination to boost the first immunization.

3.12. Blood and serum sample collection

The blood sample were collected from each group of animals for detecting humoral immune response of vaccinated and non-vaccinated cattle on day 0, 7, 14, 21, 28, and 42 days post vaccination. About 5 ml blood sample was collected from all experimentally immunized and control group in a clean sterile plain vacutainer tube from the jugular vein using individual syringe and vacutainer needle of 0.8*38 mm for each animal. Prior to collection the animals were restrained in crash and site of collection was palpated to determine the location of the jugular vein located in the lower neck and disinfected with 70 % ethanol. The vein was pressed firmly by thumb and the needle was inserted into engorged vein by free hand and the blood was collected in plain vacutainer tube. The collected blood was left at room temperature for 24 hours to clot. After 24 hours, sera were harvested aseptically into clean cryovial tubes which were labeled as day 0, 7, 14, 21, 28, and 42 as well as by a tag number of the animals and stored at -20 ^{o}C until testing. Then, the serum samples were labeled and transported to NAHDIC with the ice box for ELISA assay.

3.13. Challenge with live virus

After twenty-eight days of post vaccination the vaccinated and control group were challenged by intradermal inoculation in the upper surface of the tongue with 0.1 ml of the virus per site according to the protocol described by (Pacheco *et al*., 2005; OIE, 2018). All the challenge experiments were carried out in a highly contained environment at NVI. Locally isolated live FMDV serotype O provided by NVI and having $10^{6.4}$ $TCID_{50}$ /ml infectivity titer was used as a challenge virus. After challenge the cattle were restrained and carefully examined daily for any clinical signs of FMD and lesion, inside the mouth in the muzzle, on the tongue and feet for two weeks. Additionally, their rectal temperatures were monitored twice per day for the first seven consecutive days and recorded.

3.14. Serological assays

3.14.1. Solid phase competitive ELISA (SPCE)

Serum samples collected from the vaccinated and non-vaccinated cattle were tested for the antibody response against the three serotypes of FMDV (A, O and SAT 2). The test was carried out by using solid phase competitive ELISA (SPCE) assay for detection of structural proteins specific to the three FMDV serotypes (A, O, and SAT 2). The assay is applied to measure antibodies in serum samples of FMDV infected or vaccinated animals using a selected neutralizing anti-FMDV monoclonal antibodies (mAbs), specific for FMDV serotypes. In SPCE assay the test sera were incubated with the trapped inactivated antigen, enabling the specific antibodies present in the sample to bind to the respective antigen. Then, the ant-FMDV mAb, conjugated with peroxidase is dispensed. The reaction of conjugated antibody with homologous antigen were inhibited by antibodies of positive sample previously bound to the virus, while in the case of negative sample the conjugated mAb were bound to the virus and the reaction is appreciated by color development (OIE, 2018).

Therefore, in the present study, the SPCE kits ready to use were used to measure antibody levels elicited by FMD vaccines containing serotype A, O and SAT 2, the test was performed aseptically for each serotype according to the instructions given in the manual (IZSLER Brescia Italy). 96-wells ELISA microplates supplied pre-coated with inactivated antigen of FMDV serotypes captured by the homologous mAb were used. In the assay, 3 plates/sample coated with specific antigens of type A, O and SAT 2 were used. The first two wells A1 and B1 were used for positive control (i.e. 1/10 and 1/30 dilution of positive sera were added in A1 and B1 wells respectively). In the A1 the positive sera (7.5 µl) and ELISA buffer (67.5 µl) were mixed using mono channel micropipette and in B1, 50 µl buffer was dispensed. After mixing, 25 µl of the positive control serum was transferred from the dilution of 1/10 to the subsequent well B1 to obtain the dilution of 1/30 and mixed. Then, without changing the tip, 25 µl was pipetted from well B1 and discarded to maintain the final volume of 50 µl /well. Accordingly, 50 µl negative control was distributed in each of the four wells in positions of C1, D1, E1 and F1. The test was continued with distribution of 45 µl/well ELISA diluent buffer and 5 µl/well of test sera to gain the final dilution of 1/10 and mixed using multichannel pipette. The plates were covered and incubated at room temperature for 60 minutes to allow antigen-antibody (Ag-Ab) reaction. After incubation, 25 µl/well HRPO (Horse Reddish Peroxidase)-conjugate diluted at 1/10 dilution was added to each well. Again, the plates were covered and incubated at room temperature for 60 minutes. After 1-hour incubation, the fluid was removed from the wells. The plates were filled with 200 µl/well washing solution containing PBS-tween20 diluted in distilled water (1/10 dilution) and left at room temperature for 3 minutes without removing the solution. Washing solution was removed and washing was repeated 3 times. The last washing was left at room temperature for 5 minutes (in total of four washing cycles). Therefore, the plates were tapped firmly onto clean absorbent towel to remove residual fluids. 50 µl/well substrate/chromogen solution was added to all wells and the plates were allowed to incubate at room temperature for 20 minutes in the dark place. Finally, stop solution (50 µl/well) was added to stop the reaction between substrate and enzyme and the plates were gently shaken prior to reading. Immediately after addition of stop solution, optical density (OD) of each well was measured at wavelength of 450 nm using microplate reader. The ELISA plate absorbance was converted to the percent inhibition (PI) value. The PI 70 %

or above at 1/10 dilution, was considered the cutoff and the cattle were regarded as antibody positive.

A percentage of inhibition (PI) was calculated according to the formula described by (kit manufacturer and OIE, 2018): (100 – [mean optical density of the group/mean optical density of the negative control] × 100). The percentage of inhibition, implies the competition between antibody present in test sample and HRP conjugated guinea-pig anti-FMDV antisera for the specific FMDV antigen pre-coated on the ELISA plate (OIE, 2018). Therefore, PI is directly proportional to antibody level in the test sera.

3.14.2. Criteria for test validity

The spectrophotometric reading of the negative control wells are expected to give OD value of 1 or higher for FMDV serotype SAT 2 and O, and 0.8 OD or more reading in negative control wells for FMDV serotype A. While, in wells of the positive control serum expected to give 90 % or higher inhibition at 1/10 dilution and >50 % at 1/30 dilution for serotype A, and O, 80 % or higher at 1/10 dilution and >50 % at 1/30 dilution for SAT 2 serotype (Table 2).

Table 2: Criteria of test validity for the three FMDV serotypes

FMDV serotypes	Expected OD in negative control	Expected PI % at 1/10 dilution	Expected PI % at 1/30 dilution
A	≥0.8	≥90	>50
O	≥1	≥90	>50
SAT 2	≥1	≥80	>80

3.15. Data management and analysis

Data from SPCE ELISA result was recorded on Microsoft Excel spreadsheet. Therefore, data analysis was performed using analytical software STATA version 12. Analysis of variance (ANOVA) was used to determine statistical significance between the adjuvant

formulation with respect to their immune response. Students *t. test* was also used to compare pairwise comparisons of mean of adjuvants. p<5 % (0.05) at 95 % confidence interval (CI) was used to report the effects as statistically significant.

3.16. Ethical approval

The experimental animals were treated according to the protocol of ethical guidelines of the Animal Welfare Committee. They were kept and cared in the animal facility of NVI and used for the experiment after approval from Animal Research Ethics Review Committee (ARERC) of Addis Ababa University College of Veterinary Medicine and Agriculture (AAU-CVM) (Appendix 5).

4. RESULT

4.1. Virus titration result

The infectivity titer calculated and expressed in log_{10} of 50 % tissue culture infectious dose ($TCID_{50}$) for the three serotypes of FMDV (A, O and SAT 2) using BHK-21 cell culture is listed in (Table 3).

Table 3: The infectivity dose of the viruses expressed in log_{10} $TCID_{50}$ /ml

FMDV serotypes	Infectivity dose of the viruses in log_{10}
A	10^{7} $TCID_{50}$ /ml
O	$10^{6.4}$ $TCID_{50}$ /ml
SAT 2	$10^{7.2}$ $TCID_{50}$ /ml

TCID= Tissue culture infectious dose

4.2. Sterility and safety test result

The sterility test of the vaccine formulations showed that the vaccines were free from aerobic and anaerobic bacteria, fungal and mycoplasma contaminants. Similarly, assessment of absence of CPE on BHK-21 cells helped to confirm complete virus inactivation (Table 4). Thus, the formulated vaccines were considered to be safe for animal experimentation as per the OIE requirements for vaccine preparation (OIE, 2017).

Table 4: Summary of sterility and safety test results of the prepared trivalent FMD vaccines

Bacteriological media/ cell culture used for the test	Vaccine formulations		
	AS	Oil	AS + oil
Thioglycolate broth (for bacterial contaminants)	-ve	-ve	-ve
Tryptone Soya broth (for bacterial contaminants)	-ve	-ve	-ve
Sabouraud agar (fungal contaminants)	-ve	-ve	-ve
Mycoplasma medium	-ve	-ve	-ve
BHK-21 cell culture	no CPE	no CPE	no CPE

-ve =Negative, BHK=Baby hamster kidney cell, CPE=Cytopathic effect, AS=Aluminum hydroxide gel and saponin

4.3. Immune response result for FMDV serotype A

In experimental groups inoculated with vaccine formulations containing a mixture of aluminum hydroxide gel and saponin, aluminum hydroxide gel and saponin with a booster dose and aluminum hydroxide gel, saponin and oil, and oil alone, there was an evident increase in antibody level from day 7 to 42 post vaccination for serotype A (Table 5). On day 42 after vaccination, the percent of inhibition observed was highest in experimental group where a mixture of aluminum hydroxide gel, saponin and oil was used as an adjuvant (PI=99.26 %) followed by groups inoculated with vaccine formulation containing oil alone (PI=97.76 %) and aluminum hydroxide gel and saponin with a booster dose (PI=94.79 %). The experimental group inoculated with formulations which contains aluminum hydroxide gel and saponin alone had the lowest immune response (PI=89.57 %). There was a significant difference in humoral immune response in experimental group vaccinated with formulation containing aluminum hydroxide gel and saponin mixed with oil ($p<0.05$) as evidenced by the mean OD value and highest percentage of inhibition (Table 6) compared to other groups.

