Comparison of Immunogenicity and Safety of Selected Adjuvant Formulations for Contagious Caprine Pleuropneumonia Vaccine Improvement

Mulatu Mokonon

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Biotechnology

Office of Graduate Studies

Adama Science and Technology University

November, 2023

Adama, Ethiopia

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DECLARATION

I hereby declare that this Master Thesis entitled "**Comparison of Immunogenicity and Safety of Selected Adjuvant Formulations for Contagious Caprine Pleuropneumonia Vaccine Improvement**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citations.

Mulatu Mokonon	___________________	_______________
Name of student	Signature	Date

RECOMMENDATION OF THE ADVISORS

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled "**Comparison of Immunogenicity and Safety of Selected Adjuvant Formulations for Contagious Caprine Pleuropneumonia Vaccine Improvement**" prepared under our guidance by **Mulatu Mokonon** submitted in partial fulfillment of the requirements for the degree of Mater's of Science in Biotechnology. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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APPROVAL SHEET

We, the advisors of the thesis entitled "**Comparison of Immunogenicity and Safety of Selected Adjuvant Formulations for Contagious Caprine Pleuropneumonia Vaccine Improvement**" and developed by **Mulatu Mokonon**, hereby certify that the recommendations and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	One-way analysis of variance
APCs	Antigen-presenting cells
CCPP	Contagious caprine pleuropneumonia
DCs	Dendritic Cells
DDA	Dimethyldioctacecyl Ammonium bromide
ELISA	Enzyme-linked immunosorbent assay
FMD	Foot and mouth disease
ICE	Immuno-capture enzyme-linked immunosorbent
IFA	Incomplete Freund's adjuvant
ISA	Incomplete seppic adjuvant
KEVVAPI	Kenya Veterinary Vaccines Production Institute
Mccp	*Mycoplasma capricolum* subspecies *capripneumoniae*
MHC	Major Histocompatibility complex
NLRs	NOD Like Receptors
NOD	Nucleotide binding Oligomerization Domain
NVI	National Veterinary Institute
OIE	Office International des Épizooties (World Organization for Animal Health)
PBS	Phosphate buffered saline
PRRs	Pattern-recognition receptors
RCBD	Randomized complete block design
RIG-I	Retinoic acid-inducible Gene-I
RLRs	RIG-I like receptors
TLRs	Toll-Like-Receptors

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ABSTRACT

Contagious caprine pleuropneumonia (CCPP) is a transboundary disease of goat which is caused by the *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp). Vaccination is the most cost-effective technique in the control of CCPP than any other control measures. Selection of the effective adjuvants is crucial in ensuring the quality of vaccine and its protective efficacy. Saponin is the most commonly used adjuvant, with its shortcomings in its potential for dose related toxicity, low chemical stability, low extraction yield and irritation to laboratory workers. However, the possibility of using oil emulsion adjuvants as an alternative to saponin has not been considered, even though, oil in water adjuvant is relatively safer and cheaper. Therefore, the objective of the present study is to evaluate immunogenicity and safety of selected adjuvant formulations for CCPP vaccine improvement. For this purpose, different adjuvants (15% Emulsigen, 15% Emulsigen-D, 15%Emulsigen-P, 10% Carbigen, 3% saponin and 0.3% saponin) were evaluated on sixty-three healthy and Mccp antibody free goats by classifying in to seven study groups (six experimental groups and one control group), each group consisting of nine goats (n=9). One ml of each vaccine formulation with a different adjuvant was applied to experimental groups and sera samples were collected on days 0, 7, 14, 21, 28, 42, 49 and 56 to assess their immune response using Competitive Enzyme Linked Immune Sorbent Assay (c-ELISA). In addition, adverse effects following the administration of the vaccines were recorded. Thus, the experimental study revealed that 0.3% saponin achieved considerably highest immune response (mean percentage of inhibition [PI] value=74.45), followed by 3% saponin (66.92), Emulsigen (66.90) and Carbigen (66.45). The result did not show statistically (p>0.05, ANOVA) significant difference among them in their efficacy. Emulsigen-P and Emulsigen-D elicited sufficient immune response (mean PI=59.57 and 56.77) in comparison to non-vaccinated groups. On a different note, 3% saponin had posed irritation and high fever in some animals. Carbigen did not develop any reaction including a localized swelling seen in another groups. These findings confirmed that the oil adjuvants, particularly, Carbigen and Emulsigen are valuable to provide a sufficient immune response and better safety. Consequently, it could be concluded that these oil adjuvants can be used as an alternative to saponin in CCPP vaccine.

Keywords: *Adjuvant, CCPP, c-ELISA, goat, Mccp, PI value, vaccine*

1. INTRODUCTION

1.1. Background of the study

Contagious caprine pleuropneumonia (CCPP) is a transboundary animal disease of goat which is caused by the causative agent *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp) (Nicholas *et al.*, 2008). CCPP is a respiratory infectious disease reported first in Algeria in 1873 by Rurangirwa et al. (1997). The etiological agent, Mccp, was originally known as Mycoplasma F38, which was first isolated in Kenya from the lungs of goats with pleuropneumonia and shown to cause CCPP in 1976 (Falquet *et al.*, 2014; Nicholas *et al.*, 2008; MacOwan and Minette, 1976). Subsequently, Mccp has been isolated in other countries, such as Chad, Ethiopia, India, Oman, Sudan, Tunisia, Turkey, Uganda and Mauritius (Iqbal Yatoo *et al.*, 2019; OIE, 2018; Nicholas and Churchward, 2012; Srivastava *et al.*, 2010; Ozdemir *et al.*, 2005). It causes significant economic loss to goats.

CCPP vaccines are inactivated and adjuvanted (OIE, 2014). The antigen is composed of whole Mccp cells that are concentrated and semi-purified; with the minimum concentration of 0.15 mg of Mccp protein per dose. Due to the fastidious nature of Mccp, the production of CCPP vaccines is costly as it requires very rich media, the yield is limited, the procedure involves a purification processes, and inactivated vaccines also require larger amounts of antigen compared with live vaccines (Takele *et al.*, 2017).

The potent and safe adjuvants are required for the development of efficacious and safe inactivated vaccines due to the fact that the antigens in these types of vaccines are usually less immunogenic compared to live microbes *(*Burakova *et al.*, 2018; Kusiluka *et al.*, 2001). Adjuvants can be broadly separated into two classes based on their mechanisms of action: vaccine delivery systems and immune-stimulatory adjuvants. Vaccine-delivery systems generally are particulate (e.g., emulsions, micro-particles, iscoms, and liposomes) and function mainly to target associated antigens into antigen-presenting cells. In contrast, immune-stimulatory adjuvants are derived predominantly from pathogens and often represent pathogen-associated molecular patterns (e.g., lipopolysaccharide, monophosphoryl lipid A, CpG DNA), which activate cells of the innate immune system (Singh & O’Hagan, 2002).

In the beginning stages of CCPP immunity studies, there was always a risk of infection associated with the use of live Mccp pathogens (Mekuria *et al.*, 2008). As a result, inactivated or attenuated preparations of Mccp organisms were developed. Peyraud et al. (2014) used

sonicated antigens of the F38 strain of Mycoplasma with incomplete Freund's adjuvant, emulsified in aluminum hydroxide and found that goats immunized with this antigenic preparation developed a strong immunity. Rurangirwa et al. (1997) found that 0.15 mg of F38 Mycoplasma in saponin was the optimal formulation for inactivated Mycoplasma vaccine, providing immunity for more than a year (Rurangirwa *et al.*, 1997).

1.2. Statement of the problem

Saponin, which is an immune-stimulatory adjuvant, was widely used as adjuvant of choice for CCPP vaccine at 3 mg per dose (OIE, 2014; Diaz, 2014). However, while saponin adjuvants show great promise in vaccine development due to their ability to produce a desired immune response, continuously obtaining these compounds on a commercial scale from natural sources is challenging and the technology needed to synthetically manufacture them in a consistent manner is lacking (Shi *et al.*, 2019). It has also been noted that the stability of these adjuvants is low, with dosage-related toxicity leading to adverse effects (e.g., hemolysis) (Burakova *et al.*, 2018). QS-21 saponin is an effective immune-stimulant, but its limitations include the potential for dose-related toxicity, low chemical stability, low extraction yields (from natural sources), and an incomplete understanding for its mechanism of action (Wang, 2021).

Even though, few researches (Arguedas *et al.*, 2022; Shah *et al.*, 2017; Park *et al.*, 2016) have shown the use of emulsion oil adjuvant provides better safety and sufficient immune response against target infections, little exploration has been made towards using some oil emulsion adjuvants for CCPP vaccine as an alternative to solve the problems associated with saponin adjuvants. Moreover, since saponin is not ready to mix, it poses irritation to laboratory workers during preparation. Thus, these limitations forces CCPP vaccine manufacturers to look for better and/or alternative and safe adjuvants.

Emulsion types of adjuvants are a very attractive adjuvant for livestock vaccine preparations. These adjuvants can be formulated using inexpensive, readily available components such as mineral oil and food-grade emulsifiers (Apostólico *et al.*, 2016). Oil-based preparations such as, Montanide ISA 201 and 206, produce multiple emulsions up on gentle mixing with antigenic aqueous phase. These adjuvants demonstrate effectiveness and provide protection for different livestock species against several economically important pathogens, including

influenza and FMD viruses (Shah *et al*., 2017). Therefore, the present experimental study was aimed at evaluating immunogenicity and safety of different adjuvants which improves CCPP vaccine.

1.3. Objectives

1.3.1. General objective

To evaluate the immunogenicity and safety of selected adjuvant formulations for CCPP vaccine improvement.

1.3.2. Specific objectives

- To evaluate the immunogenicity of Emulsigen, Emulsigen-D, Emulsigen-P, Saponin and Carbigen adjuvant formulations for CCPP vaccine.
- To evaluate the safety of Emulsigen, Emulsigen-D, Emulsigen-P, saponin and Carbigen adjuvants for CCPP vaccine.

1.4. Significance of the study

The immunity and protective capability produced by vaccines can vary remarkably according to the kinds of adjuvant being used. Few researches has shown the use of emulsion oil adjuvant provides better safety and sufficient immune response against target infections (Arguedas *et al*., 2022; Park *et al*., 2016). However, no effort has been made towards using some oil emulsion adjuvants for CCPP vaccine as an alternative to solve problems associated with the utilisation of saponin. The output of research in these area helps for selection of better adjuvants, which in turn leads to vaccine improvement. It adds scientific information on the use of Emulsigen, Emulsigen-D, Emulsigen-P, saponin and Carbigen adjuvants for CCPP vaccine. Furthermore, it will provide evidence for other researchers for vaccines formulated from Mccp related pathogens for their adjuvant selection. Consequently, this will bring advancements in the area of vaccinology and related fields. In addition, pharmaceuticals or vaccine companies, customers and the country will be benefited.

1.5. Research hypothesis

The research hypothesis was stated as:

H0: Emulsigen, Emulsigen-D, Emulsigen-P, and Carbigen adjuvants can be alternative formulations to saponin for CCPP vaccine.

HA: not H0.

1.6. Limitation of the study

The present experimental study was limited to evaluate only antibody response (humoral immunity) because of lack of materials and financial support to measure cell mediated immune response. Additionally, measuring the duration of immune response developed by each treatment groups is limited due to lack of facilities and time given for this study.

2. LITERATURE REVIEW

2.1. Mycoplasma

Mycoplasma belongs to a class of bacteria named Mollicutes which characterized by its small genome size (0.58–1.35 Mb) (Razin *et al.*, 1998) and perpetually devoid of the cell wall (Abdel Halium *et al.*, 2019). They have limited biosynthetic capability and cause a number of infections in animals (Soayfane *et al.*, 2018). Different species are pathogenic to mammalian, avian species, sheep and goats, so that, economically critical in many countries. Mycoplasma species commonly associated with pneumonia in small ruminants are *Mycoplasma ovipneumoniae*, *Mycoplasma arginini*, *Mycoplasma capri*, *Mycoplasma capripneumoniae*, and *Mycoplasma capricolum* (Maniloff and Morowitz, 1972).

Mycoplasma is highly fastidious microorganism required very precise media to develop in vitro, the low ability of Mycoplasma to form macromolecules needed for their growth refers to their evolutionary development from other bacteria; it is highly suspected that many Mycoplasmas exist in nature, however, have not been isolated due to their hard growth in vitro on artificial media (Macowan & Minette, 1976; Manso-Silván & Thiaucourt, 2019). Lately, the high development of polymerase chain reaction (PCR) technique makes the detection of different species of Mycoplasma using specific primers much simpler, till now, PCR remains the most valuable and rapid method for detecting specific species of Mycoplasma (Namazi *et al.*, 2020)

These smallest microbes are resistant to common antibiotics affecting the cell wall, and the trend of frequent and prolonged use of antibiotics may lead to resistance against other antibiotic classes (Li *et al.*, 2020). Hence, the development of vaccines against mycoplasma is imperative.

