ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY, AND VETERINARY PUBLIC HEALTH

EVALUATION OF THE SAFETY AND IMMUNOGENICITY OF SAPONIN-, HEAT-, AND MINOR FORMALDEHYDE-INACTIVATED CONTAGIOUS CAPRINE PLEUROPNEUMONIA WHOLE CULTURE VACCINE

AN MSc. THESIS

BY

ABIYOT ABEBE SISAY

JUNE, 2022

BISHOFTU, ETHIOPIA

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A THESIS SUBMITTED TO THE COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE OF ADDIS ABABA UNIVERSITY IN THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN VETERINARY MICROBIOLOGY

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Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Veterinary Microbiology, Immunology, and Veterinary Public Health

Evaluation of the Safety and Immunogenicity of Saponin-, Heat-, and Minor Formaldehyde-Inactivated Contagious Caprine Pleuropneumonia Whole Culture Vaccine

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AUTHOR'S STATEMENT

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CONTENTS

LIST OF ABBREVIATIONS

ANOVA	One-way analysis of variance
AU-IBAR	African Union-Inter-African Bureau for Animal Resources
BCA	Bicinchoninic acid
BEI	Binary ethylenimine
BPL	Beta-propiolactone
c-ELISA	Competitive enzyme-linked immunosorbent assay
CCPP	Contagious caprine pleuropneumonia
CFT	Complement fixation test
CI	Confidence interval
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GIT	Growth inhibition test
IDRC	International Development Research Centre
IFA	Incomplete Freund's adjuvant
ISA	Incomplete seppic adjuvant
LAT	Latex agglutination test
Mcc	*Mycoplasma capricolum* subspecies *capri*
Mccp	*Mycoplasma capricolum* subspecies *capripneumoniae*
MLSA	Multilocus sequence analysis
NVI	National Veterinary Institute

OD	Optical density
OIE	Office International des Épizooties (World Organization for Animal Health)
OSHA	Occupational Safety and Health Administration
PCR	Polymerase chain reaction
PI	Percentage inhibition
PPR	Peste des petits ruminants
RNA	Ribonucleic acid
SBCDM	Soya bean casein digest medium
UV	Ultraviolet

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ABSTRACT

Contagious caprine pleuropneumonia (CCPP) is a fatal disease of goats imposing significant economic losses through the goat production system. Addressing effective vaccine is the most cost-effective technique in the control of the disease. In National Veterinary Institute (NVI), inactivated protein-based *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp) F-38 strain whole culture vaccine is in use since a few years ago. The vaccine is inactivated by formaldehyde (in a 0.5% proportion) and adjuvated with saponin. Despite the efficacy of the vaccine in Ethiopia, using saponin and heat inactivation has not yet been well considered. While saponin could be used both as inactivant and adjuvant simultaneously, extra formaldehyde is applied for inactivation. This could affect the immunogenicity of the vaccine, and it is not economical to invest on extra formaldehyde. Formaldehyde also has a residual effect. In addition, it is toxic to the laboratory workers, while heat treatment is relatively safer and cheaper. On the other hand, applying high amount of formaldehyde affects the immunogenicity of the vaccine. The aim of this study, therefore, was to evaluate the safety and immunogenicity of the vaccine inactivated alternatively by saponin and heat to replace formaldehyde inactivation, and by minor amount of formaldehyde to make use of such amount in the inactivation process of the vaccine. The vaccine was prepared using World Organization for Animal Health (OIE) guideline for CCPP vaccine production and the standard operating procedure of the manufacturer, NVI. The prepared Mccp culture aliquots were inactivated separately by saponin, heat treatment, and minor formaldehyde proportion. Thirty Mccp antibody-free goats were arranged into 5 groups, and a single dose of every vaccine formulation of a different inactivant was applied to each respective group. The goats were observed for 1 month for the safety and immunogenicity evaluation. All of the inactivation protocols were effective in inactivating the vaccine, and the respective vaccine preparations were safe. While the preparations inactivated by heat and 0.1% formaldehyde showed seroconversion values of equal significance with that of the conventional vaccine ($p > 0.05$), the saponin-inactivated vaccine brought unsatisfactory results. Accordingly, it was concluded that after a field trial and/or challenge study, heat and 0.1% formaldehyde, but not saponin, can be applied as alternative inactivating agents for the CCPP whole culture vaccine, which may improve the vaccine quality and occupational safety.

Keywords: *CCPP, goat, inactivant, vaccine*

1. INTRODUCTION

1.1. Background of the study

Contagious caprine pleuropneumonia (CCPP), also called ‘pleuropneumonie contagieuse caprine’ in France, ‘bou-frida’ in Algeria, and ‘abu-nini’ in Sudan, is a contagious disease that affects goats. It is caused by the bacteria *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp). It is a severe disease of goats occurring in many countries in Africa and Asia (OIE, 2021). In the acute form, it is characterised by a unilateral sero-fibrinous pleuropneumonia with severe pleural effusion (Thiaucourt and Bolske, 1996).

CCPP was first described in 1873 in Algeria. In 1881, the disease was introduced to South Africa by a shipment of Angora goats. It was eradicated by a slaughter policy for the infected goats in combination with a traditional vaccination procedure for the in-contact goats (Hutcheon, 1889). Mccp was then first isolated and shown to cause CCPP in Kenya (MacOwan and Minette, 1976; MacMartin *et al.*, 1980). Subsequently, it has been isolated in Chad, Eritrea, Ethiopia, Niger, Oman, Sudan, Tanzania, Tunisia, Turkey, Uganda, the United Arab Emirates, and more recently in Mauritius (Srivastava *et al.*, 2010), China (Chu *et al.*, 2011), and Tajikistan (Amirbekov *et al.*, 2010).

In mixed herds of goat and sheep, sheep may also be infected, which was verified by isolation of Mccp (Bolske *et al.*, 1996) or detection of anti-Mccp antibodies from clinically sick sheep (OIE, 2021). In addition, Mccp has also been isolated from healthy sheep (Litamoi *et al.*, 1990). This indicates that, sheep has a role as a reservoir for the disease (OIE, 2021). In goats, the disease is manifested by clinical signs such as anorexia, fever, dyspnoea, polypnea, cough, and nasal discharges. The acute/subacute form is characterized by unilateral serofibrinous pleuropneumonia with severe pleural effusion (OIE, 2021).

CCPP imposes significant economic losses to goat farmers over the world (Muheet Malik *et al.*, 2019; Parray *et al.*, 2019; Yatoo *et al.*, 2019a). Large losses (approximately 507 million US dollars) are faced every year in endemic areas due to morbidity, mortality, and loss of production in addition to the costs for the prevention, control, and treatment measures (Yatoo *et al.*, 2018; Parray *et al.*, 2019). In most goat production systems in Africa including Ethiopia, goats are

often important sources of income, and CCPP, therefore, negatively impacts on the ability to provide for children and family needs (Nicholas *et al.*, 2009).

To prevent the spread of CCPP, care should be taken when introducing new animals into the flock, especially in endemic areas. Flock testing, slaughter, and on-site quarantine can help in controlling the spread. In addition, antibiotic treatments and reductions in animal density to decrease contact between animals are sometimes employed. In endemic areas, exotic ungulates that are susceptible should be kept from contact with domestic goats. Vaccination is the most cost-effective technique in the control of CCPP than any other control measures. It has also been helpful in ending some outbreaks among captive wild animals (Manso-Silvan and Thiaucourt, 2019).