Table 5: Mean optical density (OD) of antibody response of cattle against FMDV serotype A

	Experimental groups					
Days post vaccination	Control (mean OD with CI)	AS (mean OD with CI)	AS boost (mean OD with CI)	Oil (mean OD with CI)	AS + oil (mean OD with CI)	P value
Day 0	1.72 (1.36- 2.0)	1.66 (1.14-2.17)	1.39 (0.84-1.94)	1.54 (1.22-1.86)	1.57 (1.17-1.96)	0.727
SE	0.13	0.20	0.21	0.12	0.15	
Day 7	1.36 (0.96-1.75)	1.02 (0.89-1.13)	0.99 (0.70-1.27)	0.89 (0.71-1.08)	0.84 (0.71-0.97)	0.0052
SE	0.14	0.04	0.10	0.07	0.05	
Day 14	1.48 (1.0-1.97)	1.48 (1.0-1.97)	0.82 (0.67-0.98)	0.63 (0.54-0.72)	0.44(0.35- 0.51)	0.0000
SE	0.17	0.17	0.06	0.03	0.03	
Day 21	1.1 (0.83-1.38)	0.62 (0.49-0.74)	0.43 (0.30-0.55)	0.32 (0.25-0.39)	0.16 (0.10-0.21)	0.0000
SE	0.09	0.04	0.04	0.02	0.02	
Day 28	1.48 (1.04-1.92)	0.40 (0.28-0.52)	0.17 (0.09-0.24)	0. 07 (0.02-0.13)	0.03 (0.01-0.04)	0.0000
SE	0.15	0.04	0.02	0.02	0.01	
Day 42	1.31 (1.09-1.53)	0.28 (0.15-0.41)	0.14 (0.06-0.20)	0.06 (0.02-0.11)	0.02 (0.01-0.02)	0.0000
SE	0.08	0.05	0.02	0.04	0.00	

AS=Aluminum hydroxide gel and saponin, AS boost=Aluminum hydroxide gel and saponin boosted, AS + oil=Aluminum hydroxide gel, saponin and oil, CI=Confidence interval, OD=Optical density, SE=Standard error

Table 6: Mean percentage inhibition of antibody response of cattle against FMDV serotype A as measured by SPCE.

Adjuvant Formulation	Percentage of inhibition (PI %)					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 42
Control	35.58	49.06	44.56	59.03	44.88	51.21
AS	37.82	61.79	70.03	76.9	85.1	89.57
AS boost	47.94	62.92	69.29	83.98	93.67	94.79
Oil	42.32	66.67	76.4	88.1	97.39	97.76
AS + oil	41.2	68.54	83.52	94.04	98.88	99.26

AS=Aluminum hydroxide gel and saponin, AS boost=Aluminum hydroxide gel and saponin boosted, AS + oil=Aluminum hydroxide gel, saponin and oil

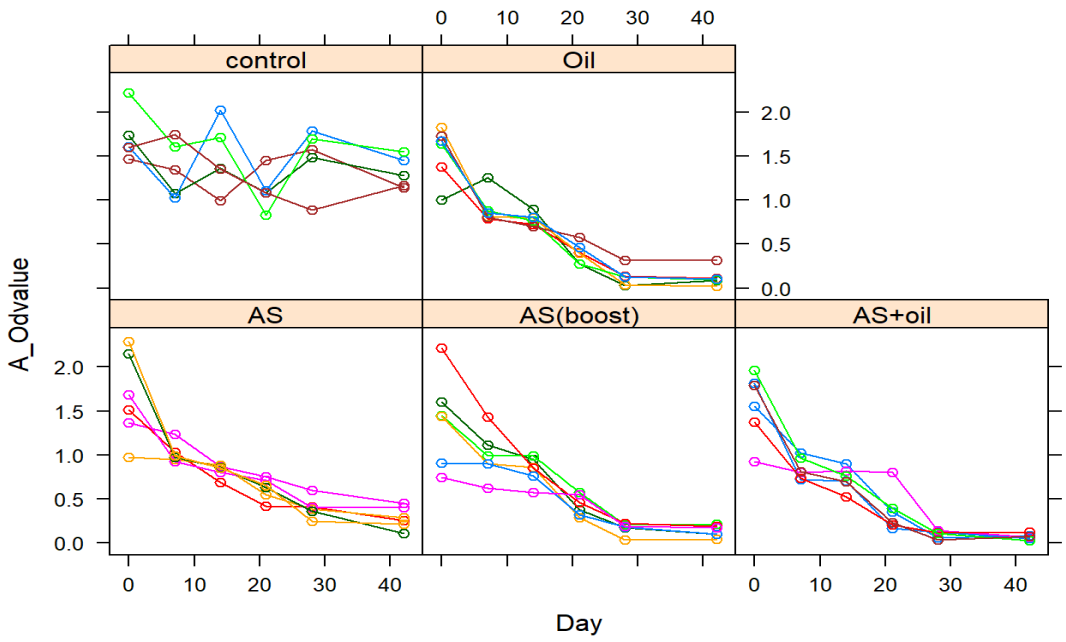

Figure 7: Line graphs displaying the antibody response of individual cattle in each experimental group throughout the study period for serotype A

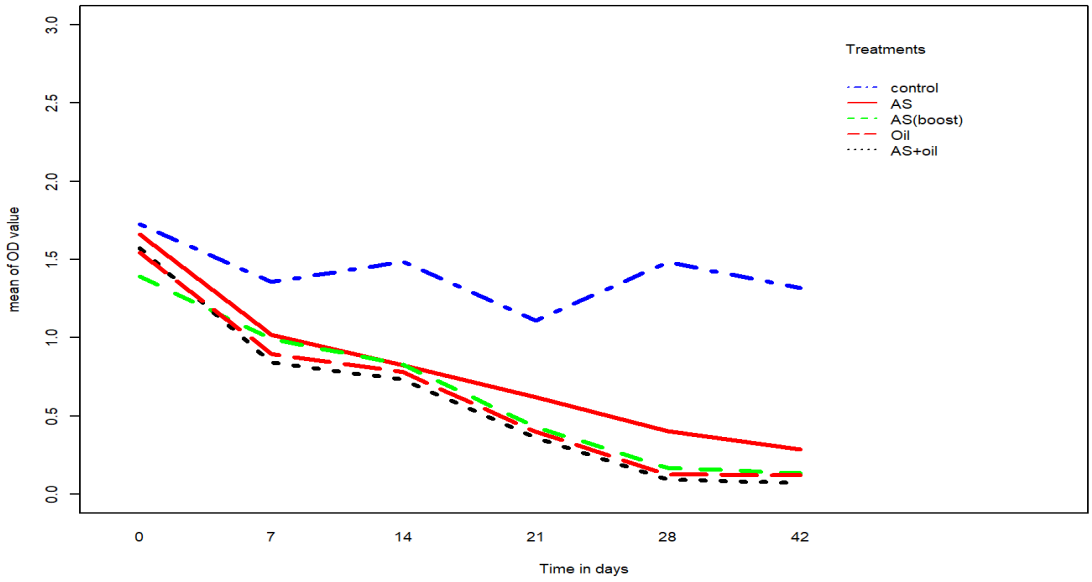

Figure 8: Line graph showing the mean antibody response of all the groups against serotype A at 0, 7, 14, 21, 28 and 42 days post vaccination

4.4. Immune response result for FMDV serotype O

In all the four experimental groups the result revealed that the mean antibody response for FMDV serotype O increased considerably over all post vaccination period (Table 7, Figure 9 and 10). Significantly higher antibody titers were recorded from day 0 to reach its maximum value at day 42 in experimental groups inoculated with formulations containing a mixture of aluminum hydroxide gel, saponin and mineral oil (1.58, 0.04), and oil alone (1.57, 0.05), ($p<0.05$). However, the titers were significantly lower in experimental groups inoculated with vaccine preparation adjuvanted with a mixture of aluminum hydroxide gel and saponin (1.62, 0.19) followed by groups inoculated with mixture of aluminum hydroxide gel and saponin with a booster dose (1.44, 0.12) at day 0 and 42, respectively compared to other groups ($p<0.05$). The maximum percent of inhibition at 42 days post vaccination was recorded in experimental groups inoculated with vaccine formulations containing a mixture of aluminum hydroxide gel, saponin and mineral oil (P=98.47 %) and mineral oil alone (P=98.08 %). The percent of inhibition in experimental groups inoculated

with vaccine formulations containing aluminum hydroxide gel and saponin with a booster dose was 95.4 %, whereas this was 92.72 % without a booster (Table 8).

Table 7: Mean optical density (OD) of antibody response of cattle against FMDV serotype O

Days post vaccination	Experimental groups					
	Control (mean OD with CI)	AS (mean OD with CI)	AS boost (mean OD with CI)	Oil (mean OD with CI)	AS + oil (mean OD with CI)	P value
Day 0	1.40 (0.8-1.99)	1.62 (1.08-2.16)	1.44 (0.69-2.19)	1.57 (1.09-2.04)	1.58 (1.02-2.14)	0.9544
SE	0.21	0.20	0.29	0.18	0.21	
Day 7	1.36 (0.73-1.98)	1.06 (0.87-1.20)	0.86 (0.57-1.14)	0.86 (0.76-0.95)	0.93 (0.7-1.08)	0.0279
SE	0.07	0.07	0.11	0.03	0.06	
Day 14	1.37 (1.1-31.61)	0.86 (0.81-0.93)	0.77 (0.50-1.04)	0.52 (0.47-0.61)	0.54 (0.39-0.69)	0.0000
SE	0.08	0.02	0.10	0.02	0.05	
Day 21	1.18 (1.01-1.34)	0.62 (0.5-0.73)	0.45 (0.37-0.53)	0.31 (0.20-0.40)	0.16 (0.10-0.22)	0.0000
SE	0.05	0.04	0.03	0.04	0.02	
Day 28	1.37 (1.19-1.55)	0.29 (0.22-0.37)	0.17 (0.06-0.26)	0.08 (0.00-0.15)	0.03 (0.01-0.03)	0.0000
SE	0.06	0.02	0.03	0.02	0.00	
Day 42	1.42 (1.16-1.66)	0.19 (0.14-0.24)	0.12 (0.08-0.15)	0.05 (0.01-0.09)	0.04 (0.02-0.05)	0.0000
SE	0.08	0.01	0.01	0.04	0.01	

AS=Aluminum hydroxide gel and saponin, AS boost=Aluminum hydroxide gel and saponin boosted, AS + oil=Aluminum hydroxide gel, saponin and oil, CI=Confidence interval, OD=Optical density, SE=Standard error

Table 8: Mean percentage inhibition of antibody response of cattle against FMDV serotype O as measured by SPCE.