2.1.1. Mycoplasma Capricolum Subspecies Capripneumonia

The four lineages of mycoplasma correspond to different geographic regions (Cottew et al. 1987). Mccp is placed in *Mycoplasma mycoides* cluster and has different species and subspecies, namely *Mycoplasma mycoides* subsp. *mycoides* large colony strains (MmmLC), *Mycoplasma mycoides* subsp. *mycoides* small colony strains (MmmSC), *Mycoplasma* species

bovine group 7 of Leach (Mbg7), *Mycoplasma capricolum* subsp. *capricolum* (Mcc), and *Mycoplasma mycoides* subsp. *capri* (Mmc) (Cottew et al. 1987; Manso-Silvan et al. 2009). Of these members, some cause similar diseases in sheep and goats but have extrapulmonary involvements also (Iqbal Yatoo *et al*., 2019).

The phenotypic and genetic traits shared in this group have their basis in conventional biochemical and immunological tests such as colony size and growth characteristics, substrate utilization, isozyme patterns, protein profiles and DNA hybridization studies (Dereje and Teshale, 2021). A striking feature of CCPP is the host and tissue specificity of the causative agent, as lesions are produced only in goat lungs (Benedetti *et al*., 2020). Although *M. capricolum* subsp. *capripneumonia* is present in high quantities in affected lungs, there is no dissemination to other organs. This may be due to a specific reaction of the lung tissue towards a mycoplasma component that leads to an exacerbated inflammatory response (Thiaucourt and Bölske, 1996).

2.1.2. Biochemical tests

Mycoplasma, due to their small genome size, has limited biosynthetic capacity, which means they lack many biochemical pathways found in Eubacteria. They are highly adapted to their host, which provides most of its nutritional requirements for growth (Abdel Halium *et al*., 2019). For this reason, there are only a few biochemical properties that can be investigated in the diagnostic laboratory. Consequently, identification of mycoplasmas is greatly reliant on serological tests based on the recognition of structural membrane proteins by specific antiserum and molecular techniques (Bölske et al., 1996).

Biochemical tests, however, are used for the initial grouping of mycoplasma. The tests most commonly used are glucose breakdown (Mccp: positive), arginine hydrolysis (Mccp: negative), 'film and spots' formation (Mccp: negative), reduction of tetrazolium chloride aerobically and anaerobically (Mccp: +/++), phosphatase activity (Mccp: negative), serum digestion (Mccp: negative) and digitonin sensitivity (Mccp: positive) (OIE, 2018). Sensitivity to digitonin differentiates cholesterol-requiring (Mycoplasmataceae and Spiroplasmataceae) from non-cholesterol-requiring (Acholeplasmataceae) Mollicutes. The genus Mycoplasma can be differentiated from Ureaplasma by its lack of urease. Within the mycoplasmas of small ruminants, biochemical tests cannot unequivocally identify an isolate (Nicolas, 2002).

2.2. Contagious caprine pleuropneumonia

Contagious caprine pleuropneumonia (CCPP) is one of the most severe diseases of goats affecting the respiratory tract (Rather *et al*., 2021; Manso-Silván & Thiaucourt, 2019; Peyraud *et al*., 2014; Iqbal *et al*., 2007). It belongs to the Mycoplasma mycoides cluster, a group of five closely related Mycoplasmas, pathogenic to ruminants and characterized by clinical signs such as fever, coughing, anorexia, dyspnoea, polypnea, nasal discharges, severe respiratory distress and high mortality (Sasikala *et al*., 2020). This disease is extremely contagious and frequently fatal; in some naive flocks where the morbidity and mortality rates can reach 100% (Kabir, 2022). CCPP causes major economic losses where it is endemic, such as Africa, Asia, and the Middle East (Manso-Silván & Thiaucourt, 2019).

When infected, animals become severely sick, they mostly die within 7–10 days. Morbidity and mortality can reach 100% and 70%, respectively (Nicholas *et al.*, 2008). The incubation period commonly ranges from 6 to 10 days. It has also been reported to range from two days to four weeks (Manso-Silvan and Thiaucourt, 2019). It is transmitted during close contact by the inhalation of respiratory droplets. There is no evidence for indirect transmission as the Mycoplasma is highly fragile in the environment (Sasikala *et al*., 2020).

2.2.1. Distribution of Contagious Caprine Pleuropneumonia

CCPP is prevalent in many countries in Africa, Asia, and the Middle East (Matios *et al*., 2014). Since Mccp is difficult to isolate from clinical materials, its presence has not been confirmed in all affected countries. In some cases, reports of its occurrence are based on clinical signs alone. After its first isolation in Kenya (MacOwan and Minette, 1976), CCPP has also emerged in newer areas, like Afghanistan, Mauritius, Tajikistan, Pakistan, India, China, Saudi Arabia, and Qatar (Hussain *et al*., 2021; Nicholas and Churchward, 2012; Srivastava *et al*., 2010; McMartin, *et al*., 1980;). It has showed an increased incidence of outbreaks in prevalent areas like Ethiopia, Kenya, Tanzania and Turkey (OIE, 2018; Takele *et al*., 2017; Ozdemir *et al*., 2005).

2.2.2. Mechanism of pathogenesis

Mycoplasmas are extracellular pathogens of mucous membranes, and it is believed that they may attach to epithelial cells (Nicolet, 1996). Adhesion of pathogen to host cells favours colonization for setting up of infection. Metabolic activity of mycoplasma releases free radicals like hydrogen superoxide and super oxide radicals, which can damage cilia or the membranes of cells (Iqbal Yatoo *et al.*, 2019). Acute disease is characterised by unilateral pneumonia and serofibrinous pleuritis with straw coloured fluid in the thorax. On cut surface, the lung is granular with copious straw-coloured 3 exudates. Varying degrees of lung consolidation or necrosis can be seen, followed by alveolar exudation and pleural fluid accumulation and pleural adhesion. The regional (bronchial) lymph nodes are also enlarged (Sadique *et al.*, 2012).

CCPP is a respiratory disease that is extremely infectious and dangerous. It is characterized by nasal discharge cough, extreme respiratory distress, difficult to walk, elevated morbidity (80-100%) and eventually spreads the disease to the entire flock. Mortality is also very high in the absence of care and can exceed 60-80% (Kabir, 2022). The pathogenesis of CCPP follows the mechanism demonstrated below (Figure 1).

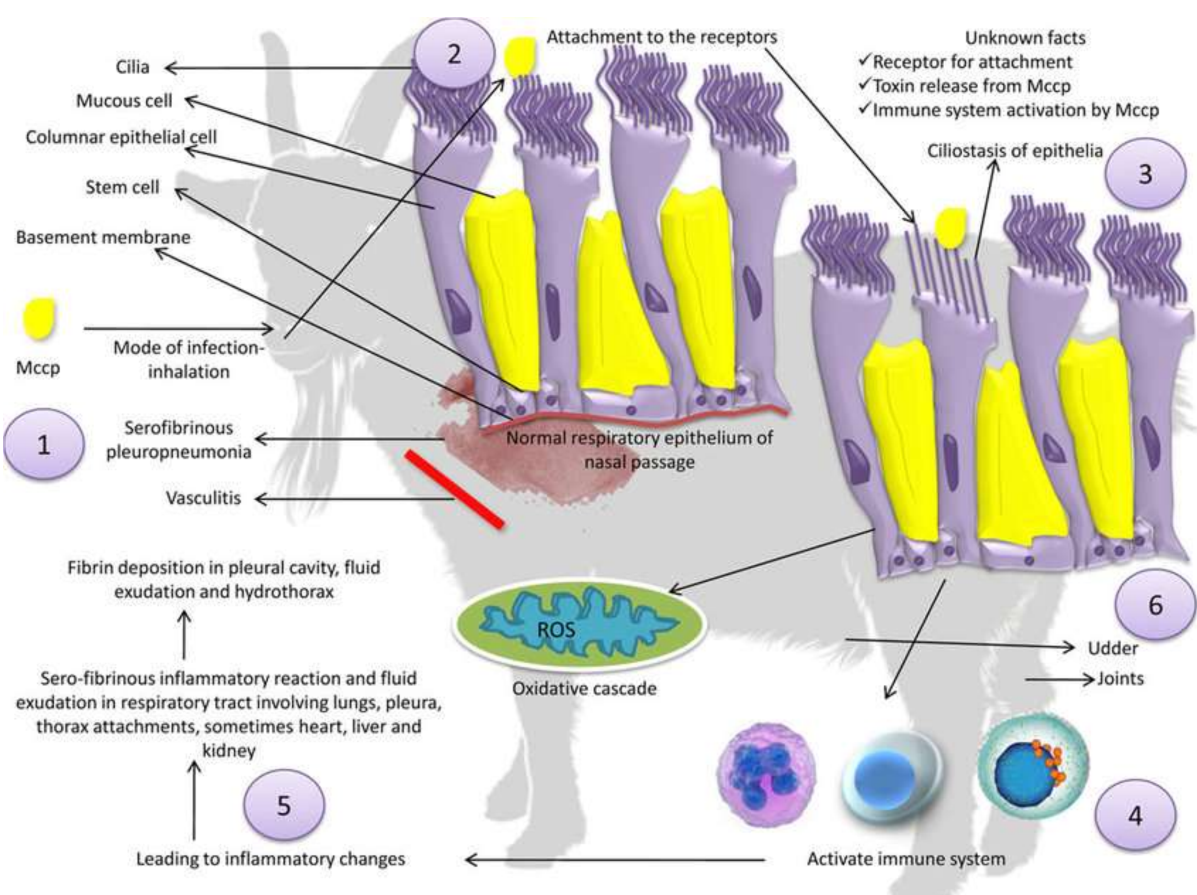

Figure 1: Mechanism of pathogenesis of Mccp:- 1: Pathogenesis of Mccp. 1) Inhalation is the commonest route of infection through aerosol transmission, (2) colonization, (3) ciliostasis of epithelia, serofibrinous pleuropneumonia, vasculitis, and fibrinocellular exudation, (4) Mycoplasmal antigens (polysaccharides, galactan, lipoprotein) activate immune system, (5) stimulation of inflammatory and oxidative cascade, (6) Mycoplasma capricolum subsp. capripneumoniae may also affect other organs like joints, eyes, and udder.

Source: (Iqbal Yatoo, 2019)

2.2.3. Diagnosis Methods

Diagnosis is one of the most important and challenging aspects of the disease as it influences prophylactic and therapeutic regimen and the control strategies for prevention of global spread (Abdollahi *et al*., 2023). Mycoplasma capripneumoniae is fastidious, grows slowly in the broth medium, and on solid media only produces minute colonies. In addition, other common mycoplasmas are sometimes overgrown (Abdel Halium *et al*., 2019). Definite diagnosis is made by culture of the causative agent from lung samples or pleuritic fluid taken at postmortem. Liquid and solid mycoplasma media are inoculated and filtered subcultures

from liquid medium may be required if there is evidence of bacterial contamination. Isolates may be identified by one the following diagnosis methods (OIE, 2018).

Diagnosis might involve microbiological, biochemical, serological and molecular techniques following a clinical tentative diagnosis. Microbiological methods include culture, isolation, and identification, which are rather conventional ones but are still considered as standard methods of detection of Mccp. However, the microbiological diagnosis of CCPP is considered difficult for two main reasons, the first being very poor in vitro growth of Mccp, and secondly, usual contamination of samples by other easily growing mycoplasmas (Thiaucourt *et al*. 1996). In addition, fastidiousness and special requirements of Mccp add to the problem of diagnostics. Hence, other diagnostic methods should be relied on (Solangi *et al*., 2023).

Several tests may be used for serological diagnosis such as complement fixation test (CFT), Latex agglutination test and competitive- enzyme-linked immunosorbent assay (c-ELISA). Among serological tests, c-ELISA tests with unique monoclonal antibodies, such that it has been a more specific test. Latex agglutination test also has been stated to be a very convenient field test for the detection of antibodies in whole blood or serum (Thiaucourt *et al.*, 1996).

To significantly improve Mccp detection, molecular based tests such as Polymerase Chain Reactions (PCR) has been used (Woubit *et al*., 2004). Specific polymerase chain reaction assays, can address the shortcomings in other diagnosis methods due to cross-reactions that occurred between the Mycoplasma mycoides family as all members of this cluster share similar biochemical and serological properties. Mccp reverse DNA sequence analysis has been successfully used to research Mccp strain genetic diversity (Kabir, 2022). Therefore, detection may be easier with specific molecular methods such as PCR and nucleic acid based tests.

2.2.4. Economic Importance of CCPP

CCPP is a serious and economically important respiratory disease which causes significant losses in goat population throughout Asia, Middle East, Europe and Africa (Xu e*t al*., 2017). In most goat production systems in Africa, goats are often important sources of income being

essential in agricultural economy by providing milk, meat and skin. Therefore, it negatively affects the livelihood of goat farmers (Nicholas *et al.*, 2009). It causes significant economic loss to goat farmers worldwide (Manso-Silván & Thiaucourt, 2019). CCPP endemic areas loose about 507 million USD every year due to the disease (Peyraud *et al*., 2014).

The CCPP poses a danger to the global goat population due to the disease's high contagiousness and propensity for fast transmission across national boundaries (Samiullah, 2013). As a result of its trans-boundary character, CCPP is classified as a List "B" illness by the Office International des Epizooties (OIE, 2017). The disease causes 100% morbidity and 80% mortality in both domestic and wild goat breeds (Ostrowski *et al*., 2011; Nicholas *et al*., 2008; Arif *et al*., 2007).

Goats are maintained largely by farmers from lower socioeconomic classes who rely on the revenue generated by their animals for their livelihood. Thus, CCPP causes severe loss to goat farmers and also sheep. These highly affect the economy and livelihood of the communities in turn. Therefore, the disease requires prevention measures of which vaccination is the most important one.