Even though few experiments have been performed to develop live vaccines, studies have focused on inactivated preparations containing saponin as an adjuvant. The dose of Mccp protein antigen determined as optimal formulation is 0.15 mg per 1 ml of preparation with 3 mg of saponin adjuvant. It provides immunity for more than a year (Rurangirwa *et al.*, 1987b). The vaccine is inactivated by formaldehyde (King, 1988). It is delivered to target animals subcutaneously in a dose of 1 ml per goat (OIE, 2021). The optimum age for vaccination of the kids should be beyond ten weeks of age due to the fact that maternal antibodies are effective up to eight weeks (King, 1988).

A few CCPP vaccines are being manufactured in Africa by the National Veterinary Institute (NVI) in Ethiopia and the Kenya Veterinary Vaccines Production Institute (KEVVAPI) in Kenya. Similarly, in the Middle East, vaccines are manufactured by the Jordan Bio-Industries Center (JOVAC) in Jordan, by Ibrize in Saudi Arabia, by Vetal in Turkey, and recently, attempts are being made by the Global Alliance for Livestock Veterinary Medicines (GALVmed) in China (Yatoo *et al.*, 2019b).

Even though more recent epidemiological investigations have to be conducted to know the current status of the disease, CCPP has been reported to be prevalent in Ethiopia. Therefore, addressing effective vaccine is necessary to reduce the morbidity and mortality of goats, and thereby to improve the country's economy (AU-IBAR, 2013). It is obvious that the effectiveness of a vaccine is dependent on the mode of the production. The way of inactivation, for instance, is

important in improving vaccine immunogenicity and potency (Jungeblut and Carrel, 1935). Currently, in NVI, as its standard operating procedure directs, the CCPP vaccine is inactivated by formaldehyde in a proportion of 0.5%, which is then adjuvated with saponin. Despite the efficacy of the vaccine in Ethiopia, using saponin and heat inactivation has not yet been well considered. Instead of using saponin both as inactivant and adjuvant simultaneously (Rurangirwa *et al.*, 1987b; OIE, 2014, 2018b, 2021), applying extra formaldehyde for inactivation is not necessary. Rather, this could impose an extra effect on the protein structure of the antigen, and hence affect the immunogenicity of the vaccine. In addition, it is not economical to invest on extra formaldehyde. Furthermore, since formaldehyde reaction is reversible, there is always some amount of residual formaldehyde in vaccines inactivated by it. As it is toxic to eukaryotic cells (Rabiei *et al.*, 2019), the residual formaldehyde needs to be removed, or neutralized with neutralizing agents such as sodium metabisulfite (Nunnally *et al.*, 2015). The other main problem in using formaldehyde for inactivation is that since it is highly volatile, it is toxic to the laboratory workers, causing irritation, dermal allergies, neurotoxicity, pulmonary function damage, nasal and lung cancer, etc. (OSHA, 1993, 2002; Kim *et al.*, 2011; Dan *et al.*, 2020), or it needs extra safety apparatus. In contrast, heat inactivation is relatively safer, and it is cheaper as well.

Since exposure to air leads to the oxidation of formaldehyde to formic acid, formaldehyde is usually formulated with 10–15% methanol to inhibit this change. Alcohols, including methanol, have a characteristic of facilitating clumping (aggregation) of proteins (Cromey, 2012). Hence, it is clear that when inactivated by formaldehyde, Mccp antigens may stick together, affecting the homogeneity of the vaccine in terms of the antigen content. There is also a concern that high formaldehyde concentration affects the immunogenicity of the vaccine through reduction of the antigenic potential of the pathogen by degrading and destructing important epitopes (Nunnally *et al.*, 2015). Hence, instead of using formaldehyde in a minor proportion (0.1%) (Rodriguez *et al.*, 2004), applying such 0.5% may result in this problem. It is also logical that higher amount of formaldehyde leaves higher residue in the vaccine and imposes higher exposure to the laboratory workers. Besides, it is not economical, even though this could be a minor concern.

Based on the above concerns, the study was aimed at addressing the issues by evaluating the safety and immunogenicity of saponin- and heat-inactivated whole culture CCPP vaccine so as to

replace formaldehyde inactivation, and by evaluating that of the vaccine inactivated by 0.1% formaldehyde proportion so as to apply the protocol if saponin and heat inactivation are found to be inappropriate.

1.2. Objectives of the study

1.2.1. General objective

The main objective of this study was to determine the safety and immunogenicity of NVI's whole culture CCPP vaccine inactivated by some alternative inactivating agents. It was aimed to replace formaldehyde inactivation by saponin and/or heat, or otherwise to make use of minor formaldehyde in the inactivation process of the CCPP vaccine.

1.2.2. Specific objectives

- To examine the effectiveness of each of the inactivating agents in inactivating the Mccp F-38 strain.
- To compare the safety and immunogenicity among saponin-, heat-, 0.1% formaldehyde-, and the existing 0.5% formaldehyde-inactivated vaccine preparation.
- To examine the dual effect of saponin: inactivation and adjuvation.

2. LITERATURE REVIEW

2.1. Contagious caprine pleuropneumonia: general overview

Contagious caprine pleuropneumonia (CCPP) is one of the most severe diseases of goats affecting the respiratory tract. This disease is extremely contagious and frequently fatal; in some naïve flocks, the morbidity and mortality rates can reach 100% and 60–70%, respectively. CCPP causes major economic losses where it is endemic, such as Africa, Asia, and the Middle East (Manso-Silvan and Thiaucourt, 2019).

When infected, animals become severely sick. Acutely affected goats generally die within 7 to 10 days (Nicholas *et al.*, 2009). However, when goats are peracutely affected, they usually die within 1 to 3 days with minimal clinical signs (Manso-Silvan and Thiaucourt, 2019). The incubation period is commonly 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 (Nicholas *et al.*, 2009).

2.1.1. Etiology and host range

There has been quite a lot of confusion regarding the exact etiological agent of the disease. It was only in 1976 that a *Mycoplasma* designated as F-38, which was called Mccp later in 1993, was confirmed as the primary cause of CCPP. Mccp belongs to the *Mycoplasma mycoides* cluster in the genus *Mycoplasma* (Nicholas *et al.*, 2009). Another organism in this cluster, *M. mycoides* subsp. *capri* (a species now containing both *M. mycoides* subsp. *capri* and the former *M. mycoides* subsp. *mycoides* large-colony type) can cause a disease that resembles CCPP, but may have extrapulmonary signs and lesions. Some reports consider *M. mycoides* subsp. *capri* to be a minor cause of CCPP; however, the World Organization for Animal Health (OIE) limits the cause of CCPP to only Mccp (Manso-Silvan and Thiaucourt, 2019).

Goats are the primary hosts for Mccp, and the only domesticated animals confirmed to be affected by this organism; however, few studies have reported the occurrence of the organism in healthy or sick sheep. There is also a possibility that this organism might have been involved in an outbreak of acute respiratory disease among goats and sheep in Ethiopia in 2002. Mccp has

caused clinical cases in some wild ruminants including wild goats (*Capra aegagrus*), Nubian ibex (*Capra ibex nubiana*), Laristan mouflon (*Ovis orientalis laristanica*), gerenuk (*Litocranius walleri*), sand gazelles (*Gazella subgutturosa marica*), Arabian oryx (*Oryx leucoryx*), and Tibetan antelope (*Pantholops hodgsonii*) with significant morbidity and mortality (Manso-Silvan and Thiaucourt, 2019).

2.1.2. Clinical signs

Clinical manifestations of CCPP include fever (body temperature reaches 41–43°C/106–109°F), coughing, anorexia, dyspnoea, polypnea, nasal discharges, and severe respiratory distress. The cough is frequent and violent. In the final stages of the disease, the goat may lose its ability to move, and it stands with its front legs wide apart. Its neck will be stiff and extended. Saliva can drip continuously from the mouth, and the animal may grunt or bleat in pain. Furthermore, frothy nasal discharge and stringy saliva may be seen. Abortion can occur in pregnant goats (Nicholas *et al.*, 2009).