Adjuvant formulation	Percentage of inhibition (PI %)					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 42
Control	51.39	52.78	52.43	59.03	47.51	45.59
AS	43.75	63.19	70.14	76.24	88.29	92.72
AS boost	50	70.14	73.26	82.76	93.49	95.4
Oil	45.48	70.14	81.25	88.12	96.93	98.08
AS + oil	45.14	67.71	81.25	93.87	98.85	98.47

AS=Aluminum hydroxide gel and saponin, AS boost=Aluminum hydroxide gel and saponin boosted, AS + oil=Aluminum hydroxide gel, saponin and oil

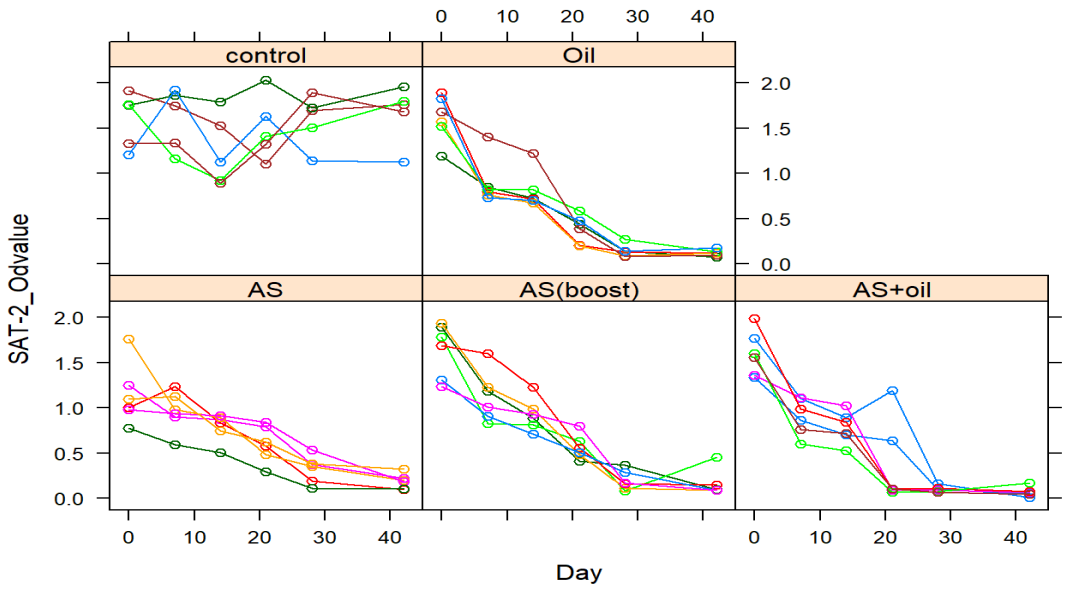

Figure 9: Line graphs displaying the antibody response of individual animals in each group throughout the study period for serotype O

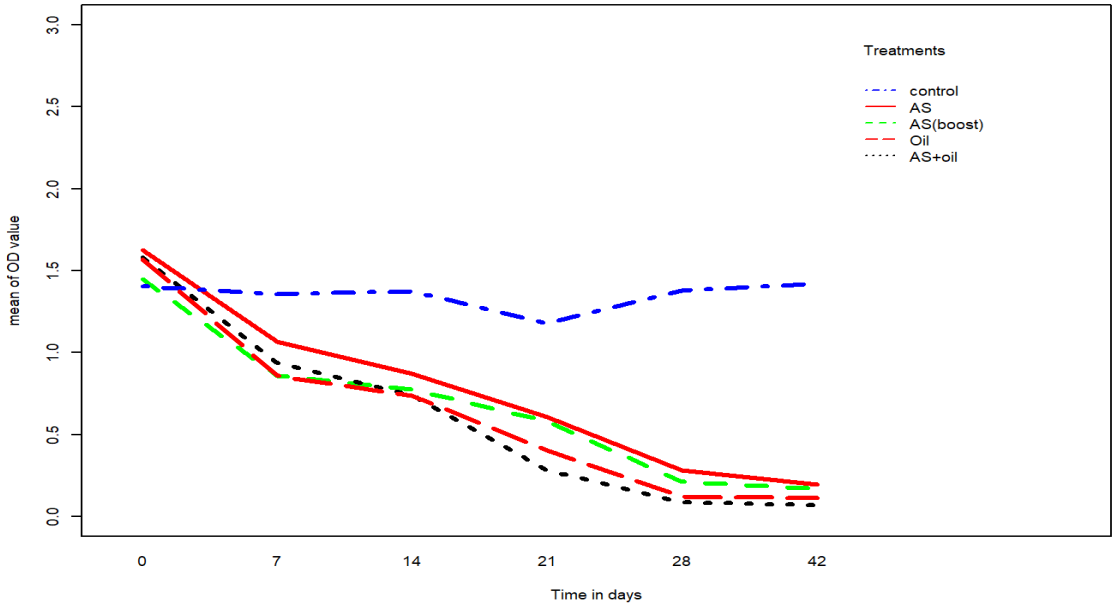

Figure 10: Line graph showing the mean antibody response of the groups against serotype O at 0, 7, 14, 21, 28 and 42 days post vaccination

4.5. Immune response result for FMDV serotype SAT 2

In similar pattern to immune responses recorded for the other two serotypes (Serotype A and Serotype O), the highest mean antibody level and percent of inhibition were observed in groups inoculated with vaccine formulation containing a mixture of aluminum hydroxide gel, saponin and oil, and oil alone (Table 9 and 10). Whereas, the results showed that groups immunized with the vaccine prepared with aluminum hydroxide gel and saponin induced less antibody response and percent of inhibition followed by groups vaccinated with aluminum hydroxide gel and saponin with booster dose.

Table 9: Mean optical density (OD) of antibody response of cattle against FMDV serotype SAT 2

	Experimental groups					
Days post vaccination	Control (mean OD with CI)	AS (mean OD with CI)	AS boost (mean OD with CI)	Oil (mean OD with CI)	AS + oil (mean OD with CI)	P value
Day 0	1.59 (1.21-1.97)	1.30 (0.82-1.78)	1.47 (1.19-1.74)	1.61 (1.35-1.87)	1.59 (1.34-1.85)	0.3123
SE	0.13	0.18	0.10	0.10	0.10	
Day 7	1.52 (1.17-1.88)	1.32 (0.94-1.71)	1.12 (0.83-1.41)	0.89 (0.63-1.16)	0.90 (0.69-1.11)	0.0039
SE	0.13	0.15	0.11	0.10	0.08	
Day 14	1.25 (0.76-1.73)	0.79 (0.63-0.95)	0.92 (0.73-1.11)	0.59 (0.42-0.76)	0.55 (0.45-0.64)	0.0001
SE	0.17	0.06	0.07	0.06	0.03	
Day 21	1.50 (1.0-1.93)	0.63 (0.47-0.79)	0.45 (0.30-0.53)	0.29 (0.16-0.40)	0.11(0.07-0.16)	0.0000
SE	0.15	0.06	0.03	0.04	0.02	
Day 28	1.59 (1.23-1.94)	0.32 (0.16-0.48)	0.16 (0.08-0.23)	0.08 (0.05-0.11)	0.04 (0.01-0.07)	0.0000
SE	0.12	0.06	0.02	0.01	0.01	
Day 42	1.66 (1.27-2.06)	0.23 (0.14-0.32)	0.16 (0.01-0.31)	0.07 (0.30-0.11)	0.02 (0.01-0.03)	0.0000
SE	0.14	0.03	0.05	0.04	0.01	

AS=Aluminum hydroxide gel and saponin, AS boost=Aluminum hydroxide gel and saponin boosted, AS + oil=Aluminum hydroxide gel, saponin and oil, CI=Confidence interval, OD=Optical density, SE=Standard error

Table 10: Mean percentage inhibition of antibody response of cattle against FMDV serotype SAT 2 as measured by SPCE.

Adjuvant formulation	Percentage of inhibition (PI %)					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 42
Control	17.19	20.23	34.89	25.37	20.89	17.41
AS	32.29	31.25	58.85	68.66	84.07	88.55
AS boost	23.44	41.67	52.08	77.01	92.04	92.04
Oil	16.15	53.65	69.27	85.57	96.01	96.52
AS + oil	17.19	53.12	71.54	94.53	98	99

AS=Aluminum hydroxide gel and saponin, AS boost=Aluminum hydroxide gel and saponin boosted, AS + oil=Aluminum hydroxide gel, saponin and oil