2.3. Prevalence and impact of CCPP in Ethiopia

In Ethiopia, the presence of CCPP was confirmed in 1990 after the isolation of Mccp from outbreaks (Thiaucourt *et al*., 1992). Although an exact epidemiological picture of the disease has not been established, outbreaks of CCPP have been recorded in different regions of the country, such as Tigray, Afar, Dire Dawa, SNNP, Oromia, Benishangul-Gumz and Amhara regional states (Nesru, 2003). Following its confirmation, several studies were undertaken to assess the status of and identify the disease in different parts of the country. These studies showed that the disease has a widespread occurrence in goats reared in sedentary, agro-pastoral and pastoral areas of the country (Ayelet *et al*., 2007). In the year 2011, the country reported 12 outbreaks with 1236 cases and 486 deaths to African Union's Inter-African Bureau of Animal Resources (Gelagay *et al*., 2007). Ninety-six (96) outbreaks were reported over four years (2007–2011) to the Ministry of Agriculture from different parts of the country (Sharew *et al*., 2005). Similarly, six outbreaks with 289 cases and 93 deaths, all affecting goats, were reported to the OIE in 2014 (OIE, 2014).

Outbreak reports and other studies that have so far been carried out to identify the etiologic agent and estimate its prevalence, though fragmented, have underscored the importance of the diseases in different parts of the country throughout the years. However, summarized information that shows the overall epidemiological status of CCPP, including the overall prevalence and major factors contributing to its widespread occurrence in the country are lacking (Asmare *et al*., 2016).

The prevalence is high, especially in areas in the immediate vicinity of endemic regions of Kenya and Sudan such as Afar, Borana and other lowlands. By 2007, Gelagay et al. reported that CCPP was becoming a very important goat disease in Borana pastoral areas (Gelagay *et al*., 2007). Asmare et al. (2016) conducted research on the status and distribution of contagious caprine pleuropneumonia (CCPP) in Ethiopia to provide a pooled prevalence estimate of CCPP in the country. Accordingly, the pooled prevalence estimate of CCPP was 25.7% (95% CI). The impact of the CCPP is a serious issue in almost all parts of the country, especially in the pastoral areas, so that, CCPP vaccine is necessary as a major control measure.

2.4. CCPP Vaccine

Strain F-38 of Mccp is used to develop attenuated/passaged broth culture vaccines. Its sonicated antigens inactivated or attenuated with incomplete Freund's adjuvant (IFA), saponin, aluminum hydroxide gel (emulsifier) or phosphate-buffered saline (PBS) provided solid immunity to challenges (Takele *et al*., 2017). The formalin-inactivated vaccine of the F-38 strain is administered as 1 ml per goat. The optimum age for vaccination is beyond 10 weeks (Münch & Wochenschr, 2015).

The specific integral membrane surface protein (p24) of the strains G22, G94/83, G108/83, and G280/80, which is identified with the help of mAb E8-18, is used as a vaccine candidate (Suschak *et al*., 2017). The Mccp strain 19/2 is used to produce subunit, capsular polysaccharide (CPS), and immune-dominant core proteins vaccine (Yatoo *et al*., 2019). Furthermore, a Kenyan isolate of Mccp was used to produce live vaccine, and showed absence of any post-vaccination reaction. It also resulted in the early appearance and longer persistence of antibodies. However, the chance of disease outbreaks was suggested (Solomon Tarekegn, 2012).

2.5. CCPP Vaccine status in Ethiopia

Ethiopia is among the countries with contagious caprine pleuropneumonia is prevalent. CCPP as it was originally known as Mycoplasma F38, was first isolated in Kenya from the lungs of goats with pleuropneumonia (Izhar *et al.*, 2022). Subsequently, Mccp has been isolated in other countries, such as Chad, Ethiopia, India, Oman, Sudan, Tunisia, Turkey, Uganda and Mauritius (Iqbal Yatoo *et al.*, 2019; OIE, 2018; Nicholas and Churchward, 2012; Srivastava, 2010; Ozdemir, 2005).

Ayelet et al. (2007) conducted a study to validate the immunity induced by inactivated F-38 antigen adjuvanted with saponin and Montanide incomplete seppic adjuvant (ISA) 50, and combined with and without anthrax vaccine. They reported that increased body temperature and local edematous reactions were seen in all the animals inoculated with saponin-adjuvanted CCPP vaccine while only 20% of the goats in the ISA 50-adjuvanted group showed local reaction. Based on the sero-conversion assessment, they reported as saponin-adjuvanted groups, in both monovalent CCPP and in the combined CCPP with anthrax (*Bacillus anthracis*) vaccine, showed a higher mean percentage of inhibition value as compared with the ISA 50-adjuvanted vaccine. Among the vaccinated groups, CCPP + anthrax + saponin were reported to show better protection. The study disclosed that the inactivated CCPP vaccine adjuvanted with saponin and ISA 50 significantly reduce morbidity and mortality of goats due to CCPP. It also indicated the importance of utilization of ISA 50 as an alternative adjuvant to minimize post-vaccinal reactions encountered in the use of saponin (Ayelet *et al.*, 2007).

In its terrestrial manual, OIE (2014) states that the CCPP vaccine antigen is composed of whole Mccp cells that are concentrated and semi-purified. Washed concentrated Mccp antigen can then be diluted to adjust the protein content. However, Takele *et al.*, (2017) evaluated the safety and immunogenicity of inactivated Kenyan isolate whole culture CCPP vaccine, and concluded that the vaccine is equally safe and immunogenic as the non-whole culture (concentrated) CCPP vaccine; rather, it is easier to produce, requires less time, and it is not capital investment-intensive. Accordingly, the authors revealed that the inactivated whole culture CCPP vaccine can be used for mass vaccination, since which, after conducting field trials and a series of validation processes, it is being produced and marketed by NVI.

The vaccine improves the health of animals. However, not sufficient enough to eradicate the disease and not fully covered all the affected areas. The immunity developed by the vaccine

lasts for around 2 years (Yatoo *et al*., 2019). As a result, a more efficacious and safer vaccine at a sufficient production scale will improve the status.

2.6. General overview of adjuvants

Adjuvants were discovered in the 1920s by Gaston Ramon, a French veterinarian. Ramon noticed that the addition of certain substances, particularly aluminum salts, to vaccines increased their efficacy (Garçon *et al*., 2011). After that, adjuvants have become an increasingly valuable component in the field of Vaccinology. The word “adjuvant” is derived from the Latin word “adjuvare” meaning “to help.” Therefore, adjuvants serve to promote and enhance immune responses to vaccine components, thereby lessening the required dose of vaccine and prolonging immunological memory. However, they are often not particularly immunogenic by themselves (Burakova *et al*., 2018).

Adjuvants can act at several different stages during the immune response, although they all influence antigen presentation directly or indirectly. They may aid in the recruitment to the site of immunization, antigen recognition, and activation of antigen-presenting and other ancilliary cells (APCs), particularly dendritic cells (DCs), resulting in the production of key cytokines, and can promote antigen presentation (Ayele, 2020).

2.6.1. Classification of adjuvants and their role in vaccine development

Adjuvants can be classified based on their physicochemical properties, origin, and mechanisms of action. Based on their mechanisms of action, adjuvants can be divided into delivery systems (particulate) and immune potentiators (immune-stimulatory) (Apostólico *et al*., 2016).

Particulate adjuvants are delivery system adjuvant which functions as carriers to which antigens can be associated. Also, they create local pro-inflammatory responses that recruit innate immune cells to the site of injection. Hence, this type of adjuvants can activate innate immunity. Examples are mineral salts, such as aluminum salts (alum), lipid particles, and microparticles. Emulsion Adjuvants: Complete Freund’s adjuvant (CFA) is a water-in-oil emulsion that contains heat-killed mycobacteria and is a classic “gold standard”

representative of this group of adjuvants. Incomplete Freund's adjuvant (IFA) is also a water-in-oil emulsion, but without mycobacteria (Garçon *et al*., 2011).

Oil-in-water emulsion adjuvant, for instance, AS03, which consists of α-tocopherol formulation stimulates the immune system by the activation of NF-κB, pro-inflammatory cytokine and chemokine production, recruitment of immune cells, mainly monocytes and macrophages, and induction of high antibody titers (Sun *et al*., 2009). An important issue is to administer AS03 with the antigen at the same injection site simultaneously to avoid a diminished response. Microparticles such as Virus-Like Particles, Virosomes, PLA/PLGA-Poly (lactic acid) (PLA) and poly (lactic-coglycolic acid) (PLGA) are biodegradable and biocompatible polymeric micro/nanoparticles that function as a delivery system by encapsulating an antigen or antigen plus adjuvant in the same particle (Haensler, 2017).

Immune Potentiators- target innate immunity signaling pathways via Pattern-recognition receptors (PRRs). PRRs consist of different classes of receptors [Toll-like receptors (TLRs), nucleotide binding oligomerization domain- (NOD-) like receptor (NLRs) and retinoic acid-inducible gene-I- (RIG-I-) like receptors (RLRs)] that are widely expressed on immune cells (Apostólico *et al*., 2016). Activation of PRRs by their agonists initiates APC activation/maturation and cytokine/chemokine production that ultimately leads to adaptive immune responses. Examples include dsRNA: Poly (I: C), Poly-IC: LC; Monophosphoryl lipid A (MPL), LPS; Flagellin; Imidazoquinolines: imiquimod (R837), resiquimod (848); CpG oligodeoxynucleotides (ODN); Muramyl dipeptide (MDP) and Saponins (QS-21). In a simplistic definition, the role of immune potentiators is to activate innate immune responses through pattern-recognition receptors (PRRs) or directly (Shi *et al*., 2019).

Adjuvants can be used to improve the immune response to vaccine antigens in several different ways: increasing the immunogenicity of weak antigens; enhancing the speed and duration of the immune response; modulating antibody avidity, specificity, isotype, or subclass distribution; stimulating CTL; promoting the induction of mucosal immunity; enhancing immune responses in immunologically immature or senescent individuals; decreasing the dose of antigen in the vaccine to reduce costs; or helping to overcome antigen competition in combination vaccines (Shah *et al*., 2017).

Generally, adjuvants perform two immunological functions: intrinsically act on the immune system to improve immune responses of antigens (immune-stimulants); and deliver and present vaccine antigens for effective uptake by antigen-presenting cells in a controlled

manner and speed to induce and/or enhance an antigen-specific immune response (vaccine delivery systems), for instance, emulsions, liposomes, virosomes, virus-like particles, and polymeric nanoparticle adjuvants (Garçon *et al*., 2011).

2.6.2. Mechanisms of action of adjuvants

Some adjuvants act by the formation of depot effect. That means, slow-release or trap of the antigen at the injection site and present a sustained supply to local APCs. 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 (Nicholls *et al*., 2010).

Generally, all the mechanisms include stimulation of APCs directly or indirectly; mainly dendritic cells (DC) (figure 1). DC activates the innate and adaptive immune system by processing the antigens and presenting them to specific T-cells. The trapped antigen is taken up through 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 (Burakova *et al*., 2018).

Additionally, the adaptive immune response is also stimulated by activated DC, via processing and presenting the antigens to specific T-cells (CD4+). MHC II and co-stimulatory molecules are also activated by DC to help interactions between DC and CD4+ T cells. Increased CD4+ is stimulated as a result of this immunological cascade, but this cascade is inadequate for CD8+ T cell stimulation, which is important for the efficacy of a vaccine against cancer and intracellular pathogens. Therefore, novel adjuvants are targeted to receptors of APCs expressed on DCs to activate the innate immune system (Münch & Wochenschr, 2015).

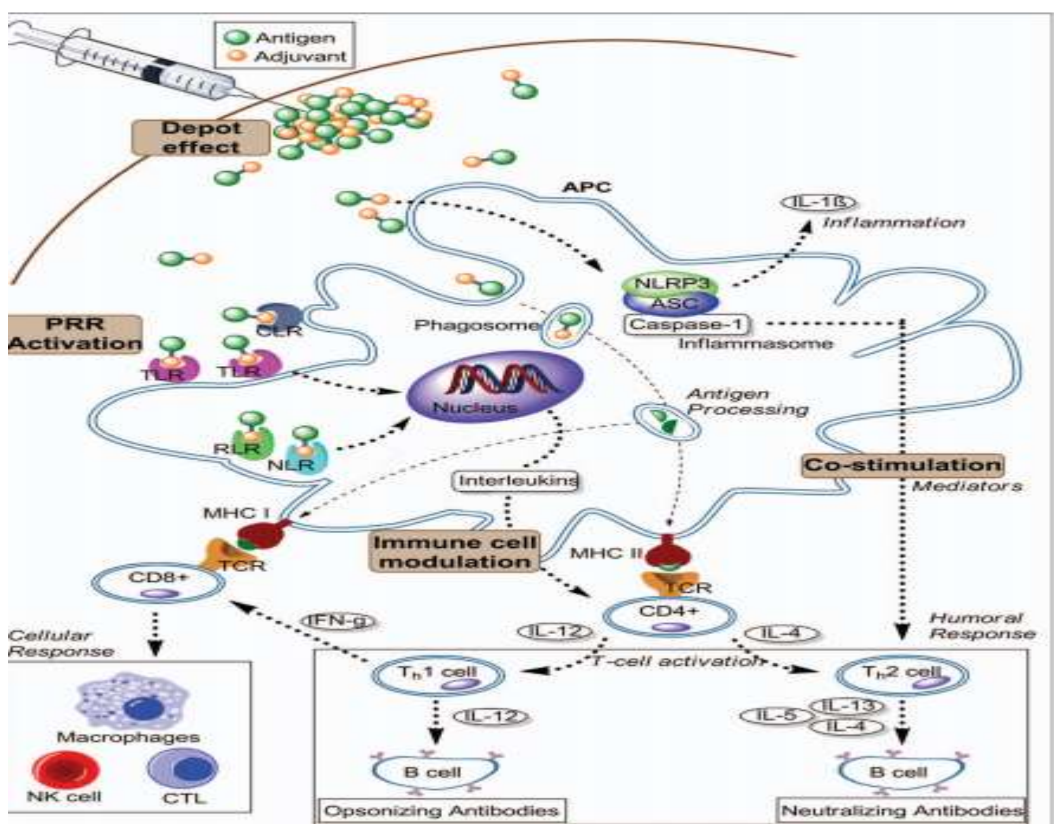

Figure 2: Mechanisms of action of adjuvants in immune systems.