Subacute or chronic cases are milder, with coughing mainly following activity. The chronic form of CCPP is characterized by a chronic cough, nasal discharge, and debilitation. Infected animals become very sick and mostly die (Nicholas *et al.*, 2009). CCPP can also be confused with peste des petits ruminants (PPR) or pasteurellosis. However, CCPP is suspected when lesions are restricted to the respiratory tract; only one lung is affected; and when animals present a conspicuous pleurisy with profuse effusion of pleural fluid (Nicholas *et al.*, 2009).

Post-mortem lesions can be considered as clinical signs, especially for diagnostic purposes. The lesions of CCPP are limited to the respiratory system. The acute form of the disease is characterized by unilateral or bilateral pneumonia and serofibrinous pleuritis with straw-colored fluid in the thorax. On cut surface, the lung is granular with copious straw-colored exudate. Pea-sized, yellow nodules surrounded by areas of congestion may be present in the lungs. Varying degrees of lung consolidation or necrosis can be seen, and the regional (bronchial) lymph nodes are enlarged. Some long-term survivors have chronic pleuropneumonia or chronic pleuritis, with encapsulation of acute lesions and numerous adhesions to the chest wall. The interlobular septa are not usually thickened in domestic goats (Manso-Silvan and Thiaucourt, 2019).

2.1.3. Prevalence and economic importance

CCPP is prevalent in many countries in Africa, Asia, and the Middle East. 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 (Manso-Silvan and Thiaucourt, 2019). More recently, CCPP has also emerged in newer areas, like Afghanistan, Mauritius, Tajikistan, Pakistan, India, China, Saudi Arabia, and Qatar. It has showed an increased incidence of outbreaks in prevalent areas like Ethiopia, Kenya, Tanzania, Turkey, and the African Union (Yatoo *et al.*, 2019a).

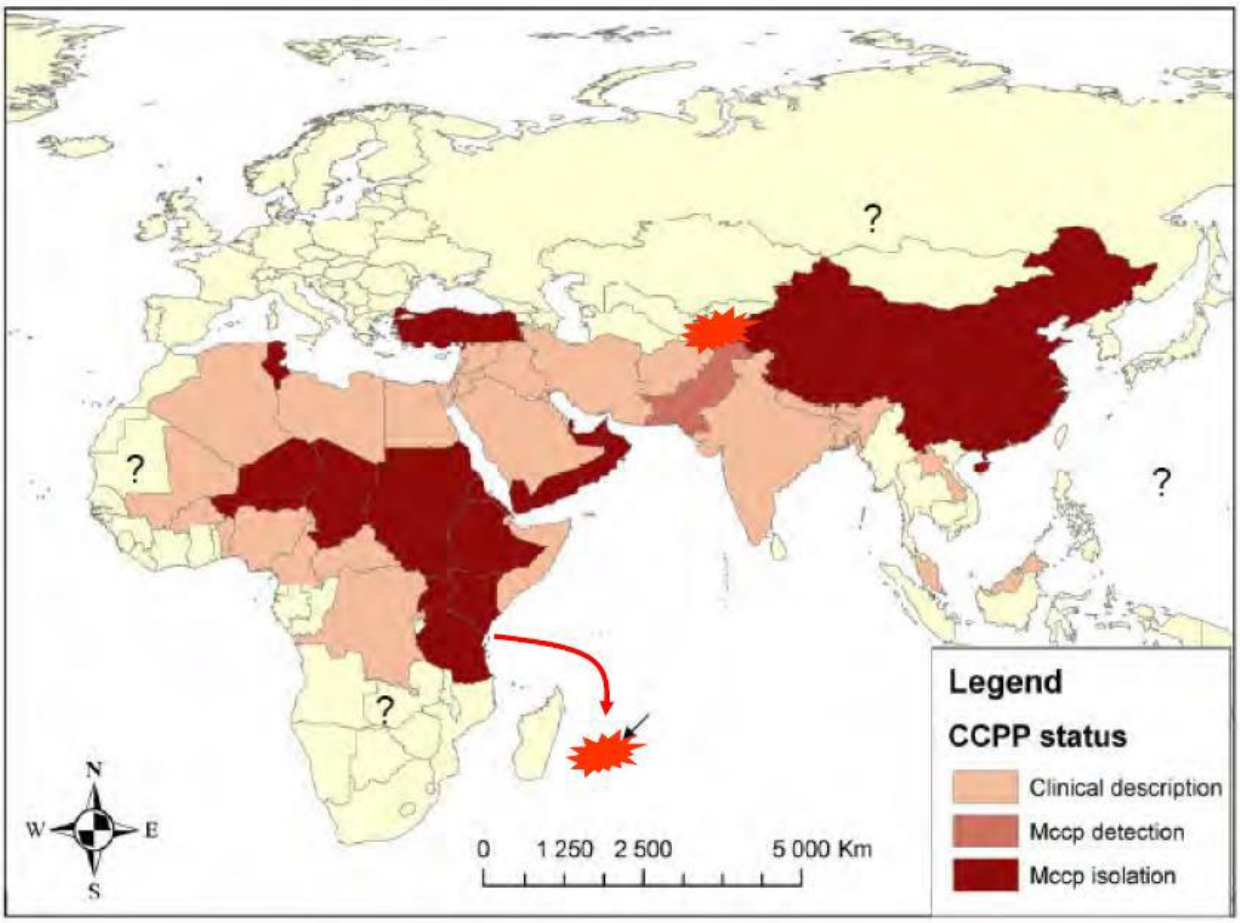

Figure 1: CCPP distribution: the spiked areas show the relatively recent outbreaks in Mauritius and Tajikistan (IDRC, 2016)

In most goat production systems in Africa, goats are often important sources of income providing meat, milk, and hides, and CCPP, therefore, has negative impacts on the ability to

provide for children and family needs (Nicholas *et al.*, 2009). CCPP causes significant economic loss to goat farmers worldwide (Muheet Malik *et al.*, 2019; Parray *et al.*, 2019; Yatoo *et al.*, 2019a). About 507 million US dollars are lost every year in endemic areas due to morbidity, mortality, and loss of production in addition to the costs for the prevention, control, and treatment measures (Yatoo *et al.*, 2018; Parray *et al.*, 2019).

CCPP has been mentioned as the most serious infection of goats in East Africa. It imposes direct and indirect effects on the goat production system. Direct losses are associated with the high morbidity and mortality rates, which result in loss of income from sales of live animals and goat products as well as decreased productivity (reduced weight gains and decreased milk production). The costs for diagnosis, treatments, and control all have a direct effect on the economy. The indirect losses are associated with the extra costs for treatments, risks associated with antibiotic residues, reproductive wastage, and trade restrictions (IDRC, 2016).

The status of the disease in Ethiopia

In Ethiopia, the occurrence of CCPP was suspected since 1980 and confirmed in 1990, since which the disease became endemic in different regions of the country. Repeated outbreaks have been reported in Tigray, Afar, Dire Dawa, Southern Region, Oromiya, Benishangul Gumuz, and Amhara. It has been reported to be more prevalent in the arid and semi-arid low land pastoral areas of the Rift Valley, Borena, South Omo, and Afar, which are home to about 70% of the country's goat population (IDRC, 2016).