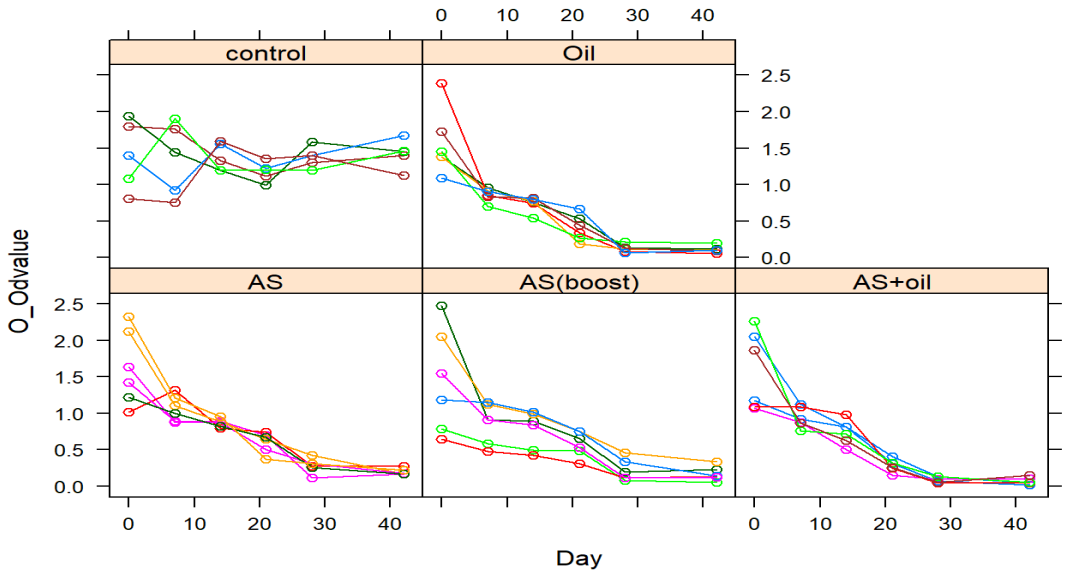

Figure 11: Line graphs displaying the antibody response of individual animals in each group throughout the study period for serotype SAT 2

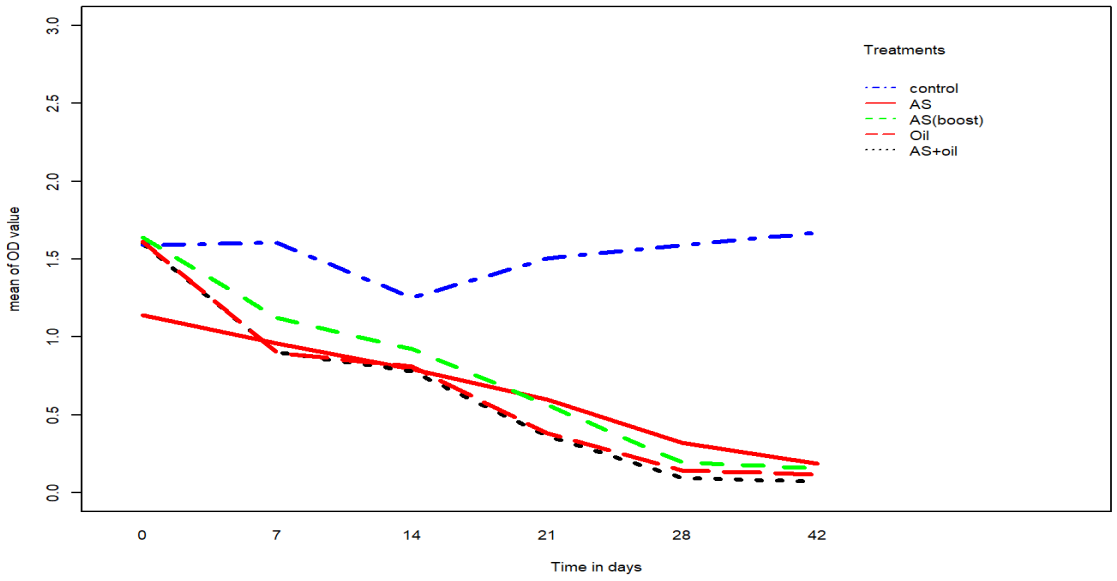

Figure 12: Line graph showing the mean antibody response of the experimental groups against serotype SAT 2 at 0, 7, 14, 21, 28 and 42 days post vaccination.

4.6. Challenge test result

The experimental cattle challenged intradermal on the upper tongue with $10^{6.4}$ TCID50/ ml of homologous FMDV serotype O at 28 days after vaccination did not develop any clinical disease and lesion of FMD. Furthermore, the body temperature of each cattle recorded daily for seven consecutive days did not exhibit any elevation in temperature (remained below 40 ^{o}C).

5. DISCUSSION

Foot and Mouth Disease (FMD) is an acute, febrile, and highly contagious vesicular disease of cloven hoofed animals caused by FMDV. It is devastating disease of livestock causes significant economic losses following an outbreak (Rodriguez and Grubman, 2009). Control of the disease particularly in endemic areas could be achieved through vaccination with the use of quality vaccine prepared with locally circulated serotypes and effective adjuvants (Tadesse *et al.*, 2017; Lyons *et al.*, 2016). Adjuvants have been used for more than 70 years even when the mode of their action had not been understood (Macedo *et al*., 2013). The use of adjuvants in inactivated FMD vaccine formulation plays a vital role to improve its performance in provision of rapid and long lasting protective immunity. Thus, the findings of the present study are imperative in contributing valuable information in the understanding of the effective formulations of FMD vaccines and selection of adjuvants used in the eradication of the disease.

Overall, in all cattle vaccinated with various experimental vaccine formulations SPCE result indicated that there was decrease in OD value of a conjugate antibody during post vaccination period. (Table 5, 7, 9 and Figure 7,8,9,10,11, and 12), which is because of the competition between antibodies in test sera and enzyme conjugated antibody for the same antigen. The decrease in OD value is indirectly proportional to antibody level in the test serum. This in turn, indicates increase in antibody titers and percentage of inhibition as antibodies available in test sera impede binding of conjugated antibody (Table 6, 8 and 10).

Evaluation of antibody responses to the serotype A, O and SAT 2, aluminum hydroxide gel and saponin, oil-based, and combination of aluminum hydroxide gel and saponin with oil-adjuvanted vaccines revealed considerable variation both in the rapidity of development and magnitude of humoral immune response (Figure 8, 10,12). The variation in immune response is related primarily to difference in formulation and booster injection to experimental animals.

In groups vaccinated with aluminum hydroxide gel and saponin formulations in all the three serotypes used in this study, a booster dose has shown a statistically significant difference in mean antibody response compared to groups without a booster dose ($p<0.05$). This is in agreement with Patil *et al.* (2012) and Cloete *et al.* (2008), who described the poor immune response elicited to inactivated FMD vaccine formulated with aluminum hydroxide gel and saponin necessitate relatively frequent revaccinations to provide protective immunity. Lyons *et al.* (2016), were also suggested that among factors that limit effective performance of inactivated FMD vaccine the requirements for repeat boosting is one factor due to short duration of protection induced by aluminum hydroxide gel and saponin without boosting. Also, similar results obtained by Çokçalışkan *et al.* (2017) in cattle, suggested that better immune response in cattle could be effects of booster dose.

The antibody response between aluminum hydroxide gel and saponin boosted and oil based group were not statistically significant for the three serotypes until day 21 post vaccination ($p>0.05$). As described by Garçon *et al*. (2011), this may be due to the reason that oil emulsions adjuvant function as delivery systems by the formation of depot effect that traps antigen at the injection site and by slowing the release of antigen to induce a long-lasting immune response. However, the mean antibody response reached to the maximum in aluminum hydroxide gel and saponin boosted group for serotype A, O and SAT 2 on day 28 and 42 were significantly lower than that of oil group ($p<0.05$). These results were concordant with those reported by Patil *et al.* (2002), who found that oil adjuvant elicited a better immune response than did aluminum hydroxide gel and saponin vaccine.

The use of combined formulation of aluminum hydroxide gel, saponin and mineral oil adjuvant is not common. However, in the present study, the mixture of aluminum hydroxide gel, saponin and mineral oil adjuvant for inactivated trivalent foot and mouth disease vaccine was evaluated. The ELISA results and statistical analysis revealed that this group showed relatively a higher post vaccination antibody response than that without mixing against the three serotypes of FMDV suggesting that the combination of aluminum hydroxide gel; saponin and mineral oil have a great impact on the immune response. This was partly in agreement with EI *et al.* (2020), in that FMD vaccine prepared with mixing

aluminum hydroxide gel and oil-based adjuvant is considered the best in the humoral immune response than oil alone. In the study by Park *et al.* (2016) conduced in mice, showed that combinations of aluminum hydroxide gel and oil-based adjuvant revealed the highest protection rate. Moreover, the results also partially corresponded to previous work conducted by Park *et al.* (2014), who reported a combination of oil with a gel enhanced the immune response in experimental animals and concluded that oil improves the effects gel of adjuvant.

On the other hand, a synergy between oil and saponin for immune response has been reported in some studies. Çokçalışkan *et al.* (2016), observed a strong antibody response in cattle vaccinated with FMD vaccine formulated with combined oil and saponin adjuvants. Recently, the other study conducted by Bazid *et al*. (2021) in guinea pigs and cattle with monovalent FMDV serotype O, also reported that oil-based FMD vaccines containing saponin induced higher antibody level than vaccine without saponin. These findings are partially corroborating with the finding of the present study that enhanced antibody response was obtained by the use of aluminum hydroxide gel and saponin in combination with mineral oil adjuvant in a FMD vaccine compared to other adjuvants.

The groups immunized with combination of aluminum hydroxide gel, saponin and mineral oil induced the higher humoral immune response in comparison to groups vaccinated with aluminum hydroxide gel and saponin. According to this finding, the higher immune response induced could be because of the effects of mineral oil adjuvant added in the formulation. As a result, tentatively it was concluded that oil adjuvant improves the effects of aluminum hydroxide gel and saponin adjuvants.

In this experimental study the virus prepared for challenge test did not develop any typical clinical signs and lesions of FMD in control group. This might be due to the serial passage of the virus used for vaccine production (laboratory adapted virus) which could lead to losing its infectivity. This is in agreement with finding of Cloete *et al.* (2008), who found that the control cattle did not develop any clinical disease. As a result, further potency test of the formulated vaccines was not carried out

6. CONCLUSION AND RECOMMENDATIONS

FMD is still considered globally as one of the most economically devastating diseases. Thus, vaccination is a very effective way of controlling and eradicating FMD from endemic regions of the world. Quality vaccine is not only depending on the antigen payload, but also on the adjuvant used in the vaccines. In Ethiopia, inactivated trivalent FMD vaccine containing aluminum hydroxide gel and saponin as adjuvants is commonly produced, but it has several limitations such as the induction of short-lived antibody responses which require frequent re-immunization. In the present study, different formulations of adjuvants were evaluated for trivalent FMD vaccine. This study demonstrated that cattle vaccinated with single injection of vaccine formulations containing aluminum hydroxide gel and saponin as an adjuvant had lower antibody response compared to cattle boosted at day 14 with the same vaccine formulation. These observations confirmed that the booster dose is necessary to enhance the immune response of aluminum hydroxide gel and saponin adjuvanted vaccine against FMD. While, trivalent FMD vaccine prepared with oil and mixture of aluminum hydroxide gel, saponin and oil adjuvant vaccine elicited better immune response than the other adjuvant formulation. Also, it could be concluded that oil-based and aluminum hydroxide gel and saponin with oil adjuvanted vaccine could replace the aluminum hydroxide gel and saponin in FMD vaccine.