Source: (Garçon *et al*., 2011)

2.6.3. Characteristics of the ideal adjuvant

Although the mechanisms of action of adjuvants are varied, there exist a number of important qualities that must be considered when designing and/or selecting new adjuvants (Nicholls *et al*., 2010). These include stimulation of an efficacious immune response (T cell and antibody responses), compatibility with antigen(s), induction of memory, reduction of antigen dose or the number of vaccination(s), a broadening of the response, stimulation of the response against combined vaccines, reduce antigen competition, stimulation of the response in young and elderly, a more rapid induction of immune response, cost effectiveness and sufficient duration of an immune response.

2.6.4. Efficacy and safety of adjuvants

Efficacy and safety are important qualities of adjuvant selection for vaccines. While some adjuvants show high immunogenicity others are less with better safety. Some can be better at

both. For example, saponin has shown more protection against CCPP but with higher post-vaccinal reactions compared with ISA 50. Increased body temperature and local edematous reactions were seen in animals inoculated with saponin adjuvanted CCPP vaccine (100%) while 20% of the goats in the ISA 50 adjuvated group showed local reaction *(*Ayelet *et al.*, 2007).

A comparison of adjuvant emulsions for their safety and ability to enhance the antibody response in horses immunized with African snake venoms has been done by Arguedas et al., (2022). They tested the performance of four commercial emulsion adjuvants (Montanide, Freund, Carbigen, and Emulsigen-D) and an experimental adjuvant (QH-769) in the antibody response of horses towards the venoms of the African snakes. According to their report Montanide, Freund and Carbigen adjuvants generated the highest immune response but induced moderate/severe local lesions at the injection site. On other side, Emulsigen-D and QH-769 adjuvants generated the lowest immune response and low incidence of local lesions. No evidence of systemic alterations was observed in the horses immunized with any of the adjuvants (Arguedas *et al*., 2022).

Emulsion-based adjuvants are a very attractive for livestock vaccine preparations. These adjuvants can be formulated using inexpensive, readily available components such as mineral oil and food-grade emulsifiers (Apostólico *et al*., 2016). Montanide ISA 201 and 206, produce multiple emulsions up on gentle mixing with antigenic aqueous phase. These adjuvants demonstrate effectiveness and provide protection for different livestock species against several economically important pathogens, including influenza and FMD viruses (Shah *et al*., 2017).

Results from the studies conducted on the FMD vaccine prepared using different adjuvants including Emulsigen®-D; Montanide ISA 206 and Emulsigen®-D (ED) with aluminum hydroxide gel (ALOH), revealed that the onset of protective antibody titer was achieved early in the Emulsigen® and Emulsigen® with ALOH gel vaccinated groups followed by Montanide ISA 206 vaccinated group. The highest peak antibody titer values were induced by Emulsigen®-D with aluminum hydroxide followed by Emulsigen®-D and lastly for Montanid ISA 206. Concerning the duration of protective immunity against the three serotypes of FMDV included in the vaccine, the results revealed that the longest duration was achieved by the Emulsigen® D alone and in combination with the ALOH adjuvant lasting for 36-week post vaccination. The protective SNT antibody titer of the vaccine with Montanide

ISA 206 adjuvant against the three serotypes lasts for 32- weeks post vaccination. Depending on these findings, they concluded that Emulsigen®-D and with aluminum hydroxide gel induce the superior immune response of sheep to the trivalent FMD vaccine over the Montanide ISA 206 adjuvated trivalent FMD vaccine (Shabana *et al.*, 2018). Below, the safety and efficacy of concerned adjuvants for this research have been reviewed.

Saponin

Saponins are naturally occurring products that possess many different biological properties as immunological adjuvants for use in vaccines. They have attracted a significant interest in activation and modulation of the immune system (Sun *et al.*, 2009). The most prominent saponin-based adjuvant is Quil-A. Quil-A is a heterogeneous mixture of water-soluble saponins extracted from Quillaja saponaria, a tree indigenous to South America (Nicholls *et al.*, 2010). Due to its toxicity, Quil-A is not suitable for human vaccines; it is widely used for veterinary applications. Although its unique potency is promising in numerous vaccine clinical trials, its inherent limitations in terms of scarcity and heterogeneity, dose-limiting toxicity, and chemical instability have hindered its further clinical advancement (Iqbal *et al.*, 2007).

Due to safety restrictions, some compounds are prohibited for use in a human vaccine (Quil-A®, mineral oil-based emulsions), but are successfully employed in vaccines for livestock species (Hagan & Vaccines, 2007). Emulsions have been employed as adjuvant systems in animal vaccines for a long time and are a good choice for animal vaccines because they are relatively simple to produce, cost-effective, and show good efficacy in the development of antibody responses. Oil-in-water (O/W) emulsions have a better safety profile. They are less irritating and toxic than water in oil emulsions (Facciol *et al.*, 2022).

Emulsions

Emulsions are the mixture of two immiscible liquids, one of which is organized into droplets, stabilized by an interfacial surfactant layer, and dispersed into the other. Thus, the dispersion of an organic liquid such as oil in water is called an oil-in-water (o/w) emulsion, while water droplets dispersed in a continuous oil phase are called water-in-oil (w/o) emulsions *(*Shah *et al.*, 2015). In addition, there are multiple or double emulsions, such as water-in-oil-in-water (w/o/w) or oil-in-water-in-oil (o/w/o). These systems are occasionally referred to as "emulsions of emulsions" as they, in the case of w/o/w emulsions, contain water droplets

within the oil droplets, which, at the same time, are dispersed into a continuous water phase (Wu & Liu, 2021).

Water-in-oil (w/o) emulsion is a dispersion of water droplets within a continuous oil phase. The antigen is entrapped in the water phase surrounded by a continuous oil phase and slowly released upon the breakdown of oil after injection. The most common example of w/o emulsion adjuvants was Freund's adjuvants. These adjuvants are two types, namely, Freund's complete adjuvant (FCA) consists of the paraffin oil, heat-killed and dried mycobacteria or without mycobacteria (Freund's incomplete adjuvant, FIA). These adjuvants were considered very efficient in inducing high-titer antibody responses. Successfully commercialized w/o emulsions are available under the brand name MontanideTM Incomplete Seppic Adjuvants (ISA) (Seppic, France). These adjuvants show effectiveness on the level of FIA, but have fewer side effects and are utilized in veterinary vaccines (Park *et al*., 2016).

Oil-in-water adjuvants for vaccines

These classes of adjuvants are formed by the dispersion of oil droplets in the aqueous phase. The oil droplets facilitate the chemokine-driven immune cell recruitment and the differentiation of macrophages and dendritic cells (DCs) (Yatoo *et al.*, 2019). O/W emulsions have a better safety profile. They are less irritating and toxic than water in oil emulsions (Fan *et al*., 2022). MF59 demonstrated better adjuvanticity in stimulating a cell-mediated immune response against influenza virus than aluminum hydroxide or calcium phosphate (Haensler, 2017). For veterinary applications, several commercially available o/w adjuvants exist under the brands of Montanide ISA, Emulsigen® (MVP Technologies, USA), and Meta-Stim® (Fort Dodge Laboratories, USA). These adjuvants are used in livestock vaccines against various economically important bacterial and viral antigens (Shabana *et al.*, 2018).

Minimum Valuable Product (MVP) laboratory provides high quality, ready-to-mix adjuvants that are free from animal origin components, including the EMULSIGEN®, CARBIGEN™ and POLYGEN™ platform systems, and also produces custom adjuvants for vaccine producers based on specific antigen and/or delivery requirements (Newswire *et al*., 2016).

EMULSIGEN® is a leading family of oil-in-water Minimum Valuable Product (MVP) adjuvants that incorporate various immune-stimulants. EMULSIGEN was the first oil-in-water adjuvant approved by USDA for use in pigs when injected both intramuscularly and

subcutaneously. EMULSIGEN is antigen-friendly in that it can be mixed with antigen at any temperature and using only mild mixing (no homogenization). Such a process can serve to enhance immunogenicity of the finished product and improve the vaccine's safety profile. It contains uniformly dispersed, micron size oil droplets which ensure maximum emulsion stability and decreased viscosity. In addition, increase the surface area available to antigens, reducing the quantity of oil required in the final vaccine.

The technology used in manufacturing EMULSIGEN reduces the undesirable side effects associated with other oil-in-water adjuvants, while still eliciting the rapid and strong immune response (PAHC, 2017). For example, in a certain study, experimental mice were divided into 4 groups and immunized with sterile buffer and VP1-PCV2bCap with different adjuvants, the immune response was monitored for 10 weeks. Robust immune response was detected after the first immunization and gradually increased after the second and third dose, especially in mice immunized by recombinant protein with Emulsigen (10%) as an adjuvant. After that the vaccine efficacy was checked in a target organism by immunizing 8-week-old piglets with VP1-PCV2bCap protein with Emulsigen (10%). The levels of anti-PCV2b specific IgG antibodies were significantly increased in piglets after the second immunization (Shah *et al*., 2015).

EMULSIGEN-D is the most significant of these newer adjuvants. It is formulated with dimethyldioctacecyl ammonium bromide (DDA) producing unique dual-adjuvant systems. These adjuvants have been demonstrated to have superior immune-stimulating and safety characteristics when compared with water-in-oil and water-in-oil-in-water adjuvants. Available data and/or publications demonstrate the value of integrating these adjuvants into inactivated vaccines for swine influenza (IAV-S), Porcine Reproductive and Respiratory Syndrome (PRRSv), Porcine Epidemic Diarrhea (PEDv), Foot and Mouth Disease (FMD) and Newcastle Disease of poultry to cite a few examples (Shabana *et al*., 2018).

EMULSIGEN-D is an oil-in-water dual adjuvant emulsion free of animal-origin ingredients. It has a milky-white appearance that creates a smooth, uniform mixture when added antigens. It contains dimethyldioctadecyl ammonium bromide (DDA) which is nanoparticle adjuvant that is incorporated into the emulsion. EMULSIGEN-D is antigen-friendly in that it can be mixed with antigen at any temperature and using only mild mixing (no homogenization). Such a process can serve to enhance immunogenicity of the finished product and improve the vaccine's safety profile (PAHC, 2017).

EMULSIGEN-D contains uniformly dispersed, micron size oil droplets which ensure maximum emulsion stability and decreased viscosity. Micron-sized oil droplets, additionally, increase the surface area available to antigens, reducing the amount of oil required in the final vaccine. EMULSIGEN-D reduces the undesirable side effects associated with water-in-oil or water-in-oil-in-water adjuvants, while eliciting the rapid and strong immunity. Oil-in-water emulsified adjuvants form a mobile depot of antigen which can target immune effector cells. The depot effect with slow release improves the presentation of antigen thus providing a significant antigen enhancement of the immune response and vaccine efficacy. The added DDA is a known T-cell immune stimulator (PAHC, 2017).

EMULSIGEN-D has the potential to induce higher levels of humoral antibody, early onset of immunity, and enhanced protection with a single vaccine dose than conventional aluminium based adjuvants. EMULSIGEN-D is an excellent adjuvant for bacterial, mycoplasma, viral, subunit, and DNA recombinant or parasite antigens. The EMULSIGEN family of adjuvants also includes EMULSIGEN-BCL and EMULSIGEN- P that incorporate other types of immuno-stimulants (Newswire *et al*., 2016).

EMULSIGEN-P is a unique, oil-in-water dual adjuvant emulsion that is free of animal origin ingredients. Its milky-white appearance creates a smooth, uniform mixture when added to veterinary antigens. EMULSIGEN-P contains uniformly dispersed, micron size oil droplets which ensure maximum emulsion stability and decreased viscosity. EMULSIGEN-P has the potential to elicit higher levels of humoral antibody, more rapid onset of immunity, and enhanced protection with a single vaccine dose as compared with conventional aluminum based adjuvants. EMULSIGEN-P is an excellent adjuvant for bacterial, mycoplasma, viral, subunit or parasite antigens (PAHC, 2017).

EMULSIGEN-P reduces the undesirable side effects, while still eliciting the rapid and strong immune response. EMULSIGEN-P's POLYGEN™ ingredient is an immune-stimulant that improves the antigenicity of virus, subunit and parasite vaccines. It has been demonstrated to stimulate T-cell responses, including interferon gamma and interleukin 12 (Park *et al*., 2016). The depot effect with slow release improves the presentation of antigen thus providing a significant antigen enhancement of the immune response and vaccine efficacy.