According to the CCPP outbreak reports from 2007 to 2011, the highest number of outbreaks was from Oromiya, Afar, Somali, and Southern Region, which are the areas that border Kenya, Somalia, and South Sudan. This phenomenon is attributed to the pastoral (extensive) nature of the rearing system; animals move freely within the regions and across the borders. Even though the outbreak reports alone do not give the true picture of the disease prevalence, their trends, when combined with serological studies and discussion with the stakeholders, gave a clear indication that CCPP is highly prevalent in the mentioned areas (IDRC, 2016).

Even though it is usually reported that goats are mostly affected by the disease, CCPP also affects sheep, but not much frequently (OIE, 2021). For instance, in their study conducted in Borena Zone, Teshome *et al.* (2019) and Ayelet *et al.* (2007a) reported a prevalence of 12.9%

and 7.4%, respectively. There is also a report showing prevalence of CCPP in sheep (18.3%) in Kefta Humera, Alamata (Tigray), and Aba'ala (Afar) (Hadush *et al.*, 2009), providing evidence for the possible role of sheep in the epidemiology of the disease.

Table 1: Prevalence of CCPP in goats in Ethiopia

Area	Prevalence (%)	Reference
Borena	31.2	Teshome *et al.* (2019)
West Amhara	8.5	Abrhaley *et al.* (2019)
Dassenech (South Omo)	87.0	Molla and Delil (2015)
Gambella	18.1	Fasil *et al.* (2015)
Borena	31.6	Lakew *et al.* (2014)
Dubti and Hadar (Afar)	14.6	Peyraud *et al.* (2014)
Dire Dawa	4.92	Yousuf *et al.* (2012)
Jijiga	32.6	Sheirf *et al.* (2012)
Borena and Guji	13.2	Bekele *et al.* (2011)
Afambo, Assayta, Dubti, Mille, Gewane, Amibara, Dewe, and Telalak (Afar)	22.5	Regassa *et al.* (2010)
Southern Ethiopia	18.6	Mekuria and Asmare (2010)
Kefta Humera, Alamata (Tigray), and Aba'ala (Afar)	32.7	Hadush *et al.* (2009)
Elfora export abattoir	48.3	Gizaw *et al.* (2009)
Hammer and Bennatsemay (South Omo)	15.5	Mekuria *et al.* (2009)
Borena	19.1	Ayelet *et al.* (2007a)
Abattoir receiving animals from Borena, Bale, Afar, and Jinka	31.0	Eshetu *et al.* (2007)
North Shoa and Wollo	23.0	Sharew *et al.* (2005)
Afar, Yabello, Metehara, Arsi, Borena, Konso, and Awash	60.0	Sharew *et al.* (2005)
Arsi	52.7	Yigezu *et al.* (2004)

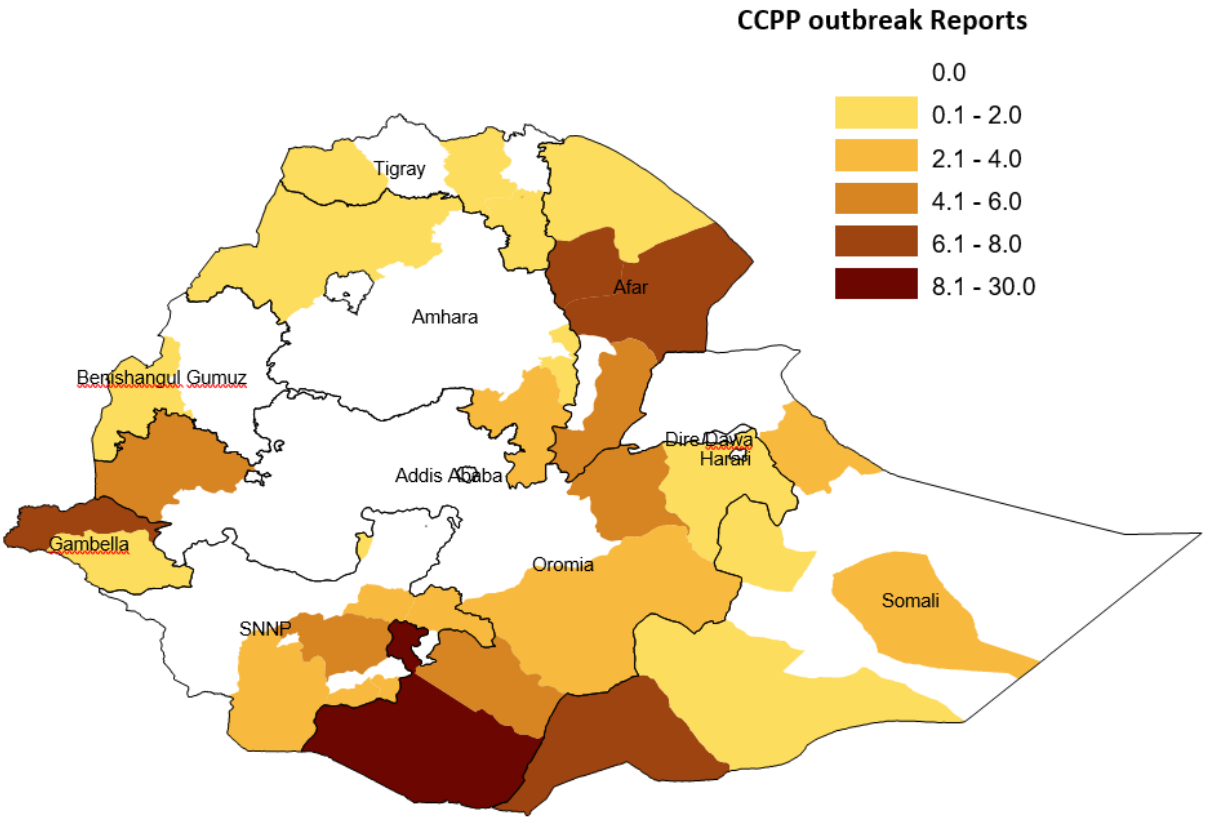

Figure 2: CCPP outbreaks (%) in Ethiopia from 2007 to 2011 (IDRC, 2016)

2.1.4. Diagnostic techniques

The diagnosis of respiratory disease in goats, particularly CCPP, is complicated, especially where it is enzootic (OIE, 2021). It has to be differentiated from other similar respiratory diseases such as PPR, pasteurellosis, and contagious agalactia syndrome (Thiaucourt and Bolske, 1996; Nicholas and Churchward, 2011).

Microscopy

Histopathologically, Mccp is characterised by an interstitial pneumonia with interstitial, intralobular oedema of the lung (Kaliner and MacOwan, 1976). When observed under dark-field microscopy *in vivo* in exudates or tissue suspensions from lung lesions or pleural fluid, it shows a branching filamentous morphology. Alternatively, smears prepared from lung lesions can be stained by the May-Grünwald-Giesma method and examined by light microscopy. The other caprine *Mycoplasma* exhibit a short filamentous or coccobacillary morphology. However, neither of these techniques provides a definitive diagnosis (OIE, 2021).

Culturing

The necropsy samples to be taken include lung lesions (particularly from the interface between consolidated and unconsolidated areas), pleural fluid, and mediastinal lymph nodes (OIE, 2021). Media used to culture Mccp include goat liver broth and goat serum (MacOwan and Minette, 1976); WJ medium (Jones and Wood, 1988); modified Hayflick's medium and modified Newing's tryptose broth (Kibor and Waiyaki, 1986). Media enriched with 0.2–0.8% sodium pyruvate perform considerably better, both for primary isolation and antigen production (Mohan *et al.*, 1990; Thiaucourt *et al.*, 1992). Pleuropneumonia-like organisms (PPLO) broth with horse (alternatively pig or donkey) serum is also used for isolation of Mccp (OIE, 2021).