Therefore, based on the above conclusion, the following recommendations are forwarded:

- Since the aluminum hydroxide gel and saponin is poorly immunogenic indicating the need for a booster dose and administration with booster dose should be used for control of the disease.
- Considering the antibody response induced by mineral oil and combination of aluminum hydroxide gel and saponin with oil these adjuvants should be encouraged to replace the aluminum hydroxide gel and saponin in FMD vaccine production.
- Further experimental research on evaluation of these adjuvants should be performed to establish the validity of the study.
- Regarding the challenge virus, further work should be carried out on biological characterization of viral infectivity.

7. REFERENCES

Agrawal, A., Owais, M., and Singh, U. P. (2013): Novel Vaccine Adjuvants. *BioMed Research I.*

Ahlberg, V., Hjertner, B., Wallgren, P., Hellman, S., Bengtsson, K. L., and Fossum, C. (2017): Innate immune responses induced by the saponin adjuvant Matrix-M in specific pathogen free pigs. *Veterinary Research*, **48**(1): 30.

Alemayehu, G., Zewde, G., and Admassu, B. (2014): Seroprevalence of foot and mouth disease (FMD) and associated economic impact on Central Ethiopian cattle feedlots. *Journal of Veterinary Medicine and Animal Health*, **6**(5): 154-158

Aman, E., Molla, W., Gebreegizabher, Z., and Jemberu, W. T. (2018): Spatial and temporal distribution of foot and mouth disease outbreaks in Amhara region of Ethiopia in the period 1999 to 2016. *BMC Veterinary Research*, **16**(1): 1-8.

Arzt, J., Belsham, G. J., Lohse, L., Bøtner, A., and Stenfeldt, C. (2018): Transmission of foot-and-mouth disease from persistently infected carrier cattle to naive cattle via transfer of oropharyngeal fluid. *Msphere*, **3**(5).

Ashenafi, B. (2012): Costs and benefits of foot and mouth disease vaccination practices in commercial dairy farms in Central Ethiopia. *Wageningen University MSc thesis*.

Auty, H., Mellor, D. J., Gunn, G. J., and Boden, L. (2019): The risk of Foot and Mouth Disease transmission posed by public access to the countryside during an outbreak. *Frontiers in veterinary science*, **6**: 381.

Awate, S., Babiuk, L. A. B., and Mutwiri, G. (2013): Mechanisms of action of adjuvants. *Frontiers in immunology*, **4**: 114.

Ayebazibwe C, Mwiine FN, Tjornehoj K, Balinda SN, Muwanika VB, Ademun OkurutAR. (2010): The role ofAfrican buffalos (Syncerus caffer) in the maintenance of foot-and-mouth disease in Uganda. BMC Vet Res **2010**:6-54.

Ayelet, G., Gelaye, E., Guitian, J., Sahle, M., Knowles, N.J., Mahapatra, M. (2008): The Status of Foot and Mouth Disease (FMD) in Ethiopia. The Global control of FMD - Tools, ideas and ideals – Erice, Italy Erice, Italy

Ayelet, G., Mahapatra, M., Gelaye, E., Egziabher, B. G., Rufeal, T., Sahle, M., and Knowles, N. J. (2009): Genetic characterization of foot-and-mouth disease viruses, Ethiopia, 1981–2007. *Emerging infectious diseases*, **15(**9): 1409.

Ayelet, G., Gelaye, E., Negussie, H., and Asmare, K. (2012): Study on the epidemiology of foot and mouth disease in Ethiopia. *Rev Sci Tech*, **31**(3): 789-98.

Azmi, F., Ahmad Fuaad, A. A. H., Skwarczynski, M., and Toth, I. (2014): Recent progress in adjuvant discovery for peptide-based subunit vaccines. *Human vaccines and immunotherapeutics*, **10**(3): 778-796.

Bastos, A. D. S., and Sangare, O. (2001): Geographic distribution of SAT-2 type foot-and-mouth disease virus genotypes in Africa. *Proceedings of the southern African Society for Veterinary Epidemiology and Preventive Medicine, Onderstepoort, South Africa*, **1011**, 2026.

Bari, F.D., Parida, S., Asfor, A.S., Haydon, D.T., Reeve, R., Paton, D.J., and Mahapatra, M. (2015): Prediction and characterization of novel epitopes of serotype Afoot-and-mouth diseases viruses circulating in East Africa using site-directed mutagenesis. The Journal of general virology, **96**(Pt 5),1033.

Barteling, S. J., and Cassim, N. I. (2004): Very fast (and safe) inactivation of foot-and-mouth disease virus and enteroviruses by a combination of binary ethyleneimine and formaldehyde. *Developments in biologicals*, **119**: 449-455.

Bayissa, B., Ayelet, G., Kyule, M., Jibril, Y., and Gelaye, E. (2011): Study on seroprevalence, risk factors, and economic impact of foot-and-mouth disease in Borena pastoral and agro-pastoral system, southern Ethiopia. *Tropical animal health and production*, **43**(4): 759-766.

Bazid, A. H. I., El-Alfy, H. A., El-Didamony, G., Elfeil, W. K., El-Sayed, M. M., and Fawzy, M. (2021): Adjuvant effect of saponin in an oil-based monovalent (serotype O) foot-and-mouth disease virus vaccine on the antibody response in guinea pigs and cattle. *Archives of Virology*, 1-8.

Birhanu, T. (2014): Prevalence of the major infectious animal diseases affecting livestock trade industry in Ethiopia. **4**(17).

Brito BP, Rodriguez LL, Hammond JM, Pinto J, Perez AM. (2015): Review of the global distribution of foot-and-mouth disease virus from 2007 to 2014. *Transbound*

Emerg Dis, **64**: 316–32

Cao, Y. (2014): Adjuvants for foot-and-mouth disease virus vaccines: recent progress. *Expert review of vaccines*, **13**(11): 1377-1385.

Carvalho Gomes, P. A. D. (2000): Surface plasmon resonance as a tool in the functional analysis of an immunodominant site in foot-and-mouth disease virus. Universitat de Barcelona.

Central Statistical Agency (2012): The Federal democratic republic of Ethiopia central statistical agency, agriculture in figure key findings of the 2008/09-2010/11 agricultural sample surveys for all sectors and seasons country summary, FDRECSA Addis Ababa, Ethiopia, 14-18.

Choi, J. H., You, S. H., Ko, M. K., Jo, H. E., Shin, S. H., Jo, H., and Park, J. H. (2020): Improved immune responses and safety of foot-and-mouth disease vaccine containing immunostimulating components in pigs. *Journal of Veterinary Science*, **21**(5).

Cloete, M., Dungu, B., Van Staden, L. I., Ismail-Cassim, N., and Vosloo, W. (2008): Evaluation of different adjuvants for foot-and-mouth disease vaccine containing all the SAT serotypes. *Onderstepoort Journal of Veterinary Research*, **75**(1): 17-31.

Çokçalışkan, C., Türkoğlu, T., Sareyyüpoğlu, B., Uzunlu, E., Babak, A., Özbilge, B. B., and Gülyaz, V. (2016): QS-21 enhances the early antibody response to oil adjuvant foot-and-mouth disease vaccine in cattle. *Clinical and experimental vaccine research*, **5**(2): 138-147.

Çokçalışkan, C., Türkoğlu, T., Uzunlu, E., Sareyyüpoğlu, B., Hancı, İ., İpek, A.,and Gülyaz, V. (2017): Influence of vaccine potency and booster administration of foot-and-mouth disease vaccines on the antibody response in cattle with maternal antibodies. *Journal of veterinary science*, **18**(Suppl 1), 315.

De Gregorio, E., Caproni, E., and Ulmer, J. B. (2013): Vaccine adjuvants: mode of action, *Front. Immunol*. **4**(2013) 214.

Doosti, M., Nassiri, M., Nasiri, K., Tahmoorespur, M., and Zibaee, S. (2019): Immunogenic evaluation of FMD virus immuno-dominant epitopes coupled with IL-2/FcIgG in BALB/c mice. *Microbial pathogenesis*, **132**: 30-37.

EI, E. S., WG, M., and AI, H. (2020): Immunomodulating efficacy of different adjuvants informulation of Foot and Mouth disease vaccine relative to its immunogenicity. *Journal of Applied Veterinary Sciences*, **5**(3): 14-30.

FAO. (2014). FAOSTAT. Food and Agriculture Organization of the United Nations.

Fernández-Tejada, A., Tan, D. S., and Gin, D. Y. (2016): Development of improved vaccine adjuvants based on the saponin natural product QS-21through chemical synthesis. *Accounts of chemical research*, **49(**9):1741-1756.

Francis, M. J. (2018): Recent advances in vaccine technologies. *The Veterinary Clinics of North America. Small Animal Practice*, **48**(2): 231.

Gao, Y., Sun, S. Q., and Guo, H. C. (2016): Biological function of Foot-and-mouth disease virus non-structural proteins and non-coding elements. *Virology journal*, **13(**1): 1-17.

Garçon, N., Leroux-Roels, G., and Cheng, W. F. (2011): Vaccine adjuvants. *Perspectives in Vaccinology*, **1**(1): 89-113.

Gelaye, E., Ayelet, G., Abera, T., and Asmare, K. (2009): Seroprevalence of foot and mouth disease in Bench Maji zone, Southwestern Ethiopia. *Journal of Veterinary Medicine and Animal Health*, **1**(1): 005-010.