CARBIGEN and POLYGEN are MVP's polymer-type adjuvants. Its muco-adhesive properties make CARBIGEN to be particularly applicable for presenting inactivated antigens to mucosal membranes. Intranasally, inactivated antigens with CARBIGEN have been used

successfully in horses, pigs and small animals. It has also shown exceptional performance in adjuvating PCV2 antigens (Arguedas *et al*., 2022).

CARBIGEN is a terminally-sterilized, carbomer-based adjuvant suspension containing a proprietary emulsified component and is free of animal origin ingredients. Its milky-white appearance forms a smooth, uniform mixture when added to veterinary vaccine. The polymer used to make Carbigen is a pharmaceutical grade, cross-linking polymer that encapsulates the antigen, providing a slow release as well as a depot effect. The depot effect with slow release improves the presentation of antigen to effector cells providing a significant enhancement of the immune response and efficacy of the vaccine. CARBIGEN has been used intranasally, by spray and immersion to produce IgA and IgG responses against various disease organisms (Shi *et al*., 2019).

CARBIGEN has the potential to elicit higher levels of humoral antibody and cellular immunity, more rapid onset of immunity and enhanced protection with a single vaccine dose as compared with conventional aluminium based adjuvants. It can be used in combination with bacterial and viral antigens, either parenterally or intranasally. CARBIGEN has been evaluated as an adjuvant for PCV2 vaccines and displays an excellent efficacy and safety profile (PAHC, 2017).

POLYGEN has an inherent ability to stimulate cell mediated immunity and is especially useful in small animal and bovine vaccines where increased T-cell responses are required. It has been shown to stimulate Ɣ-interferon and IL-12 when used in subunit and/or parasite vaccines (PAHC, 2017).

3. MATERIALS AND METHODS

3.1. Description of the Study area

The study was conducted from December 2022 to July 2023 in the laboratory of National Veterinary Institute (NVI) found at Bishoftu, Ethiopia (Figure 3). Bishoftu is located at 45 km southeast of Addis Ababa at 9°N and 40° E (https://en.db-city.com/Ethiopia--Oromia--East-Shewa-Zone--Bishoftu). It has a bimodal rainfall season: the long rainfall season from late June to late September and the short season from February to April, with a mean annual rainfall range of 246 mm to 1,083 mm. The temperature range is 14°C to 27°C with the average relative humidity of 79% (Adea Woreda report, 2020).

The site from which the animals were purchased was Bulbula, East shewa zone, Oromia region, Ethiopia. According to the livestock survey result obtained from central statistical agency of Ethiopia, the livestock population for the country was estimated to be 70 million cattle, 52.5 million goats and 42.9 million sheep being found in the country. From the total goat population of the country, 431,649 goats are found in East Shewa zone. Out of these, Bulbula, one of the East Shewa towns, is thought to have a large goat population (CSA, 2021). It is located at 184 km southeast of Addis Ababa at 7°N and 38°E with an altitude of 1602m above sea level.

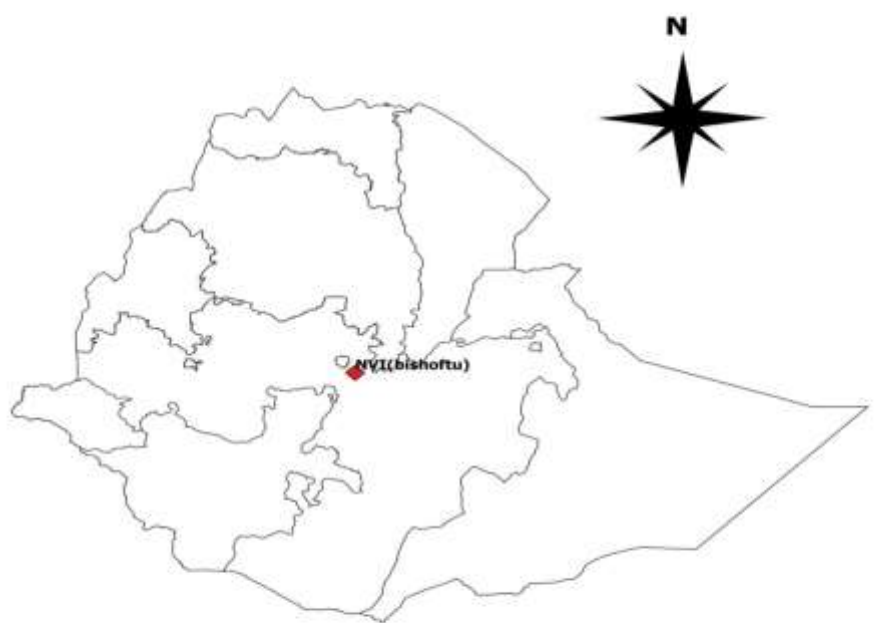

Figure 3: Location of study site. **Source:** (QGIS 3.10 version)

3.2. Study animals

The animals used for this study, goats, were purchased from local livestock market, Bulbula, and maintained in the animal facility of NVI. During the purchase, clinical health of the goats and physiological status were checked by the professionals (from NVI) involved in the work. All the study animals were managed properly with adequate provision of water and feed. Sixty apparently healthy male goats were purchased and designed for the experimental study according to the simple random sampling technique.

3.3. Study Design

Experimental study design was used to conduct the present study with the following components.

3.3.1. Husbandry of the experimental animals and Antibody Screening

Clinically healthy goats were purchased and were kept in clean sheds. Sixty-three goats older than six months of approximately equal age and negative for Mccp-specific antibodies were used for this experiment. Sixty purchased goats and 10 goats (for contingency) from NVI, totally 70 goats were screened for Mccp antibody by using competitive ELISA (c-ELISA). Sixty-three animals tested negative for CCPP were treated by albendazole and oxytetracycline, and left for two weeks for adaptation. They were offered appropriate feeds like wheat bran, maize, Alfalfa, and adlibetum clean water.

3.3.2. Culture preparation

Mccp stock seed, used for the production of CCPP vaccine, were taken from NVI vaccine seed bank, and passaged three times at weekly interval in an inoculum media (Hayflick broth). The culture showing adequate change in pH (6.65–6.95) and free from contamination were allowed to pass to the next passage. Inoculum from the last (third) passage were seeded into CCPP production media containing 20% horse serum, in a proportion of 20%. The mixture was incubated with continuous slow agitation in spinner bottle (90–100 rpm) at 37ºC for 7 to 10 days until the desired turbidity and pH (6.4–6.8) achieved. Sample from the grown

culture were taken aseptically and observed for purity (because, Mccp lacks cell wall, it can't stain under light microscope) by Gram staining technique (OIE, 2014).

3.3.3. Adjuvant preparation

Four oil adjuvants: Emulsigen, Emulsigen-D and Emulsigen-P were prepared at 15% v/v, whereas, Carbigen was at 10%v/v as per the manufacturer's instruction (PAHC, 2017) to be compared with 3% and 0.3% saponin. On the other hand, saponin adjuvant was prepared by boiling 100 gm of saponin powder in 1 liter distilled water (10%), filtered by 0.22 μm pore filter sheet for sterilization and sterility test (OIE, 2014).

3.3.4. Inactivation of the whole culture and vaccine formulation

Inactivation of Mccp culture and formulation of the vaccine with adjuvants were performed according to the protocol used by CCPP vaccine production laboratory at NVI**.** The culture was inactivated with 0.5% formaldehyde as per the recommendation of OIE (2014).

The Mccp culture that has protein antigen content greater or equal to 0.15 mg per ml of whole culture was subjected to vaccine formulation. Emulsigen, Emulsigen-P and Emulsigen-D were added to the culture at 15% v/v whereas Carbigen at10%; saponin at 3% and 0.3%.

3.3.5. Vaccine quality control

Purity test

The inactivated culture was checked for the presence of bacterial and fungal contaminants by culturing samples from the culture on sterility test media: tryptose agar, Sabouraud dextrose agar, tryptose soya broth/SBCDM broth, and thioglycollate broth. The cultured samples were checked by Gram staining for bacterial contaminants OIE (2014).

Safety test

Safety of the vaccine with each of the adjuvants used was checked by injecting 3 goats with 2 ml by subcutaneous route, and 2 goats were kept as controls. The vaccinated goats were examined daily for the presence of CCPP clinical signs and reactions at the injection site. Rectal temperature was recorded twice daily for 12 days in the morning and afternoon.

3.3.6. Immunogenicity and safety tests

Experimental animals grouping and vaccination

Mccp antibody-free goats were selected based on the c-ELISA result used during screening for immunization and adjuvant evaluation tests. Totally 63 goats were randomly assigned and grouped into seven experimental groups each of them consisting of 9 animals. Accordingly, Group-1 received Carbigen (10%) adjuvanted CCPP vaccine; Group-2 with 3% saponin; Group-3 with 0.3% saponin (the usual vaccine of NVI used as a positive control); Group-4 with Emulsigen (15%); Group-5 with Emulsigen-D (15%) and Group-6 with Emulsigen-P (15%) adjuvanted vaccine, while the final group (Group-7) was used as negative control without vaccination.

Follow up and blood sample collection

All experimental groups of goats were followed on daily basis for clinical signs and adverse reaction. The rectal temperature of vaccinated and control group goats was recorded for 12 days twice daily (in the morning and afternoon). Five ml of blood was collected from each experimental goat once per week on days 0, 7, 14, 21, 28, 35, 42, 49 and 56 post-vaccination using plain vacutainer tubes. Collected blood was allowed to clot at room temperature. Clear serum was separated in another sterile tube and was stored at -20 ^{o} C until used for the subsequent process/assay.

Serological assay by Enzyme-linked immunosorbent assay (ELISA)

ELISA, typically c-ELISA, was used to determine the Mccp-specific antibody response that reacts with the Mccp antigen coated on microtiter plates, i.e. sero-conversion. Sera collected from each group of goats immunized by the same vaccine of different adjuvant were analyzed for measuring the antibody level. The test was performed by using antibody detection kits (IDEXX, France) as per the manufacturer’s instruction (Mccp antibody detection kit manual).

Microplates are coated with a purified Mccp lysate. Samples to be tested are premixed with a specific monoclonal antibody detection solution (detection solution MAb) in a separate plate (“pre-plate”) and then transferred into the coated microplate. Any Mccp specific antibodies

present in the sample formed an immune-complex with Mccp antigen coated on the microplate competing with the Mab in the detection solution for the specific epitopes. After washing away unbound material, an anti-mouse antibody enzyme conjugate that binds the MAb in the detection solution were added.

In the presence of immune-complexes between Mccp antigen and antibodies from the sample, the MAb cannot bind to the specific epitopes and the conjugate is therefore prevented from binding. Conversely, in the absence of Mccp antibodies in the test sample, the Mab can bind to its specific epitopes and the conjugate were free to bind to it. Unbound conjugate was washed away and an enzyme substrate (TMB) was added. In presence of the enzyme, the substrate oxidized and developed a blue compound becoming yellow after blocking. Subsequent color development was inversely proportional to the amount of anti-Mccp antibodies in the test sample. The optical density (OD) of each well was measured at wavelength of 450 nm using microplate reader. The results were expressed in "percentage of inhibition" (PI) by comparing the optical density in the test well with the optical densities in the MAb control wells.

A percentage of inhibition was calculated according to the formula described by kit manufacturer (IDEXX, France): {100 x (MAb control mean OD – samples' OD) / (MAb control mean OD – conjugate control mean OD)}. The percentage of inhibition implies the competition between antibody present in test sample and HRP conjugated anti- mouse antisera for the specific Mccp antigen pre-coated on the ELISA plate (OIE, 2018). Therefore, PI was directly proportional to antibody level in the test sera.

Validity criteria of the test

The test was considered valid on the basis of the readings of controls used for c-ELISA. The mean Percentage Inhibition value of negative controls shall be PI < 35%; mean positive control PI value shall be between 50% to 80%; whereas, strong positive controls PI value shall be 60% to 90% for the readings of the samples to be valid. Accordingly, the samples with PI $\geq$ 55 were considered sero-positive (Appendix 1).

3.4. Data management and analysis

Collected data were entered into the Microsoft Excel Spread sheet program to create database. To determine whether there are any statistically significant differences among each adjuvanted vaccine formulation, Repeated Measure analysis of variance (ANOVA) and Mixed Model Analysis were used in Prism version 9.5.1(733). Statements of statistical significance were made based on $p < 0.05$.

3.5. Ethical considerations

The experiment was carried out according to the ethics and guidelines of laboratory animal experimentation. Ethical clearance was obtained from National Veterinary Institute (Appendix 2). The animals were used only for evaluation of efficacy (humoral immune response) and safety.

4. RESULTS

4.1. Vaccine purity test result

The sterility test of the vaccine formulations showed that the vaccines were free from aerobic and anaerobic bacteria, fungal and mycoplasma contaminants. Thioglycollate broth was used for anaerobic bacterial contaminants; tryptose soya broth and tryptose agar were for aerobic bacterial contaminants; whereas, sabouraud agar was for fungal contaminants. The result of the test showed negative in all test media. i.e., no any microbial growth was seen on the purity test media.

Similarly, assessment of absence of Mccp growth helped to confirm complete inactivation of the bacteria. Thus, the formulated vaccines were considered to be safe for animal experimentation.