Mccp cultures are incubated at 37°C. It is best to incubate plate cultures in a humid atmosphere of 5% CO_2, 95% air or nitrogen gas, or in a candle jar with a source of moisture. The cultures can also be incubated anaerobically. Broth cultures should be examined daily for evidence of growth: color change and the appearance of floccular material, whereas plate cultures should be examined up to 15 days at every 1–3 days interval using a stereo microscope. In broth, growth of Mccp is visible within 4–15 days. Sometimes, Mccp exhibits comet-like growth in unshaken broth cultures. Turbidity is always very faint; if there is gross turbidity, it indicates bacterial contamination (OIE, 2021).

Colony morphology of Mccp varies with the medium used, its passage level, and the age of the culture. In early stages, many *Mycoplasma* species, including Mccp, produce colonies of bizarre morphology, often small, centreless, and of irregular shape. This phenomenon is often associated with the use of marginally suitable medium. When passaged, on agar media, the isolates demonstrate conventional 'fried egg' colonies of size 0.1–0.5 mm (OIE, 2021).

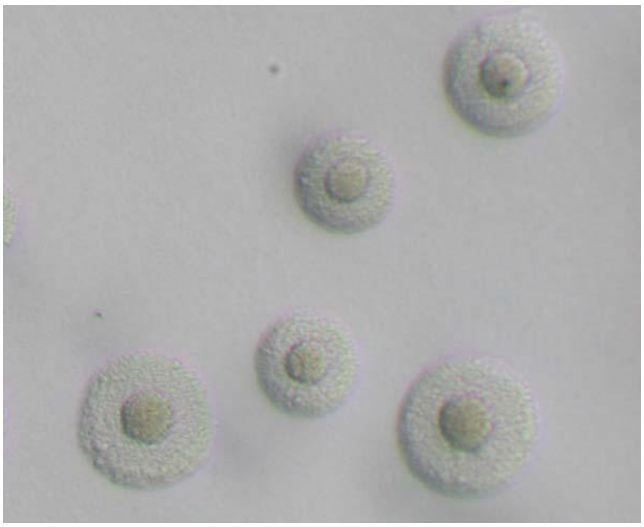

Figure 3: Colonies of *Mycoplasma* under a stereomicroscope (Waites *et al.*, 2013)

Biochemical test

The most commonly used biochemical tests are glucose breakdown test (positive for Mccp), arginine hydrolysis test (negative for Mccp), 'film and spots' formation test (negative for Mccp), test for reduction of tetrazolium chloride aerobically and anaerobically (+/++ for Mccp), phosphatase activity test (negative for Mccp), serum digestion test (negative for Mccp), and digitonin sensitivity test (positive for Mccp) (OIE, 2021).

Gel precipitin test

Gel precipitin test helps to detect Mccp antigen in tissue specimens (OIE, 2021). Mccp has been reported to release an antigenic polysaccharide to which a specific monoclonal antibody (mAb) (WM-25) is produced (Rurangirwa *et al.*, 1987c). The mAb immunoprecipitates with the polysaccharide in an agar gel. The test is used particularly when specimens are no longer suitable for culture because of deterioration during transit. It is possible to replace the mAb by goat CCPP convalescent sera provided they are precipitating, i.e. they contain IgMs. This test is not completely specific, and could give some crossreactions, notably with *M. leachii* (OIE, 2021).

Growth inhibition test

Among the diagnostic tests available, the growth inhibition test (GIT) is the simplest and most specific test. The principle of GIT is direct inhibition of growth on a solid medium by a specific hyperimmune serum, and it primarily detects surface antigens (OIE, 2021).

Serologically, Mccp is highly homogeneous, and wide zones of inhibition free of 'breakthrough' colonies are observed with antiserum to the type strain, regardless of the source of the test strain (Jones and Wood, 1988). An mAb specific for Mccp in the GIT has been produced as Mccp cross-reacts with *M. leachii* (PG-50), *M. equigenitalium*, and *M. primatum* in the GIT when polyclonal antisera are used (Rurangirwa *et al.*, 1987c). The mAb reagent, WM-25, is specific for Mccp by the disc growth inhibition method, which will exclude *M. agalactiae*, *M. capricolum* subspecies *capri* (Mcc), and the other members of the *Mycoides* cluster associated with goats. A small proportion of Mccp isolates also cross-react with antiserum to Mcc (OIE, 2021).

Fluorescence antibody test

Fluorescent antibody tests (FAT), including direct and indirect, are the most effective of the various serological methods for identifying most *Mycoplasma*. They are simple, rapid, sensitive, and economical. The best and most commonly used is the indirect fluorescent antibody test (IFAT) applied to unfixed colonies on agar. Antiserum against a single strain is sufficient to identify field isolates of that species, and antisera are diluted before use (OIE, 2018b).

Complement fixation test

Seroconversion to Mccp in experimentally infected animals is observed by the complement fixation test (CFT) to start 7–9 days after the appearance of clinical signs, to peak between days 22 and 30, and to decline rapidly thereafter (OIE, 2021). As many *Mycoplasma* belonging to the *Mycoides* cluster are expected to induce cross-reactions, it is recommended to perform additional tests when finding CFT-positive titres in a CCPP-free region. Suspicious sera should be tested in parallel with antigens prepared with various *Mycoplasma* species, most importantly *M. capricolum*, *M. mycoides* subsp. *mycoides*, *M. leachii*, and *M. mycoides* subsp. *capri*. The

antigen yielding the highest titre should indicate which species was infecting the animal/herd (OIE, 2021).

Latex agglutination test

Latex agglutination test (LAT) is a rapid, specific, and relatively sensitive test developed in Kenya. It uses a carbohydrate extracted from Mcc linked to latex particles, which agglutinate in the presence of specific antibodies in the blood of affected goats (March *et al.*, 2000). Latex beads sensitized with the Mccp polysaccharide present in the culture supernatant are used in a slide agglutination test (Rurangirwa *et al.*, 1987a).

LAT is very useful in time of an outbreak, because it can be performed at the penside using a drop of whole blood. March *et al.* (2000) also described a LAT for circulating antigen, which could provide earlier detection in affected animals before antibodies appear. This test is sensitive at an early stage of the disease as long as IgM persists in the serum. It has no a well-characterized specificity. Cross-reactions may occur as polysaccharides of Mccp are similar to that of *M. leachii* and *M. capricolum* subsp. *capricolum* (Bertin *et al.*, 2015) and some other bacteria (OIE, 2021).

Competitive enzyme-linked immunosorbent assay

Competitive enzyme-linked immunosorbent assay (c-ELISA), a type of enzyme-linked immunosorbent assay (ELISA), has been proved as both specific and sensitive. This test has been reformatted as a kit containing pre-coated plates and ready-made reagents, including mAb 4/52. The new c-ELISA kit has been re-validated to establish its cut-off value, a percentage inhibition (PI) value of 55%, to obtain 99.9% specificity. It allows the detection of positive sera in CCPP-infected herds. Due to the fact that it is highly specific, it can be applied to evaluate herd status using targeted sampling of recovered animals in the tested herds, which should greatly enhance its sensitivity without any problem in the specificity (OIE, 2021).

As the seroconversion measured one and two months post-vaccination is proportional to the Mccp antigen, c-ELISA can be applied to evaluate the quality of a CCPP vaccine. However, the correlation between c-ELISA titre and protection efficacy is not established (OIE, 2021).