Grazioli, S., Moretti, M., Barbieri, I., Crosatti, M., and Brocchi, E. (2006): Use of monoclonal antibodies to identify and map new antigenic determinants involved in neutralisation on FMD viruses type SAT 1 and SAT 2. *Report of the Session of the Research Foot and Mouth Disease, Group of the Standing Committee of the European commision of Foot and Mouth disease, Paphos, Cyprus: FAO*, 287-297.

Grubman, M. J., and Baxt, B. (2004): Foot-and-mouth disease. *Clinical microbiology reviews*, **17**(2): 465-493.

Hassan, A. I. (2016). Effect of different culture systems on the production of foot and mouth disease trivalent vaccine. *Veterinary world*, **9**(1): 32.

Haensler, J. (2011): Oil emulsions as vaccine adjuvants. Future Medicine Ltd.

He, P., Zou, Y., and Hu, Z. (2015): Advances in aluminum hydroxide-based adjuvant research and its mechanism. *Human vaccines and immunotherapeutics*, **11**(2): 477-488.

HogenEsch, H., O'Hagan, D. T., and Fox, C. B. (2018): Optimizing the utilization of aluminum adjuvants in vaccines: you might just get what you want. *npj Vaccines*, **3**(1): 1-11.

Ibrahim, E. E. S., Gamal, W. M., Hassan, A. I., Mahdy, S. E. D., Hegazy, A. Z., and Abdel-Atty, M. M. (2015): Comparative study on the immunopotentiator effect of ISA 201, ISA 61, ISA 50, ISA 206 used in trivalent foot and mouth disease vaccine. *Veterinary world*, **8**(10): 1189.

Iyer, A. V., Ghosh, S., Singh, S. N., and Deshmukh, R. A. (2000): Evaluation of three 'ready to formulate'oil adjuvants for foot-and-mouth disease vaccine production. *Vaccine*, **19(**9-10): 1097-1105

Jamal, S. M., and Belsham, G. J. (2013): Foot-and-mouth disease: past, present and future. *Veterinary research*, **44**(1): 116.

Jemberu, W. T., Mourits, M. C., Woldehanna, T., and Hogeveen, H. (2014): Economic impact of foot and mouth disease outbreaks on smallholder farmers in Ethiopia. *Preventive veterinary medicine*, **116**(1-2): 26-36.

Jemberu, W. T., Mourits, M., Rushton, J., and Hogeveen, H. (2016): Cost-benefit analysis of foot and mouth disease control in Ethiopia. *Preventive Veterinary Medicine*, **132**: 67-82.

Jemberu, W. T., Molla, W., and Fentie, T. (2020): A randomized controlled field trial assessing foot and mouth disease vaccine effectiveness in Gondar Zuria district, Northwest Ethiopia. *Preventive Veterinary Medicine*, **105:**136**.**

Kallel, H., Jouini, A., Majoul, S., and Rourou, S. (2002): Evaluation of various serum and animal protein free media for the production of a veterinary rabies vaccine in BHK-21 cells. *Journal of biotechnology*, **95**(3): 195-204.

Karber, G. (1931): Beitrage zur kille kiven behand–lung pharmakologis reihenversuche naunyn schiede berg. *Arch. Exp. Path. Pharmak*, **126**: 280-283.

Kasem, S., Fatah, S. A., Khodier, M., and Desouky, A. (2017): Evaluation of the efficacy of simultaneous vaccination of cattle against rabies and foot and mouth disease viruses. *Glob Vet*, **18**: 226-233.

Kim, H., Kim, A. Y., Kim, J. S., Lee, J. M., Kwon, M., Bae, S., and Ko, Y. J. (2019):

Determination of the optimal method for the concentration and purification of 146S particles for foot-and-mouth disease vaccine production. *Journal of virological methods*, 269: 26-29.

Knight-Jones, T. J. D., and Rushton, J. (2013): The economic impacts of foot and mouth disease What are they, how big are they and where do they occur? *Preventive veterinary medicine*, **112**(3-4): 161-173.

Knight-Jones, T. J. D., Edmond, K., Gubbins, S., and Paton, D. J. (2014): Veterinary and human vaccine evaluation methods. *Proceedings of the Royal Society B: Biological Sciences*, **281**(1784).

Kool, M., Fierens, K., and Lambrecht, B. N. (2012): Alum adjuvant: some of the tricks of the oldest adjuvant. *Journal of medical microbiology*, **61**(7): 927-934.

Lee, S., and Nguyen, M. T. (2015): Recent advances of vaccine adjuvants for infectious diseases. *Immune network*, **15**(2): 51-57.

Longjam, N., Deb, R., Sarmah, A. K., Tayo, T., Awachat, V. B., and Saxena, V. K. (2011): A brief review on diagnosis of foot-and-mouth disease of livestock: conventional to molecular tools. *Veterinary medicine international*, **2011**.

Lubroth, J., Rweyemamu, M. M., Viljoen, G., Diallo, A., Dungu, B., and Amanfu, W. (2007): Veterinary vaccines and their use in developing countries. *Revue scientifique et technique (International Office of Epizootics)*, **26**(1):179-201.

Lyons, N. A., Lyoo, Y. S., King, D. P., and Paton, D. J. (2016). Challenges of generating and maintaining protective vaccine-induced immune responses for foot-and-mouth disease virus in pigs. *Frontiers in veterinary science*, **3:** 102.

Macedo, L. B., Lobato, Z. I. P., Fialho, S. L., Viott, A. D. M., Guedes, R. M. C., and Silva-Cunha, A. (2013): Evaluation of different adjuvants formulations for bluetongue vaccine. *Brazilian Archives of Biology and Technology*, **56**(6): 932-941.

Maree, F. F., Kasanga, C. J., Scott, K. A., Opperman, P. A., Chitray, M., Sangula, A. K., andPaton, D. J. (2014): Challenges and prospects for the control of foot-and-mouth disease: An African perspective. *Veterinary Medicine: Research and Reports*, **5**: 119.

Maree, F. F., Nsamba, P., Mutowembwa, P., Rotherham, L. S., Esterhuysen, J.,and Scott,

K. (2015): Intra-serotype SAT2 chimeric foot-and-mouth disease vaccine protects cattle against FMDV challenge. *Vaccine*, **33**(25): 2909-2916

Maughan, C. N., Preston, S. G., and Williams, G. R. (2015): Particulate inorganic adjuvants: recent developments and future outlook. *Journal of Pharmacy and Pharmacology*, **67**(3): 426-449.

Mastelic, B., Ahmed, S., Egan, W. M., Del Giudice, G., Golding, H., Gust, I., and Lambert, P. H. (2010): Mode of action of adjuvants: implications for vaccine safety and design. *Biologicals*, **38(**5): 594-601.

Megersa, B., Beyene, B., Abunna, F., Regassa, A., Amenu, K., and Rufael, T. (2009): Risk factors for foot and mouth disease seroprevalence in indigenous cattle in Southern Ethiopia: the effect of production system. *Tropical animal health and production*, **41**(6): 891-898.

Mekonen, H., Beyene, D., Rufael, T., Feyisa, A., and Abunna, F. (2011): Study on the prevalence of foot and mouth disease in Borana and Guji Zones, Southern Ethiopia. *Veterinary World*, **4**(7): 293-296.

Mesfine, M., Nigatu, S., Belayneh, N., and Jemberu, W. T. (2019): Sero-Epidemiology of foot and mouth disease in domestic ruminants in Amhara region, Ethiopia. *Frontiers in veterinary science*, **6**: 130.

Mohan, T., Verma, P., and Rao, D. N. (2013): Novel adjuvants and delivery vehicles for vaccines development: a road ahead. *The Indian journal of medical research*, **138**(5): 779.

Mohamoud, A., Tessema, E., and Degefu, H. (2011): Seroprevalence of bovine foot and mouth disease (FMD) in Awbere and Babille districts of Jijiga zone, Somalia Regional State, Eastern Ethiopia. *African Journal of Microbiology Research*, **5**(21): 3559-3563.

Molla, B., Ayelet, G., Asfaw, Y., Jibril, Y., Ganga, G., and Gelaye, E. (2010): Epidemiological study on foot-and-mouth disease in cattle: seroprevalence and risk factor assessment in South Omo zone, south-western Ethiopia. *Transboundary and emerging diseases*, **57**(5): 340-347.

Morse, S. A., and Meyer, R. F. (2017). Viruses and Bioterrorism. *Reference Module in Life Sciences*.

Mosad, W., Elsayed, E., Hassanin, A. I., Mohamed, A. A., and Shabana, W (2019): Trials for Improvement of the Immune Response of Foot and Mouth Disease Vaccine Using Oil/Gel Mixture as an Adjuvant *ARC Journal of Animal and Veterinary Sciences*, **5**:1-9

Nazzarodel Cañizo, A. A. N., Mazza, M., Bellinzoni, R., and Cascone, O. (1997): Foot and mouth disease virus concentration and purification by affinity chromatography. *Applied biochemistry and biotechnology*, **61**(3): 399-409.

OIE (2008): 'Foot and mouth disease (Version adopted by the World Assembly of Delegates of the OIE in May 2009): *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008.*

OIE (2018): Foot and mouth disease (infection with foot and mouth disease virus) (NB: Version adopted in May 2017): OIE terrestrial manual 2018.

Pacheco, J. M., Brum, M. C., Moraes, M. P., Golde, W. T., and Grubman, M. J. (2005): Rapid protection of cattle from direct challenge with foot-and-mouth disease virus (FMDV) by a single inoculation with an adenovirus-vectored FMDV subunit vaccine. *Virology*, **337**(2): 205-209.

Park, J. H. (2013): Requirements for improved vaccines against foot-and-mouth disease epidemics. *Clinical and Experimental Vaccine Research*, **2**(1), 8.

Park, M. E., Lee, S. Y., Kim, R. H., Ko, M. K., Lee, K. N., Kim, S. M., and Park, J. H. (2014): Enhanced immune responses of foot-and-mouth disease vaccine using new oil/gel adjuvant mixtures in pigs and goats. *Vaccine*, **32**(40): 5221-5227.

Park, M. E., Lee, S. Y., Kim, R. H., Ko, M. K., Park, J. N., Lee, K. N., ... and Park, J. H. (2016). Altered adjuvant of foot-and-mouth disease vaccine improves immune response and protection from virus challenge. *Trials in Vaccinology*, **5**: 97-104.