4.2. Efficacy of the vaccines

Throughout the experimental period (for 56 days) the sero-conversion of the goats categorized under the 7 treatment groups were analysed. Consequently, the PI value of sero-conversion for the duration of each of the 7 treatment groups is presented in (Table 1). Among the 6 vaccine preparations, 0.3% Saponin showed the highest mean percentage of inhibition (PI) value (74.45) followed by 3% Saponin (66.92), Emulsigen (66.90), Carbigen (66.45), Emulsigen-P (59.57) and Emulsigen-D (56.77). Except Emulsigen-P, in all others the highest mean PI value was recorded on day 28.

Table 1: Mean percentage of inhibition (PI) value of experimental groups

Testing period (days)		Treatment group						
		Control	Carbigen	3%Saponin	0.3%Saponin	Emulsigen	Emulsigen-D	Emulsigen-P
0	**M±SEM**	**34.08**±0.92	**37.17**±0.64	**35.83**±1.20	**36.01**±1.22	**34.80**±1.48	**35.08**±1.30	**36.06**±1.39
	95%CI	31.97-36.19	37.70-38.64	33.06-38.61	33.21-38.82	31.37-38.22	32.08-38.08	32.85-39.28
7	**M±SEM**	**35.15**±1.62	**68.79**±3.11	**76.37**±2.86	**74.81**±2.55	**68.17**±4.28	**63.34**±3.27	**69.26**±2.15
	95%CI	32.24-38.06	61.01-75.33	69.78-82.97	68.93-80.69	58.31-78.04	55.80-70.89	64.30-74.23
14	**M±SEM**	**34.91**±1.13	**72.39**±4.73	**73.44**±2.75	**72.06**±4.42	**67.48**±4.18	**62.16**±1.46	**64.84**±3.20
	95%CI	32.30-37.52	61.48-83.30	67.09-79.80	61.86-82.26	57.86-77.11	58.79-65.54	57.45-72.22
21	**M±SEM**	**35.92**±1.26	**67.13**±4.38	**66.35**±4.23	**78.22**±2.89	**68.63**±4.75	**61.87**±2.01	**66.80**±1.31
	95%CI	33.01-38.82	57.04-77.23	56.59-76.10	71.55-84.88	57.67-79.59	57.22-66.51	63.71-69.88
28	**M±SEM**	**35.83**±1.06	**78.01**±2.60	**79.42**±4.20	**85.77**±2.74	**75.44**±4.50	**67.78**±3.52	**67.16**±1.56
	95%CI	33.38-38.28	72.01-84.01	69.72-89.12	79.46-92.08	65.05-85.82	59.46-76.10	63.48-70.84
35	**M±SEM**	**37.01**±1.28	**65.07**±3.34	**67.48**±3.92	**76.04**±3.40	**67.64**±3.87	**57.49**±3.22	**58.75**±1.33
	95%CI	34.07-39.96	57.38-72.77	58.45-76.52	66.82-85.25	58.73-76.56	49.86-65.11	55.61-61.89
42	**M±SEM**	**36.74**±0.93	**65.36**±2.86	**56.65**±4.46	**71.34**±4.10	**62.54**±3.85	**49.17**±3.52	**54.74**±2.23
	95%CI	34.60-38.88	58.77-71.95	46.35-66.95	61.88-80.80	53.67-71.42	40.84-57.49	49.46-60.01
49	**M±SEM**	**34.23**±0.96	**61.08**±3.11	**59.18**±3.43	**70.30** ±5.45	**63.52**±4.88	**45.99**±4.25	**51.81**±2.71
	95%CI	32.02-36.45	53.90-68.26	51.29-67.08	57.42-83.18	52.27-74.77	35.58-56.40	45.18-58.45
56	**M±SEM**	**31.99**±0.94	**54.41**±3.40	**56.45**±3.16	**67.01**±6.48	**61.78**±5.52	**46.35**±5.41	**43.17**±3.55
	95%CI	29.83-34.15	46.56-62.26	48.97-63.93	51.70-82.33	49.05-74.52	33.10-59.60	34.48-51.86

PI= percentage of inhibition; SEM= standard error of the mean; CI= confidence interval

Throughout the experimental period, over 56 days, the PI value was significantly different within each treatment group from the first week to the next nine weeks (p<0.05) for all vaccinated groups except control group. The highest statistically significant difference was observed between day-0 and day-7 as well as day 28 to other weeks (Table 2).

Table 2: Statistical variation within each vaccine formulation over the experimental period

Treatment groups	Statistical values		Significance variation
	P value	**F (DFn, DFd)**	
Control	P = 0.08	F (2.787, 22.30) = 2.615	No
Carbigen	P<0.0001	F (3.656, 29.25) = 20.50	Yes
3% saponin	P<0.0001	F (3.700, 29.14) = 34.15	Yes
0.3% saponin	P<0.0001	F (1.955, 15.15) = 25.01	Yes
Emulsigen	P<0.0001	F (3.213, 25.70) = 16.91	Yes
Emulsigen-D	P<0.0001	F (2.658, 18.94) = 25.26	Yes
Emulsigen-P	P<0.0001	F (3.028, 21.19) = 36.69	Yes

The table shows the statistical variation within each treatment in mean PI value throughout the duration of the experiment. F= degree of freedom; DFn=degree of freedom numerator; DFd=degree of freedom denominator.

In all groups mean PI value was significantly increased starting from day 7, until day 28 except for the Emulsigen –P where the PI value was highest at day 7 (69.26) than at day 28 (67.16). After day 28, in all groups mean PI value started to drop gradually (Figure 4).

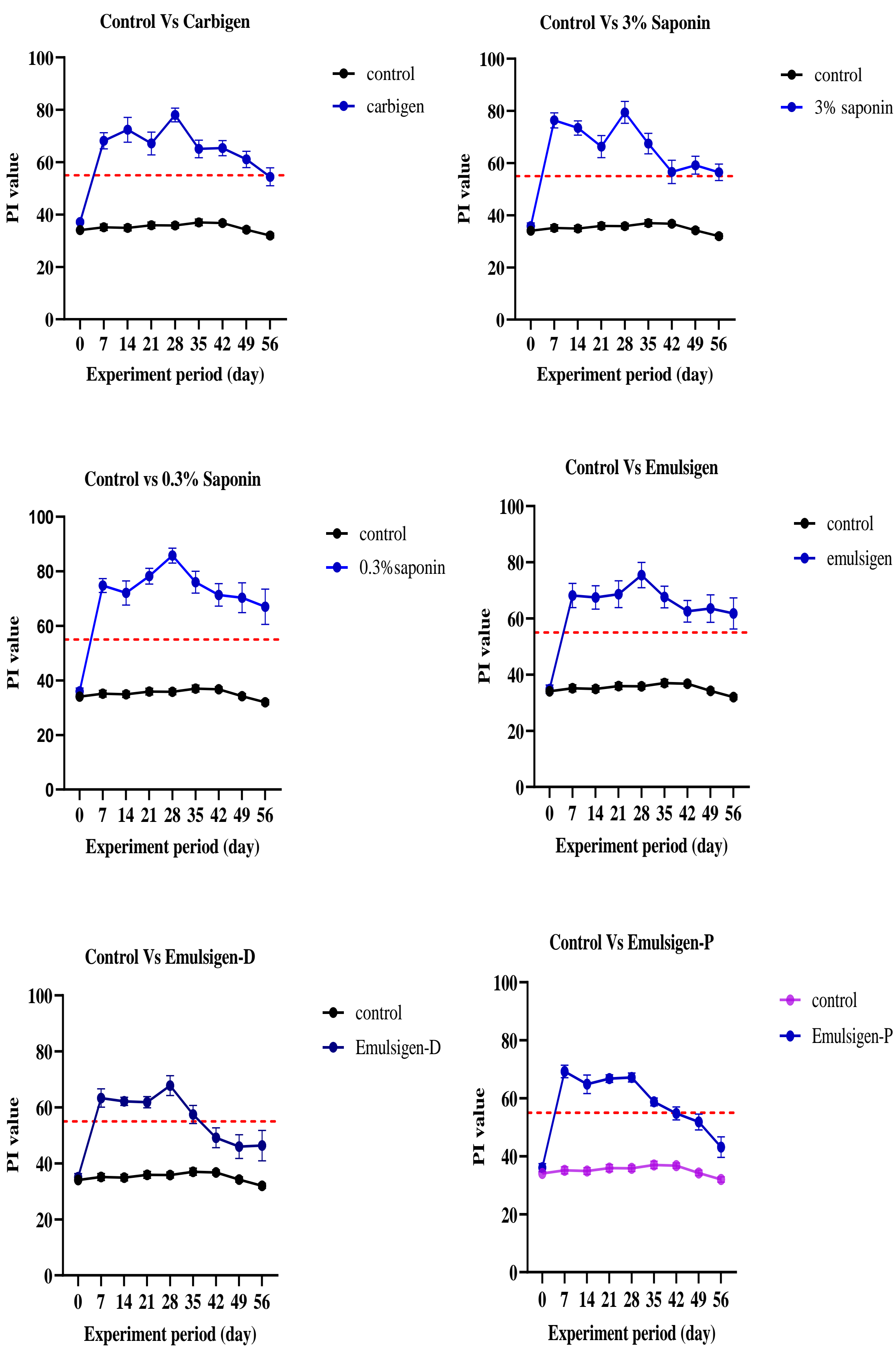

Figure 4: Trends of each treatment in mean PI value

4.3. Multiple Comparison of control with other treatment groups

Multiple grouped comparison of control group was made to each of the vaccinated groups. As a result, all groups showed significant differences in sero-conversion in comparable to the control groups except at day 0, i.e., before vaccination (Table 3).

Table 3: Mean difference in PI value of control group to other groups

Period	Mean difference (control - treatment group)						
		Carbigen	Saponin (3%)	Saponin (0.3%)	Emulsigen	Emulsigen-D	Emulsigen-P
BV	Mean difference	-3.09	-1.75	-1.94	-0.72	-1.00	-1.99
	P value	0.1253	0.9368	0.8965	0.9999	0.9991	0.9284
PV	Mean difference	-31.23	-31.70	-39.23	-31.68	-21.55	-24.35
	P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	F(DFn, DFd)	F(8,128) = 15.93	F(8, 127) =27.01	F (8,126) =20.69	F (8, 128) = 13.88	F (8, 121) =19.77	F (8, 120) =27.01

BV- before vaccination; PV- post vaccination; DFn/d- degree of freedom numerator/denominator

4.4. Multiple comparison of all groups

Based on the multiple comparison of all groups made, statistically ($F (48, 430) = 5.524$; $p<0.0001$)) significant differences of mean PI value were observed between control group and treatment groups (Figure 5).

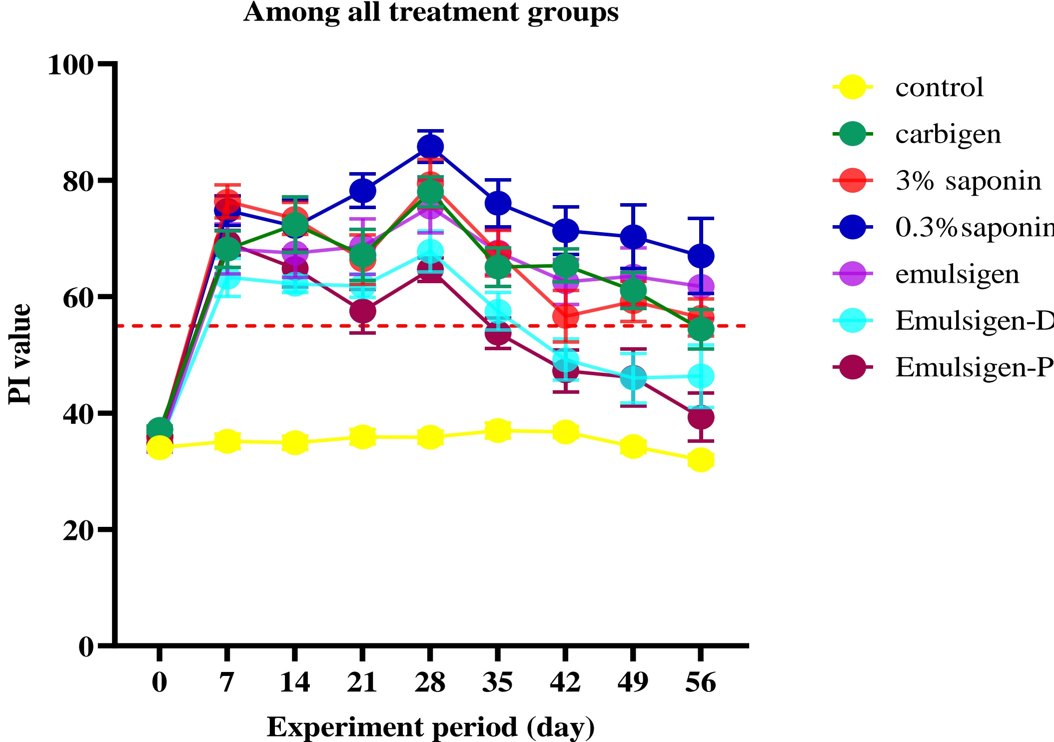

Figure 5: Comparison among all treatments: at the beginning, before vaccination control groups had no significant differences compared to treatment groups, post vaccination significant variations were observed in all comparisons.