Polymerase chain reaction and sequencing

Molecular techniques are used for the rapid and specific identification of Mccp. Polymerase chain reaction (PCR) and sequencing can be used to establish the molecular epidemiology of CCPP (OIE, 2021). In addition, a multilocus sequence analysis (MLSA) method is available (Manso-Silvan *et al.*, 2011). MLSA revealed two main lineages comprising five groups from a representative set of Mccp strains. Because of its rapidity and reliability, identification of Mccp strains by PCR has now superseded all the other techniques. However, since PCR reaction is sensitive to contamination, it must be performed with great care (OIE, 2021).

For the specific identification of Mccp, two PCR assays have been published. The first one is based on the 16S ribosomal ribonucleic acid (rRNA) gene amplification. The PCR product is then analyzed by restriction enzyme cleavage for the identification of the Mccp amplicon (Bascunana *et al.*, 1994). The second PCR assay, on the other hand, is based on a specific amplification (Woubit *et al.*, 2004). These PCR techniques can be used directly on lung tissue, pleural fluid, and other clinical materials (Bolske *et al.*, 1996). Even though isolation of Mccp remains the confirmatory test, PCR is the preferred technique for the diagnosis of CCPP since isolating Mccp is so difficult (OIE, 2021).

2.1.5. Control and prevention

Disease reporting: a quick response is vital for containing outbreaks in CCPP-free regions. Veterinarians should follow national and/or local guidelines for disease reporting. It is important to report any cases and suspects timely in order to address the preventive measures before the disease spreads (Manso-Silvan and Thiaucourt, 2019).

Due to the poor survival of *Mycoplasma* in the environment, CCPP is most likely to enter a country through infected animals. CCPP outbreaks can be eradicated through flock testing, on-site quarantine, movement controls, slaughter of infected and exposed animals, and cleaning and disinfection of the premises. In endemic areas, exotic ungulates that are susceptible should be kept from contact with domestic goats. Furthermore, reductions in animal density (to decrease contact between animals) can be sometimes employed (Manso-Silvan and Thiaucourt, 2019). Antibiotic treatments can also be considered; antibiotics such as tetracyclines, fluoroquinolones,

and the macrolide family are generally clinically effective if used early enough. In remote areas and/or nomadic herds, long-acting formulations are preferred in order to achieve a complete treatment (Nicholas *et al.*, 2009).

Vaccination is the most cost-effective technique in the control of CCPP than any other control measures. It has also been helpful in ending some outbreaks among captive wild animals (Manso-Silvan and Thiaucourt, 2019). Most vaccines against CCPP are available in the form of inactivated preparations (Rurangirwa *et al.*, 1987b).

2.2. Vaccines against contagious caprine pleuropneumonia

Initially, goats were vaccinated with lung extracts obtained from infected animals subcutaneously (Hutcheon, 1889), and attenuated high passage broth cultures of *Mycoplasma* strain F-38 were used (MacOwan and Minette, 1978). In the beginning stages of immunity studies for CCPP (Muthomi and Rurangirwa, 1983; Perreau *et al.*, 1984), there was always a risk of infection associated with the use of live Mccp antigens. Hence, inactivated and attenuated preparations of Mccp organisms were started to be developed (Rurangirwa *et al.*, 1984; Rurangirwa *et al.*, 1987b; Litamoi *et al.*, 1989).

MacOwan and Minette (1978) developed an attenuated live vaccine, but it did not progress. Since then, a number of different preparations have been produced, including a vaccine composed of sonicated antigens that did not progress either (Nicholas *et al.*, 2009). Despite such and other few experiments have been performed to develop live vaccines, studies have focused on inactivated preparations (Rurangirwa *et al.*, 1987b). CCPP vaccine is inactivated by formaldehyde (King, 1988). A 1 ml of the vaccine preparation should contain a dose of 0.15 mg Mccp protein antigen with 3 mg of saponin as an adjuvant (Rurangirwa *et al.*, 1987b).

Quantification of the Mccp protein antigen is used for batch potency evaluation of the final vaccine. The assay that has been used for Mccp antigen quantification in the CCPP vaccine is the bicinchoninic acid (BCA) assay. This assay is used to quantify the total protein in a sample, and is not specific to the Mccp antigen. Contaminants that may be present in the final product may affect the amount of the Mccp antigen evaluated in the vaccine, which is one of the challenges for the evaluation of the potency of the CCPP vaccine (OIE, 2021). Baziki *et al.* (2020)

developed and evaluated an immuno-capture ELISA (ICE) using an mAb as a capture for the specific detection and quantification of the Mccp protein antigens in the CCPP vaccine.

One ml of the formulated vaccine is delivered per goat subcutaneously (OIE, 2021). The optimum age for vaccination of the kids is recommended to be beyond 10 weeks of age due to the fact that maternal antibodies are effective up to eight weeks (King, 1988). The vaccine has been proven to provide immunity for more than one year (Rurangirwa *et al.*, 1987b). The inactivated Mccp strain F-38 saponin-adjuvated vaccine showed full (100%) protection against natural CCPP infection (Litamoi *et al.*, 1989).

2.2.1. Candidates of contagious caprine pleuropneumonia 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. Saponin and IFA were similar in their immune-potentiating ability, and were superior to aluminum hydroxide (Rurangirwa *et al.*, 1984). Formalinized (formalin-inactivated) vaccine of the F-38 strain is administered as 1 ml per goat. The optimum age for vaccination is beyond 10 weeks (King, 1988). The immune response, typically the humoral immune response, to polysaccharide vaccines of strain F-38 was evaluated by Rurangirwa *et al.* (1990).

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 (Rurangirwa *et al.*, 1997). The Mccp strain 19/2 is used to produce subunit, capsular polysaccharide (CPS), and immunodominant core proteins vaccine (March and Jones, 1998; March *et al.*, 2000; March *et al.*, 2002). Furthermore, a Kenyan isolate of Mccp was used to produce live vaccine, and showed absence of any post-vaccination reaction. It also resulted in early appearance and longer persistence of antibodies. However, chance of disease outbreaks was suggested (Tarekegn *et al.*, 2012).

2.2.2. Contagious caprine pleuropneumonia vaccine status in Ethiopia

Ayelet *et al.* (2007b) conducted a study to validate the immunity induced by inactivated F-38 antigen adjuvated 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-adjuvated CCPP vaccine while only 20% of the goats in ISA 50-adjuvated group showed local reaction. Based on the seroconversion assessment, they reported as saponin-adjuvated 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 ISA 50-adjuvated vaccine. Among the vaccinated groups, of CCPP + anthrax + saponin was reported to show better protection. The study disclosed that inactivated CCPP vaccine adjuvated 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.

OIE (2021) explains 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, Tesgera *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. They also stated that it, rather, is easier for production; 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.

2.3. Methods of inactivation for killed vaccines

Bacterial vaccines had been used to treat allergic and non-allergic rhinitis since 1913. They can be classified into different groups: live attenuated vaccines, killed whole cell vaccines, toxoids, and subunit vaccines. The inactivated vaccines contain killed bacterial cells. The inactivation is achieved by chemical, heat, or radiation treatment of the pathogenic bacteria (Giesker and Hensel, 2014). Inactivation of the pathogen by heat or chemical destroys the pathogen's ability to replicate, but keeps it intact so that the host's immune system can still recognize it. It is critical to maintain the epitope structure on the antigen during inactivation (Dai *et al.*, 2019).

For the inactivation of bacterial cells and preserve their surface structures, so that the host's immune system can still recognize them and produce responses against them, different fixation methods have been demonstrated, such as inactivation by chemical agents (Urbaniak *et al.*, 2014), heat (Cantwell and Kelso, 1981), sonication (Chandrasekaran *et al.*, 2006), and UV irradiations (Wang *et al.*, 2016). Researches indicate that the amount of resistance to each of these methods is different between Gram-negative and Gram-positive bacteria. In addition, based on images acquired by atomic force microscopy (AFM), after fixation, the morphology of the bacterial cells is different depending on the method utilized (Rabiei *et al.*, 2019).