Patil, P. K., Bayry, J., Ramakrishna, C., Hugar, B., Misra, L. D., and Natarajan, C. (2002): Immune responses of goats against foot-and-mouth disease quadrivalent vaccine: comparison of double oil emulsion and aluminium hydroxide gel vaccines in eliciting immunity. *Vaccine*, **20**(21-22): 2781-2789.

Patil, P. K., Bayry, J., Ramakrishna, C., Hugar, B., Misra, L. D., Prabhudas, K., and Natarajan, C. (2012): Immune responses of sheep to quadrivalent double emulsion foot-and-mouth disease vaccines: rate of development of immunity and variations

among other ruminants. *Journal of clinical microbiology*, **40**(11), 4367.

Rajput, Z. I., Hu, S. H., Xiao, C. W., and Arijo, A. G. (2007): Adjuvant effects of saponins on animal immune responses. *Journal of Zhejiang University Science B*, **8**(3): 153-161.

Reed, S. G., Orr, M. T., and Fox, C. B. (2013): Key roles of adjuvants in modern vaccines. *Nature medicine*, **19(**12): 1597-1608.

Rimaniol, A. C., Gras, G., Verdier, F., Capel, F., Grigoriev, V. B., Porcheray, F. and Dormont, D. (2004): Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type. *Vaccine*, **22**(23- 24):3127-3135.

Rodriguez, L. L., and Grubman, M. J. (2009): Foot and mouth disease virus vaccines. *Vaccine*, **27**: 90-96.

Saeed, A., Kanwal, S., Arshad, M., Ali, M., Shaikh, R. S., and Abubakar, M. (2015): Foot-and-mouth disease: overview of motives of disease spread and efficacy of available vaccines. *Journal of animal science and technology*, **57**(1): 1-7.

Selim, A., Abouzeid,N., Aggour,A.M., andSobhy,N.M. (2010): Comparative study for immune efficacy of two different adjuvants bivalent FMD vaccines in sheep. *J Am Sci*, **6**: 1292-8.

Shah, R. R., Brito, L. A., O'Hagan, D. T., and Amiji, M. M. (2015): Emulsions as vaccine adjuvants. In *Subunit Vaccine Delivery* (pp. 59-76). Springer, New York, NY.

Song, X., Bao, S., Wu, L., and Hu, S. (2009). Ginseng stem–leaf saponins (GSLS) and mineral oil act synergistically to enhance the immune responses to vaccination against foot-and-mouth disease in mice. *Vaccine*, **27**(1): 51-55.

Spickler, A. R., and Roth, J. A. (2003): Adjuvants in veterinary vaccines: modes of action and adverse effects. *Journal of Veterinary Internal Medicine*, **17**(3): 273-281.

Sulayeman, M., Dawo, F., Mammo, B., Gizaw, D., and Shegu, D. (2018): Isolation, molecular characterization and sero-prevalence study of foot-and-mouth disease virus circulating in central Ethiopia. *BMC veterinary research*, **14**(1): 110.

Sun, H. X., Xie, Y., and Ye, Y. P. (2009): Advances in saponin-based adjuvants. *Vaccine*, **27**(12): 1787-1796.

Tadesse, B., Alamir, K., and Demssie, E. (2017): Review on the Control of Foot and Mouth Disease by Vaccination.

Tadesse. B. (2018): Review On the Evaluation of Efficacy of Foot and Mouth Disease Vaccine. Rep Opinion, **10**(2):55-6.

Tesfaye, J. (2019): Review on the Epidemiology and Economic Impact of Foot and Mouth Disease in Ethiopia. *Agricultural Journal*, **14**(5): 79-93.

Tritto, E., Mosca, F., and De Gregorio, E. (2009): Mechanism of action of licensed vaccine adjuvants. *Vaccine*, **27**(25-26):3331-3334.

Vogel, F. R. (2000): Improving vaccine performance with adjuvants. *Clinical Infectious Diseases*, **30**.

Wael M. Gamaledin, Ehab Elsayed, Amr I. Hassanin, Assem A. Mohamed, Walaa Shabana, (2019): Performance of Aluminium Hydroxide Gel and ISA Oils Adjuvanted Foot and Mouth Disease Vaccines. *ARC Journal of Animal and Veterinary Sciences*, **5**(3):1-9

World Reference Laboratory from https://www.wrlfmd.org/foot-and-mouth-disease/occurrence

Xiao, C., Rajput, Z. I., Liu, D., and Hu, S. (2007): Enhancement of serological immune responses to foot-and-mouth disease vaccine by a supplement made of extract of cochinchina momordica seeds. *Clinical and Vaccine Immunology*, **14**(12): 1634-1639.

Yahya, M. Hailemariam, Z., Amare, L. B., and Rufael, T. (2013) 'Seroprevalence of foot and mouth disease in traditionally managed cattle in East and West Hararghe zones, Ethiopia. *Revue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux*, **66**(1).

Yirgalem, M., Dawo, F., Gizaw, D., Mamo, B., Bilata, T., and Shegu, D. (2020): Phylogenetic and Sequence Variability Analyses of Vp1 Protein Of Foot And Mouth Disease Viruses In Cattle In Amhara Region Of Ethiopia.

Zerabruk, G., Romha, G., and Rufael, T. (2014): Sero-epidemiological investigation of foot and mouth disease in cattle managed under extensive husbandry system in Tigray, northern Ethiopia. *Global Veterinary*, **13**: 112-116.

Zhou, B. (2019): Classical swine fever in China-an update Minireview. *Frontiers in*

veterinary science, **6**: 187.

8. LIST OF APPENDICES

Appendix 1: Preparation of BHK-21 cell culture and FMD vaccine procedure

a) Cell culture

- *Materials and equipment*
  - Biological freezer -20 ^{o}C, and -80 ^{o}C
  - Centrifuge
  - Inverted microscope
  - Tissue culture flasks (25cm^2, 75cm^2)
  - Laminar air flow
  - Incubator
  - Sterile pipette 5ml and 10ml
  - water bath
  - Sterile gauze
  - Falcon tube 50 ml
  - Pipette bulbs, pear shape 125 ml
  - Reagent bottle 500 ml
- *Reagents and Biologicals*
  - Minimum essential media (MEM) sterilized with 0.22µm pore size filter sheet
  - Sterile calf serum
  - Tryptose Phosphate Broth(TPB) autoclaved at 121^{0}c for 15minutes
  - Denature ethanol alcohol 70%
  - Antibiotics and antifungal
  - Phosphate Buffered Saline(PBS)
- *Procedure*
  - Take cryopreserved BHK-21 cell and place directly on to warm water bath at +37 ^{o}C
  - Wait the vial content until the suspension has thawed completely
  - Disinfect the cryovial with 70% ethanol alcohol and place in the biosafety cabinet

- Transfer the thawed cell into falcon tube of 50 ml and centrifuge at the speed of 1500 rpm for 10 minutes and discard the supernatant.
- Re-suspend the cell in fresh complete sterile MEM medium containing 10% calf serum, 10% fetal calf serum, 10% TPB and antibiotics inside the laminar air flow hood.
- Transfer to flask of 25 cm^2 and incubate at 37 ^{0}C
- Then follow the growth of the cell line and for any contamination using inverted microscope.
- Pre-warm both Trypsin /EDTA and phosphate saline solution to activate for Sub culturing working
- Take cell culture flask with confluent monolayer from the incubator
- Decant old media and wash 2-3 times with sterile PBS to remove the residual of culture medium (which would inactivate the trypsin).
- Add Trypsin /EDTA and observe the action of trypsin/ EDTA for three minutes
- Decant the trypsin/ EDTA, wait by placing the flask inverted until the cell detached from it inside the laminar flow hood
- Add complete growth medium and pipette vigorously with Pipette Bulbs (Trypsinization), mechanically until monolayer become dispersed to a single cell.
- Add the final amount of the complete media and dispense to other three 75cm^2 tissue culture flask and then incubate at 37^0 C in 5% Co_2 incubator.
- Follow the growth of the cell line for confluence and for any contamination using inverted light microscope

b) Production of FMD vaccine

- *Materials and equipment*
  - Laminar air flow
  - Incubator inverted microscope
  - Incubator
  - Petridish
  - Sterile pipette 10ml
  - Pipette bulbs

- Flask 75 cm^2
- Sterile reagent bottle
- Labeling marker
- Biological freezer -20 ^{o}C

➢ *Reagents and Biologicals*

- Virus suspension containing serotype A, O and SAT 2
- Maintenance media
- Antibiotic
- BHK-21 cell line
- PBS
- Denature alcohol 70% ethanol

➢ *Procedure*

- Place the cell, media and other materials that are used in inoculation into biosafety cabinet after disinfection with 70% ethanol.
- Discard the old media from the monolayer cell and wash the cell gently with pre warmed PBS.
- Inoculate 100ml of virus sample to the cell in each 75 cm^2 tissue culture flasks aseptically for the three serotypes and shake the flask gently to distribute inoculum uniformly over the monolayer cell.
- Incubate the flasks at 37^0 C for 17-24 hours
- Monitor the inoculated flasks daily for the development of CPE.
- Observe carefully CPE and harvest the culture supernatants of virus grown on BHK-21 cell monolayers after 24 hours post-infection and poured
- Collect the virus into sterile bottle aseptically and label the bottles with serotype of the virus.
- Concentrate the virus using 50% of ammonium sulfate and stirred at low speed overnight.
- Purify the virus by using high-speed centrifugation at 3000 rpm for 30 minutes to remove nonstructural proteins
- Dilute the concentrated virus by using PBS and inactivate with formaldehyde