Four vaccine formulations with the adjuvant Carbigen, 3% Saponin, 0.3% Saponin and Emulsigen were found to be significantly different to the control group until day 56 post vaccination, but not to each other. The rest two formulations, Emulsigen-D and Emulsigen-P were significantly different from control group until day 35 post vaccination. After day 42, they became non-significant to control group. On the other hand, Emulsigen-D and Emulsigen-P showed statistically significant differences when compared to the other four treatment groups during some weeks (Table 4).

Table 4: Vaccinated groups showing statistical variation

Days	Groups showing variation		Mean difference	P value
D14	3% saponin	Emulsigen-D	11.28	0.0406
D21	0.3% saponin	Emulsigen-D	16.35	0.0052
		Emulsigen-P	20.67	0.0092
D28	Carbigen	Emulsigen-P	13.35	0.0151
	0.3% saponin	Emulsigen-D	17.99	0.0169
		Emulsigen-P	21.11	0.0003
D35	0.3% saponin	Emulsigen-D	18.55	0.0333
		Emulsigen-P	22.29	0.0057
D42	Carbigen	Emulsigen-D	16.20	0.0380
		Emulsigen-P	18.13	0.0202
	0.3% saponin	Emulsigen-D	22.18	0.0131
		Emulsigen-P	24.11	0.0073
D49	0.3% saponin	Emulsigen-D	24.31	0.0460
D56	0.3% saponin	Emulsigen-P	27.70	0.0435

D – represented the days at which statistically significant difference occurred among the vaccinated groups. It should be noticed that the table presents only the point where the variations occurred. Hence, except at these testing points there was no significant difference among the vaccine groups.

4.5. Safety of the vaccines

4.5.1. Temperature post vaccination

During the follow up period, the body temperature was recorded for 12 days, twice a day, post vaccination. Accordingly, the body temperature of all the animals treated with different adjuvants vaccine formulation, in general, remained between 37 to 39.7°C (Figure 6 and 7). However, statistically significant differences were observed ($F (66, 616) = 1.96$; $P<0.0001$)) for two days (Appendix 3 and 4). Generally, the variation in the animal's body temperature was due to 3% saponin and Emulsigen-P (Table 5).

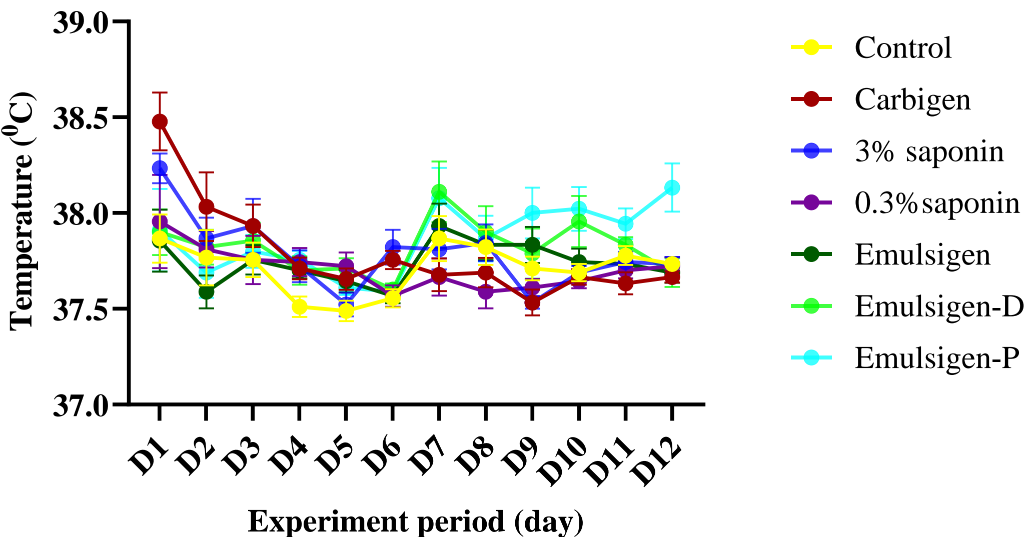

Figure 6: Morning body temperature: the figure shows the mean body temperature of the animals in the morning. D - represents the days on which temperature was recorded. The variation was due to the difference between control and Emulsigen-P at day 4 (i.e., p=0.0459).

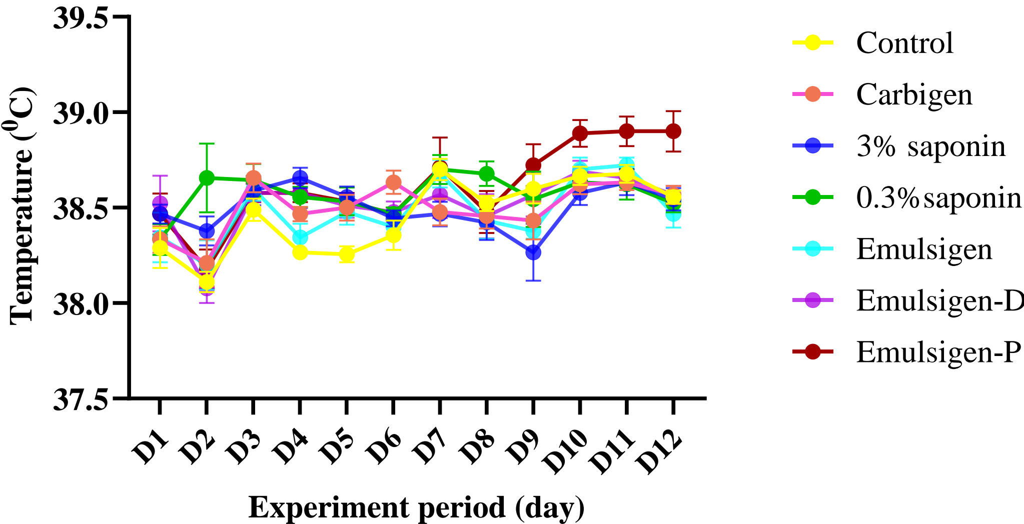

Figure 7: Afternoon body temperature: D- days: the figure shows the mean body temperature of the animals in the afternoon. The variation was due to the differences presented in the table 5 below.

Table 5: Body temperature variation among the experimental animals for few days

Days	Groups		Mean difference	P value
Day 4	Control	Carbigen	-0.20	0.0098
	Control	3% saponin	-0.39	0.0004
	Control	0.3% saponin	-0.29	0.0056
	Control	Emulsigen	-0.08	0.9394
	Control	Emulsigen-D	-0.31	0.0458
	Control	Emulsigen-P	-0.31	0.0017
	3% saponin	Emulsigen	0.31	0.0396
Day 5	Control	3% saponin	-0.30	0.0085
	Control	Emulsigen-P	-0.28	0.0044

The table depicts the days at which body temperature of animals treated with different vaccine formulations has shown statistically significant variation ($P<0.05$).

4.5.2. Other Adverse Reactions

During the follow up periods, the vaccinated goats showed no significant abnormal conditions. However, a localized swelling was developed at the injection site in all vaccinated groups except Carbigen group. The swelling was subsided after two weeks first in 0.3% Saponin, next in 3% Saponin and lastly in Emulsigen family. Additionally, while 3% saponin causes immediate irritation which lasted until 3 days, Emulsigen and Emulsigen-P groups induced alopecia in some animals (Appendix 5).

5. DISCUSSION

The use of adjuvants in inactivated CCPP 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 CCPP vaccines and selection of adjuvants used in the prevention of the disease.

In all animals vaccinated with various experimental vaccine formulations c-ELISA assay result indicated that there is an increment in PI value during post vaccination period, which was due to the competition between antibodies in test sera and enzyme conjugated antibody for the same antigen. This in turn, indicated increase in antibody titers as antibodies available in test sera impede binding of conjugated antibody.

In the present experimental study, evaluation of antibody responses to the CCPP vaccine formulations with different adjuvants: 10% Carbigen, 3% Saponin, 0.3% Saponin, 15% Emulsigen, 15% Emulsigen-D and 15% Emulsigen-P revealed considerable variation both in the safety and magnitude of humoral immune response. The variation in immune response is related primarily to difference in formulation of vaccines (Arguedas *et al*., 2022; Park *et al*., 2016).

Generally speaking, all vaccinated groups has developed significant antibody response ($p<0.05$) compared to un-vaccinated or control group (Figure 3) where the mean PI value differed (Table 1). However, statistically ($p<0.05$) significant variation has been observed among the vaccine groups (Figure 5). The formulations with 10% Carbigen, 3% Saponin, 0.3% Saponin and 15% Emulsigen have significantly developed superior antibody response than 15% Emulsigen-D and 15% Emulsigen-P ($p<0.05$) but not to each other ($p>0.05$) (Table 4). Among the first four groups, except 0.3% Saponin which showed the highest mean PI value (74.45), 3% Saponin (66.92), Emulsigen (66.90) and Carbigen (66.45) were almost the same in their PI value. Even though, no previous report on comparison of all these selected adjuvants together, the finding in this experimental study is partially in line with what has been reported by Arguedas et al. (2022) where the oil adjuvants Carbigen and Emulsigen-P provide highest and moderate immune response, respectively.

Moreover, in all the vaccinated groups, robust immune response was generated starting from day 7 (Figure 4). This result is supported by PAHC (2017) who described that these oil

adjuvants elicit rapid immune response providing a significant vaccine efficacy. Also, similar finding was achieved for Emulsigen from an experimental study done by Shah et al. (2015) where mice and target organism were immunized by recombinant protein, VP1-PCV2bCap with Emulsigen and a significant antibody level was obtained. Collectively, the observations from this experimental work disclosed that Carbigen, Emulsigen, Emulsigen-D and Emulsigen-P could provide a significant antigen enhancement of the immune response and CCPP vaccine efficacy. By this juncture, the study has partly proved what PAHC (2017) and Newswire et al. (2016) described: "Carbigen, Emulsigen, Emulsigen-D and Emulsigen-P are excellent adjuvant for bacterial, mycoplasma, viral, subunit, and DNA recombinant or parasite antigens".

Regarding safety of the vaccines, all vaccinated groups, except Carbigen group, induced localized swelling at the site of injection. 3% saponin posed irritation for 3 days and more transient feverish reaction for 2 days (Table 5) than 0.3% saponin. This is associated with the pro-inflammatory effect described by Burakova et al. (2018) and dose related toxicity of saponin (Wang, 2021). On the other side, some animals vaccinated with Emulsigen and Emulsigen-P adjuvant groups generated alopecia following a local lesion at the site of injection. The localized swelling in Emulsigen, Emulsigen-D and Emulsigen-P subsided later than that of the Saponin. According to the investigation of Shah et al. (2017) this could be because of the nature of the adjuvants and their delivery system in contrary to Saponin which is direct immune-stimulant (Apostólico *et al*., 2016).

Overall, the result of the present study revealed that all animals with saponin formulation at both concentrations have shown better antibody response, showing an incidence of local lesion and some irritation in the case of 3% saponin. This result is similar with what previously done by Ayelet et al. (2007) where saponin has shown more protection against CCPP but with increased body temperature and local edematous reactions seen in animals vaccinated with saponin adjuvanted CCPP vaccine.

Carbigen adjuvated CCPP vaccine has shown high antibody response in goats inoculated with it, almost equal to that 3% of saponin adjuvated vaccine. But, no any adverse reaction and even swelling at the injection site unlike the saponin adjuvants. This outcome is partly in agreement with what Arguedas et al., (2022) did in which adjuvant emulsions compared for their safety and ability to enhance the antibody response of horses immunized with African

snake venoms where the Carbigen adjuvants generated the highest immune response but induced moderate local lesions at the site of injection.

Concerning Emulsigen-D and Emulsigen-P vaccine groups, the present result demonstrated that although they have provided higher immune response compared to control groups, they induced lower mean antibody titer among the all vaccine preparations. On the other hand, a localized swelling was seen similarly in both groups, except the alopecia seen in Emulsigen-P. These results were concordant with the work of Arguedas et al. (2022) who compared Montanide, Freund, Carbigen, and Emulsigen-D for the safety and ability to enhance the antibody response of horses towards venoms of the African snakes and reported that Emulsigen-D adjuvant generated the lowest immune response and low incidence of local lesions. On the other side, the result disagrees with the report of Shabana et al. (2018) who prepared FMD vaccine with different adjuvants and found that Emulsigen achieve early onset of antibody titer, while higher antibody was induced lately by Emulsigen-D, probably due to genetic variability, environmental variations or others. In addition, it was founded that the duration of protective immunity was higher for Emulsigen-D.

This research only measured the antibody response induced by each vaccine adjuvant formulations, so that, rather than humoral immunity, cannot represent the cell mediated immunity that each the CCPP vaccine could provide. This is due to the lack of materials, financial support and cost of the technology required. Furthermore, because challenge test has not been done, the work is limited to determine the protective efficacy of the vaccines fully. Additionally, measuring the duration of immune response developed by each treatment groups was limited due to the time given for this study. Unavailability of similar previous works using these selected adjuvants for CCPP vaccine was also another issue while discussing the result indicating the need for further research on CCPP vaccine and other vaccines to build consistent generality in the use of these adjuvants.