2.3.1. Heat inactivation

Heat (thermal) inactivation is one of the most historical and important preservation methods. It is proven that microorganisms are more sensitive to wet (moist) heat than to dry heat. The main mechanisms of wet heat inactivation are posing damage to the cell membranes; degrading nucleic acids; and denaturing enzymes and other essential proteins. On the other hand, oxidation of cell constituents and protein denaturation are more likely in dry heat inactivation (Cantwell and Kelso, 1981).

The inactivation temperature and duration vary depending on the type of the pathogen to be inactivated, usually 56–80°C and from 30 minutes to 16 hours, respectively (Tola *et al.*, 1999; Rodriguez *et al.*, 2004). As a non-thermal alternative to conventional thermal approaches, sonication is mostly used coupled with pressure and/or heat for microbial inactivation. Since spores of bacteria are relatively resistant to this method, prolonged period of ultrasonication is required (Chandrasekaran *et al.*, 2006).

2.3.2. Chemical inactivation

The most widely used chemical fixatives include 2.5% glutaraldehyde, 5% binary ethylenimine (BEI), 10% formalin, 4% paraformaldehyde, methanol, acetone, ethanol, and acetic acid solutions. All of these solutions are appropriate in preserving bacterial cell morphology, but aldehyde-based solutions are preferred to alcohol ones, because alcohol results in detachment of the surface ultrastructures (pilli and flagella). Chemical inactivation by formaldehyde or formalin has been successful (Urbaniak *et al.*, 2014; Dai *et al.*, 2019). According to Rurangirwa *et al.*

(1987b) and OIE (2021), saponin also acts as an inactivating agent for Mccp and as an adjuvant for the inactivated CCPP vaccine.

Some common chemical inactivants and their mechanism of action

i) Formaldehyde

Formaldehyde is the most widely used inactivant for vaccine production purposes (Nunnally *et al.*, 2015). It is used as a sterilant (inactivant) in both its liquid and gaseous forms (Tulis, 1972). It is commercialized and used principally as a water-based solution called formalin, which is 37% formaldehyde weight by volume (w/v), i.e. 37% w/v formaldehyde is equivalent to 100% formalin. The aqueous solution of formaldehyde is a bactericide, tuberculocide, fungicide, virucide, and sporicide (Klein and DeForest, 1963).

Long-term exposure to formaldehyde to low levels in the aerosol or on the skin can cause asthma-like respiratory problems and skin irritations, such as dermatitis and itching. Due to these reasons, laboratory workers should have limited contact with formaldehyde. These considerations limit its role in inactivation, sterilization, and disinfection activities (OSHA, 1993).

Regarding the mechanism of action, formaldehyde inactivates microorganisms by alkylating the amino and sulfhydral groups of proteins and ring nitrogen atoms of purine bases in the nucleic acids (Favero and Bond, 1991). Formaldehyde has also been shown to form amino acid (primarily the residues of the basic amino acid lysine) and protein-deoxyribonucleic acid (DNA) cross-links by creating covalent bonds in between; protein-DNA cross-links inhibit DNA synthesis (Permana and Snapka, 1994). Different concentrations of aqueous form of formaldehyde act on a wide range of microorganisms. Poliovirus can be inactivated by an 8% concentration of formalin in 10 minutes, but all the other viruses are found to be inactivated by 2% formalin (Klein and DeForest, 1963). Four percent formaldehyde is a tuberculocidal agent, inactivating 10^4 *Mycobacterium tuberculosis* in two minutes (Rubbo *et al.*, 1967), and 2.5% formaldehyde inactivates about 10^7 *Salmonella typhi* in 10 minutes in the presence of organic matter (McCulloch and Costigan, 1936). In comparative sporicidal action tests with 4% aqueous formaldehyde and 2% glutaraldehyde against the spores of *Bacillus anthracis*, formaldehyde is

slower than glutaraldehyde. To achieve an inactivation factor of 10^4, formaldehyde requires two hours of contact, while glutaraldehyde requires only 15 minutes (Rubbo *et al.*, 1967).

Methods of inactivation by formaldehyde vary greatly between vaccines. The differences include formalin concentration (from 0.08% to 0.009% w/v), time of inactivation (from days to months), and temperature (usually 37°C). Generally, higher formalin concentration and temperature result in faster inactivation, but higher formalin concentration may affect the immunogenicity of the vaccine through degradation and destruction of important epitopes. The inactivation time must be sufficient to ascertain complete inactivation, but it should not be too long as to destroy the immunogenicity of the vaccine. Therefore, the immunogenicity of the inactivated sample should be monitored during the inactivation process to ensure no loss of the antigenic potential (Nunnally *et al.*, 2015).

ii) Beta-propiolactone (BPL)

BPL is widely used for the inactivation of viruses. It is mostly used in the production of influenza and rabies vaccines. The rapid hydrolysis into non-toxic and non-carcinogenic products will completely eliminate BPL levels from the reaction within 2 hours at 37°C. This is the main advantage of BPL inactivation over that of formaldehyde, where residual formalin must be removed. All of the BPL reactions are rapid and stable. Alkylation or acylation reactions with interacting nucleophiles (including nucleotides), which are repeatedly present in large biological molecules such as nucleic acids, are irreversible (Nunnally *et al.*, 2015).

The primary mechanism of action by which BPL inactivates pathogens is direct interaction with nucleic acids (Mate *et al.*, 1977). BPL mainly reacts with the nitrogen-7 atom of guanosine, and to a lesser extent with adenosine at the N-1 position (Hemminki, 1981). Then, the BPL-modified guanine is misread by the polymerase as an adenine, therefore for every alkylated guanosine, a GC-AT transition mutation is incorporated (Segal *et al.*, 1981). These mutations in combination with BPL-induced DNA double helix cross-linking (Perrin and Morgeaux, 1995) can make the genomes dysfunctional, making the pathogen unable to replicate, which in turn leads to its complete inactivation (Nunnally *et al.*, 2015). As BPL primarily interacts with DNA or RNA, it is thought that the immunogenic epitopes of the protein would remain intact, which entails that BPL-inactivated pathogens would maintain their high immunogenicity (Nunnally *et al.*, 2015).

The impact of BPL on the immunogenicity of the vaccine varies with the type of the pathogen, but is lower than that of formalin as BPL interacts with protein moieties to a lesser extent (Nunnally *et al.*, 2015). Its performance in the inactivation process is directly correlated with its initial concentration, the nature of the pathogen, and the temperature (Budowsky *et al.*, 1991). Generally, these parameters are set at 4°C for 18–24 hours with a BPL concentration of 0.1–0.25% (Lawrence, 2000).

When compared to formaldehyde inactivation, which needs days to months, BPL inactivation takes significantly shorter time: minutes to hours. The other advantage of inactivation by BPL is the lower inactivation temperature, which prevents thermal degradation of important epitopes. Moreover, protein moieties are less likely to be altered by BPL as its primary reaction is with nucleic acids. Despite the evident advantages of BPL, formaldehyde, rather, is more widely used for inactivation, perhaps due to its historical use and years of experience which has paved the regulatory pathways for the licensure (Nunnally *et al.*, 2015).

iii) Binary ethylenimine (BEI)

BEI is an aziridine compound (Delrue *et al.*, 2012) that has been used to inactivate mainly foot-and-mouth disease virus (Bahnemann, 1975; Sarkar *et al.*, 2017) as well as many other viruses such as infectious bursal disease virus (Habib *et al.*, 2006), rabies virus (Mondal *et al.*, 2005), Newcastle disease virus (Razmaraii *et al.*, 2012), and Japanese encephalitis virus (Yang *et al.*, 2012). It is preferred over BPL for the inactivation of rabies and other viruses in veterinary vaccine production. It inactivates rabies virus very effectively, and the rabies tissue culture vaccine inactivated with BEI is potent and stable (Larghi and Nebel, 1980). At a concentration of 0.1 M, BEI (1.5–5.0%) has also been reported to inactivate some *Mycoplasma* (Rodriguez *et al.*, 2004).