Appendix 2: Tests for sterility and safety of vaccine

a) Sterility test

- *Material and equipment*
  - Test tube
  - Sterile pipette
  - Laminar air flow
  - Incubator
  - Petri dish
  - Water bath
- *Media and reagents*
  - Thioglycollate broth
    - ✓ Pancreatic digest of casein 15 gm
    - ✓ Yeast extract 5 gm
    - ✓ Dextrose 5.5 gm
    - ✓ L-Cysteine 0.5 gm
    - ✓ Sodium thioglycollate 0.5 gm
    - ✓ Resazurin 1 gm
    - ✓ Distilled water 1000ml
    - ✓ Mix all the ingredients in distilled water and heat to dissolve completely
    - ✓ Adjust PH 7.1 ± at 25 ^{o}C.
    - ✓ Dispense 10 ml/ test tubes and sterilize at 121 ^{o}C for 15 minutes
  - Tryptone Soya broth
    - ✓ Tryptic soya broth 30 gm
    - ✓ Distilled water 1000 ml
    - ✓ Dissolve all the ingredients in water and dispense in 10 ml/ tube
    - ✓ Sterilize at 121 ^{o}C for 15 and incubate at 37 ^{o}C
  - Sabouraud agar
    - ✓ Peptone 10 gm
    - ✓ D-glucose 20 gm

- ✓ Agar 22 gm
- ✓ Distilled water 1000ml
- ✓ Mix all the ingredients in distilled water and heat to dissolve completely
- ✓ Dispense in 20 ml/ test tube and sterilize by autoclaving at 121 ^{o}C for 15 minutes
- ✓ Distribute in Petri dish (l tube/ Petri dish) and incubate at 37 ^{o}C
- o Mycoplasma medium
- ✓ PPLO broth base 21 gm
- ✓ Horse serum 300ml
- ✓ Distilled water 700ml
- ✓ Dissolve PPLO broth in distilled water and autoclave at 121 ^{o}C for 15 minutes
- ✓ Cool at 45-50 ^{o}C in water bath and aseptically add horse serum
- ✓ Incubate the prepared media at 37 ^{o}C

➢ Procedure

o Clean the exterior of the vials and tubes with disinfectant

o Inoculate 1ml of vaccine into each prepared media/ tube in laminar air flow except control media

o Incubate the inoculated bacteriological media at 37 ^{o}C and the other media for fungi at room temperature for seven consecutive days to detect bacteria and fungi contamination respectively.

➢ Interpretation of the results

o Pass if no growth of microorganisms is observed in any of the broths/media.

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a) Safety test using BHK-21 cell culture

➢ Inoculate prepared vaccine on confluent BHK-21 cell culture to confirm complete inactivation of the virus

➢ Incubate at 37 ^{o}C in 5% Co_2 incubator for 48 hours

➢ Follow for the presence of CPE

➢ Interpretation of the result

o No development of CPE indicates complete inactivation of the virus.

Appendix 3: Virus titration

- *Materials and equipment*
  - Laminar air flow
  - Incubator
  - Water bath
  - Tissue culture plate of 96 wells
  - Multichannel micropipette
  - Micropipette tips
  - Sterile graduated pipette
  - Pipette bulbs
  - Plasma bottle
- *Reagents and Biologicals*
  - Growth medium
  - Trypsin and EDTA
  - Denature ethyl alcohol
  - PBS
- *Procedure*
  - Check the cells growth under microscope
  - Warm the media and trypsin in water bath at 37^{o}C
  - Put the confluent cell and other materials used for virus titration in laminar air flow after disinfection.
  - Remove old media from the cell culture
  - Wash the cell 2-3 times with PBS and add trypsin and EDTA to dissociate attached cell and observe for 3 minutes until the cell detached from the flask
  - Re-suspend the cell in complete growth medium and mix thoroughly by pipetting
  - Fill 100µl of the cell suspension using multichannel pipette into 88 wells of 96-wells tissue culture plates for the three serotypes, leave column number 11 of the plate empty and 12 as negative control
  - Incubate at 37 ^{o}C for 48 hours.
  - Then, after 48 hours of incubation observe complete growth of the cell on the plates

- Inoculate tenfold serially diluted FMDV serotypes aseptically
- Incubate at 37 ^{o}C in 5% Co2 incubator and follow for development of CPE
- Calculate the titer of the virus using Spearman karber method as follow:

Log titer m= antilog x + d(Er/n-0.5)

x= The highest dilution giving 100% positivity

d= The logarithm difference between dilutions

Er= Total number of positive wells

n= Number of wells used for each dilutions

Appendix 4:Evaluation of antibody response by Solid phase competitive ELISA (SPCE)

- *Materials and equipment*
  - ELISA microplate pre-coated with FMDV serotype specific MAb
  - Multichannel micropipette
  - Monochannel micropipette
  - Microplate ELISA reader
  - Desktop computer
  - Clean towel
  - Plastic sealer
- *Reagents*
  - FMDV positive control
  - FMDV negative control
  - Diluent Buffer
  - Horse Reddish Peroxidase (HRO) conjugate
  - PBS Tween20 10x concentrated
  - Distilled water
  - Substrate (chromogen solution)
  - Stop solution (sulfuric acid)

a) Preparation of conjugate, substrate and stop reagents

- Determine the amount of each solution needed for the to be done according instruction of test kit
- For conjugate calculate 2.5 ml per each microplate and add 0.5 ml to the total
- For substrate and stop solution calculate 5 ml per each microplate and add 1 ml to the total

b) Preparation of washing solution

- Dilute the PBS Tween20 10x concentrated 1/10 in distilled water

c) Solid Phase Competitive ELISA (SPCE)

- Open the sealed ELISA microplate start with control sera distribution
- Dispense 50µl negative control in four wells in position C1, D1, E1, and F1
- Dispense 67.5µl diluent buffer in wells A1 and B1
- Add 7.5µl of the positive control serum in well A1 and mix with single channel micropipette
- Transfer 25µl positive control from A1 to B1 to obtain 1/30, mix using single channel micropipette and discard 25µl without changing the tip from well B1
- Add 45µl/well ELISA diluent buffer and 5µl/well sample sera (1/10 dilution)
- Mix by using multichannel micropipette
- Cover and incubate the plate for 1 hour at room temperature

- **Conjugate**
  - After incubation without washing add 25µl/well the diluted HRPO-conjugate
  - Again cover and incubate the plate for 60 minutes at room temperature
- **Washing**
  - Empty the wells and tap hard to discard all remaining residual fluid
  - Fill the wells with 200µl/well of washing solution (PBS Tween20) and incubate for 3 minutes at room temperature
  - Then, empty the wells and repeat the washing 3 times leaving the last washing for 5 minutes at room temperature, Total of 4 washing cycle

- Discard residual fluid from the plate by tapping on clean absorbent towel

➢ **Substrate/ Chromogen solution**

- Dispense 50µl/well of the substrate or chromogen solution to all wells
- Cover and keep the plate at room temperature for 20 minutes in the dark place (start timing when first well is filled)

➢ **Stopping the reaction/Blocking**

- Stop the reaction by adding 50µl/well of the stop solution and gently shake the wells prior to reading

➢ **Reading**

- After stopping the reaction immediately read the Optical Density (OD) of each wells at 450 nm wavelength

➢ **Calculation of results**

- The percentage of inhibition produced by the positive control and by test sera is calculated as follows:
- % inhibition= 100-(serum OD/reference OD) *100

Reference OD = Mean OD of four wells processed with the negative control

➢ **Criteria for test validity**

- Spectrophotometric readings must be as follows**:**

FMDV serotypes	OD value in Negative control	% inhibition of positive control at 1/10 dilution	% inhibition of positive control at 1/30 dilution
A	≥0.8	≥90	>50
O	≥1	≥90	>50
SAT 2	≥1	≥80	>50

➢ **Interpretation of antibody response**

- Positive when producing an inhibition ≥ 70% at the 1/10 dilution
- Negative when producing an inhibition <70% at the 1/10 dilution

Appendix 5: Ethical clearance certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
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Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/27/06/13/2021

Name of Applicant: **Getu Ayele (BSc in Vet. Lab. Tech, MSc fellow)**

Address: Department of Microbiology, Immunology & Vet. Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Evaluation of different adjuvant formulations of inactivated trivalent FMD vaccine in calves*

Date of application: **December, 2020**

Nature of the project: **Invasive**
Target animal species: **cattle/calves**
Number of animals involved: **29**
Study area: **National Vet. Institute, Ethiopia**

Minutes No. and date of review: **VM/ERC/06/13/021, 28/03/2021**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary

Getachew Terefe (DVM, PhD, Professor of Vet. Parasitology)
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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Please quote Our Ref. No. When replying

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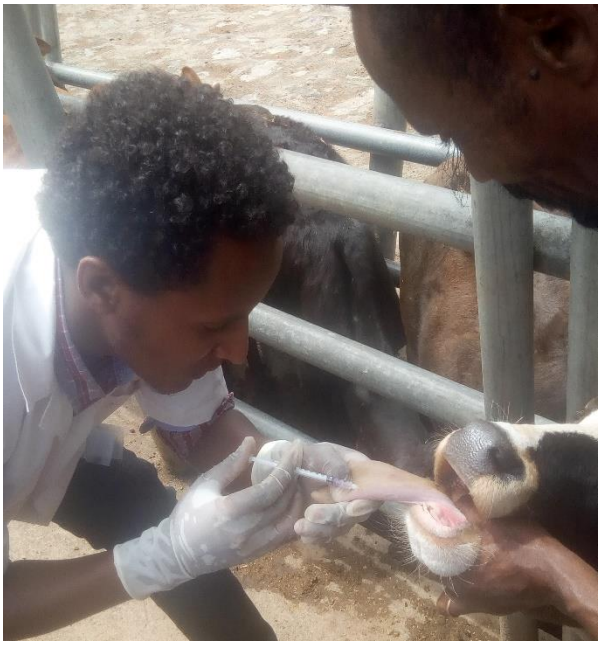
Fax 251-11-4339933 Tel. +251 114338450 P.o.x. Box}34 Bishoftu, Ethiopia

Appendix 6: Experimental cattle used for the study and miscellaneous pictures during the study

Cattle used for the study

Blood collection from jugular vein of cattle


Inactivated trivalent FMD vaccines used for immunization of the cattle, formulated with oil, AS and combinatination of AS and oil.

Challenge test done on 28 days post vaccination by intradermal inoculation in the upper surface tongue of the cattle

Titration of the virus using BHK-21 cell culture