6. CONCLUSION AND RECOMMENDATIONS

In the present study, saponin and different oil adjuvants were evaluated for CCPP vaccine formulation. The study figured out that though 0.3% saponin achieved considerably highest humoral immune response, Carbigen and Emulsigen were not shown statistically ($p>0.05$) significant difference with 0.3% saponin and were observed to be equally immunogenic with 3% saponin. Additionally, Carbigen had not developed any reaction including a localized swelling seen in another groups. Moreover, Emulsigen-D and Emulsigen-P had elicited sufficient immune response in comparison to non-vaccinated groups. These findings confirmed that the oil adjuvants, particularly, Carbigen and Emulsigen are valuable to provide sufficient humoral immune response and better safety. Consequently, it could be concluded that these oil adjuvants can be used as alternatives to saponin in CCPP vaccine.

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

- Considering the safety and antibody response induced by Carbigen, Emulsigen, Emulsigen-D and Emulsigen-P, these adjuvants should be encouraged as an alternative to saponin in CCPP vaccine production.
- Further experimental research on evaluation of these adjuvants should be performed on another goat breeds from other parts of the country to establish the validity of the observed results.
- To generate further evidence about these adjuvants, assessment of cell mediated immunity is advised.
- Duration of protective immunity and protective efficacy should be further evaluated on larger sample sizes
- Evaluation under field condition/field test should be performed before large scale production.

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8. APPENDICES

Appendix 1: Calculation of the c-ELISA plate reading

Controls

Conjugate control mean OD ($CC\bar{x}$) and Mab control mean OD ($MabC\bar{x}$) were calculated as follows:

$$CC\bar{x} = (CC1\ OD(450) + CC2\ OD(450)) / 2$$

$$MabC\bar{x} = (MabC1\ OD(450) + MabC2\ OD(450) + MabC3\ OD(450) + MabC4\ OD(450) / 4$$

Samples and controls

The percentage of inhibition (S PI) for each sample and control was determined as:

$$S\ PI\% = 100 \times \{MabC\bar{x} - S\ OD(450)\} / (MabC\bar{x} - CC\bar{x})$$

Validity criteria

$0.50 \leq MabC\bar{x} \leq 2.00$ $CC\bar{x} < 0.30$

Mean NC PI ≤ 35 $50\% \leq$ Mean PC PI $\leq 80\%$ $60\% \leq$ Mean SPC PI $\leq 90\%$

Where,

$MabC\bar{x}$ is Mab control mean absorbance or OD

$CC\bar{x}$ is conjugate control mean absorbance or OD

NG – negative control

PC – positive control

SPC – strong positive control

PI – percentage of inhibition

Interpretation

- ❖ Negative, if S PI% < 55%
- ❖ Positive, if S PI% ≥ 55%

Appendix 2: Letter of ethical clearance

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NATIONAL VETERINARY INSTITUTE

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Ref. No NVI\1392
ቀን
Date: 16\06\2023

To: Mulatu Mokonon

Subject: Letter of Ethical Clearance

The research ethical committee of the National Veterinary institute reviewed and discussed your research project entitled **"Comparison of selected adjuvants for improvement of safety and efficacy of contagious caprine pleuropneumonia vaccine"** on Dec, 2022. After discussion and review of your project proposal, it is found scientifically and ethically sound from relevance, originality and technical competence point of view.

Hence, the project proposal is allowed to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviations, variations or changes may be made only in consultation between and reported to the committee.
2. All comments given by the committee should be considered and fulfilled by the researchers.
3. The project activity is open for occupational supervision by the committee whenever this is deemed necessary.

The committee expects to be informed about the progress of the study with any changes in the protocol.

Yours sincerely,

Bekecha Bayisa (Dr)
Operational Deputy
Director General

The Federal Democratic Republic ★ Ethiopia National Veterinary Institute

Cc:

- General Manager
- Research & Development Directorate Director

NVI

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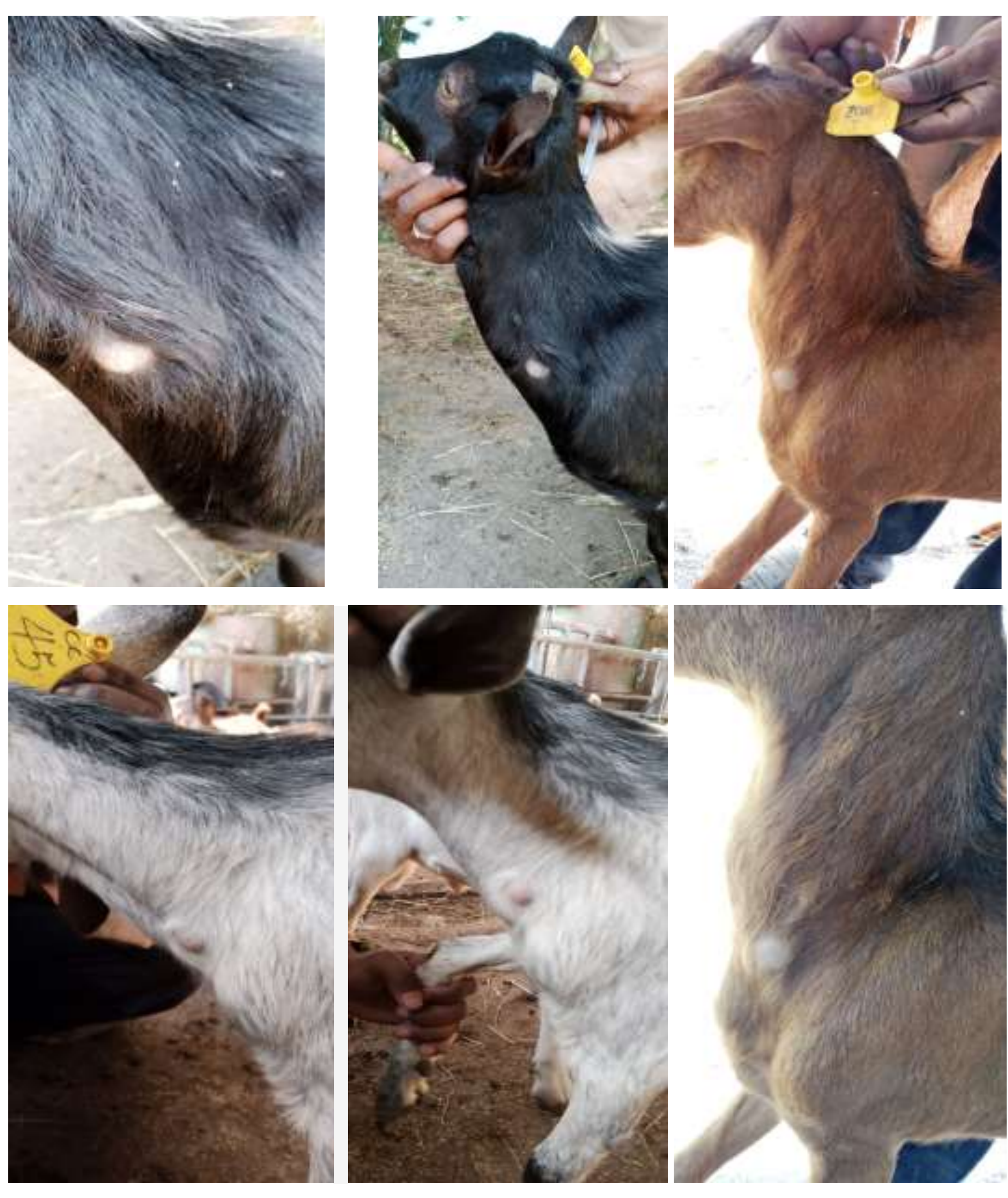
Appendix 3: Morning body temperature of the animals by their experimental groups

Treatment groups	Measurements (°C)				P value
	Minimum	Maximum	Difference	Mean	
Controls	37.10	38.60	1.50	37.71	
Carbigen adjuvanted vaccine	37.20	39.40	2.20	37.73	
3% saponin adjuvanted vaccine	37.10	39.30	2.20	37.76	P<0.0001
0.3% saponin adjuvanted vaccine	37.10	38.90	1.80	37.70	
Emulsigen adjuvanted vaccine	37.20	38.90	1.70	37.74	
Emulsigen-D adjuvanted vaccine	37.40	38.80	1.40	37.82	
Emulsigen-P adjuvanted vaccine	37.20	39.10	1.90	37.87	

Appendix 4: Afternoon body temperature of the animals by their experimental groups

Treatment groups	Measurements (°C)				P value
	Minimum	Maximum	Difference	Mean	
Controls	37.70	39.10	1.40	38.46	
Carbigen adjuvanted vaccine	37.70	38.90	1.20	38.50	
3% saponin adjuvanted vaccine	37.90	39.90	2.00	38.54	P<0.0001
0.3% saponin adjuvanted vaccine	37.40	39.40	2.00	38.50	
Emulsigen adjuvanted vaccine	37.40	39.30	1.90	38.48	
Emulsigen-D adjuvanted vaccine	37.80	39.40	1.60	38.52	
Emulsigen-P adjuvanted vaccine	37.60	39.50	1.90	38.62	

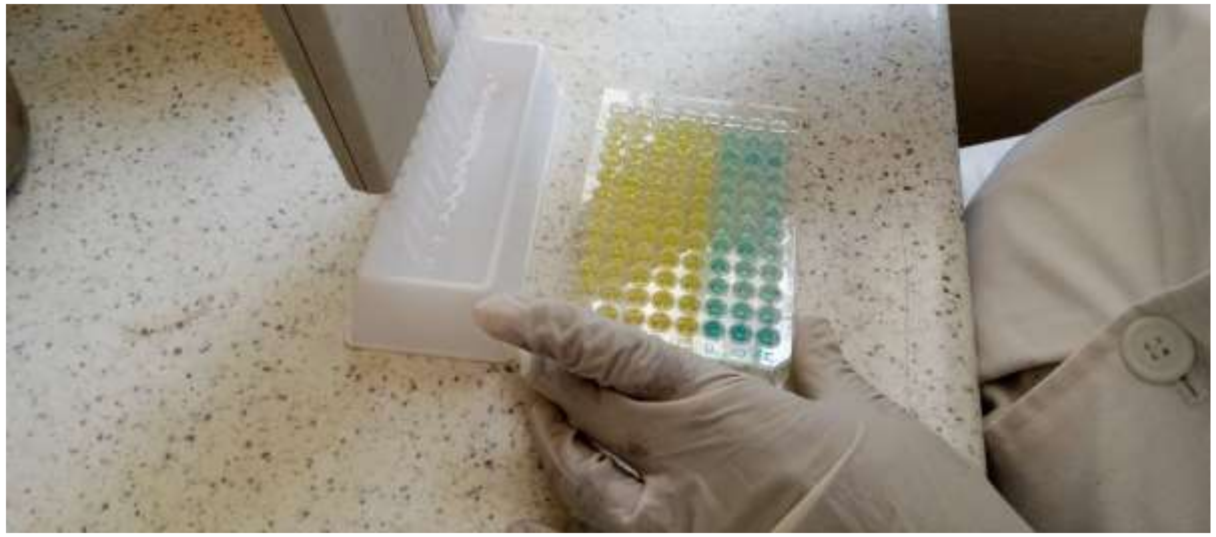
Appendix 5: pictures of goats showing alopecia

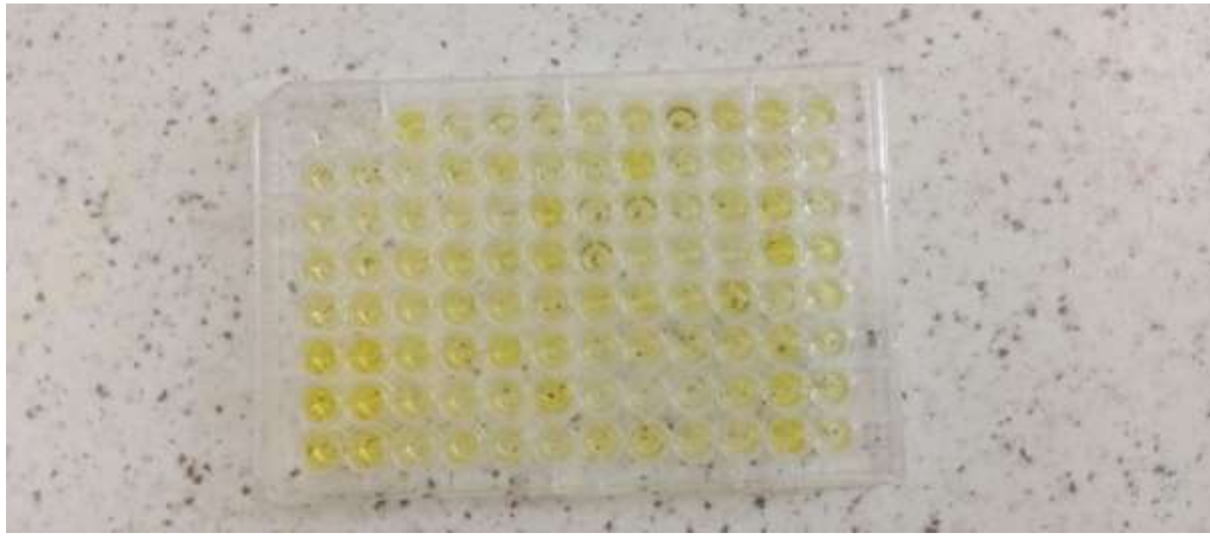


Alopecia in picture

Appendix 6: miscellaneous photos during experiment

Blood collection from jugular vein and serum collection





Color change before and after adding stop solution