BEI reacts with nucleic acids of pathogens while maintaining the conformation and accessibility of the epitopes to a much greater degree than formaldehyde and BPL do (Delrue *et al.*, 2012). It inactivates viruses by binding mainly to guanine nucleotide, or to small amount of adenine nucleotide, in DNA or RNA molecules to form alkylated nucleotides, without interfering with the antigenicity of the antigen (Delrue *et al.*, 2012). The parameters for inactivation by BEI vary with the type of the pathogen to be inactivated. For instance, at a temperature of 37°C, a 5%

proportion of 0.1 M of BEI inactivates *Mycoplasma*, even without a significant protein damage (Rodriguez *et al.*, 2004), while its 1.6 mM concentration inactivates rabies virus (Mondal *et al.*, 2005).

BEI is inexpensive and easy to prepare, as well as the inactivation protocol is simple (Razmaraii *et al.*, 2012). Since it does not react with protein moieties, the BEI-inactivated vaccines of many DNA and RNA viruses have been reported to be more antigenic (give higher antibody titers) than those inactivated by formaldehyde (Kai and Chi, 2008). However, like BPL and formaldehyde, BEI is a carcinogenic agent. Hence, many vaccine producers have been directing their research to find a natural agent (such as ascorbic acid) that provides a safe and complete inactivation (Rawatt *et al.*, 1995; Madhusudana *et al.*, 2004).

iv) Saponins

Saponins, also referred specifically to as triterpene glycosides, are toxic plant-derived organic chemicals that have a foamy characteristic when agitated in water (Hostettmann and Marston, 1995). They include chemicals like glycyrrhizin, hederaginin, quillaia (aka quillaja), and Quil-A (Hostettmann and Marston, 1995; Lorent *et al.*, 2014). Since they are amphipathic (amphiphilic), they act as surfactants with a potential ability to interact with cell membrane components, such as cholesterol and phospholipids (Lorent *et al.*, 2014). Saponins have also been used as immune-potentiating (immune stimulator) adjuvants in the development of vaccines, especially for subunit vaccines and vaccines directed against intracellular pathogens (Sun *et al.*, 2009), even though their mechanism of action is not well understood (Ahlberg *et al.*, 2017).

Tensoactivity and many other biological properties of saponins have been ascribed to their action on membranes (Babu *et al.*, 1997; Bottger *et al.*, 2012; Gilabert-Oriol *et al.*, 2013). Studies about saponin action on membranes demonstrate that saponins have a membrane lytic activity (Baumann *et al.*, 2000). The lytic action is due to the interaction of saponin aglycone with membrane sterols (particularly cholesterol and ergosterol), and it leads to the rearrangement of membrane lipids, formation of long-lasting pores in the membranes, permanently increased cell membrane permeability, and finally lysis of cells (Bangham *et al.*, 1962; Simons *et al.*, 2006). Saponins also have the ability to affect the fluidity of the cell membrane. They can increase ATPase activity through their interaction with the membrane cholesterol, displacing it from the

immediate environment of ATPases. Removal of cholesterol from the cell membrane results in an increased membrane fluidity which can facilitate conformational changes that ATPases undergo during their transport activity (Sparg *et al.*, 2004). The effect of saponins on the membrane fluidity may alter ion transport, and activity of membrane proteins and enzymes, which shows the mechanism of action of saponins on cellular functions (Ma and Xiao, 1998). However, there are some microorganisms that have enzymes with an anti-saponin activity. These enzymes hydrolyze saponins, and avoid their antimicrobial activity, making inactivation by saponin difficult (Bernards *et al.*, 2011).

According to Rurangirwa *et al.* (1987b), Rodriguez *et al.* (2004), and OIE (2014, 2018b, 2021), saponins (3 mg/ml) can be used as inactivating agents for *Mycoplasma* in vaccine production. These authors reported that at a temperature of 37ºC and within a duration of 24 hours, they effectively inactivate some *Mycoplasma*.

v) Phenols

Published reports show that phenols are bactericidal, fungicidal, virucidal, and tuberculocidal compounds (Prindle, 1983; Goddard and McCue, 2001). A 0.5% dilution of phenol (2.8% ortho-phenylphenol and 2.7% ortho-benzyl-para-chlorophenol) inactivates HIV (Martin *et al.*, 1985), and a 2% solution of phenol (15% ortho-phenylphenol and 6.3% para-tertiary-amylphenol) inactivates most fungi (Terleckyj and Axler, 1987). According to Rodriguez *et al.* (2004), phenols have also been shown to inactivate some *Mycoplasma* when they are applied at a proportion of 0.25–0.5%.

At high concentrations, phenols act as gross protoplasmic poisons, penetrating and disrupting the cell wall and precipitating the cell proteins. Higher phenol concentration also causes coagulation of cytoplasmic constituents, which in turn results in irreversible cellular damage (Hugo, 1991). Low concentrations of phenols and higher molecular-weight phenolic derivatives cause bacterial death by inactivation of essential enzyme systems as well as by leakage of intracellular constituents (including K^+ ion) and essential metabolites from the cell wall (Prindle, 1983). Phenols have also been reported to possess membrane-active properties (impose membrane damage) (Denyer, 1995).

2.3.3. Irradiations

Irradiations, such as γ-radiation and ultraviolet (UV) ray, can be applied in microbial inactivation. UV (specifically UV-C) light is germicidal; it kills fungi, bacteria, viruses, and protozoa as it deactivates/damages their DNA by forming covalent bonds between adjacent bases (by inducing formation of thymine dimers), and such bonds prevent the DNA from being unzipped for replication, which in turn makes them unable to multiply. When the organism tries to replicate, it dies. Bacteria are generally easier to inactivate by UV than viruses, with fungi and spores being harder to be inactivated (Russell, 1999; ClorDiSys, 2019).

3. MATERIALS AND METHODS

3.1. Study site

The entire study was conducted in the CCPP, molecular, and serology laboratory of NVI located at Bishoftu, Central Ethiopia. NVI is the sole laboratory in Ethiopia producing veterinary vaccines and drugs. Currently, it produces over 22 quality veterinary vaccines and 12 veterinary drugs. It also provides services like disease diagnosis, diagnostic kit supply, feed analysis, and laboratory techniques training.

3.2. Experimental design

3.2.1. Experimental animals

Thirty young and clinically healthy local breed goats of approximately equal age (about 6 months to 1 year) and negative for Mccp-specific antibodies (screened using c-ELISA) were used for this experiment. The goats were kept in clean sheds. They were treated by albendazole and oxytetracycline, and left for 1 month for adaptation. The goats were offered appropriate feeds like wheat bran coarse, hay, alfalfa, and clean water.

3.2.2. Culture preparation

Mccp stock seed was 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 was passed to the next passage. Inoculum from the last (third) passage was seeded into CCPP production media in a proportion of 20%. The mixture was incubated with continuous slow agitation (90–100 rpm) at 37ºC for 7 to 10 days until the desired turbidity and pH (6.4–6.8) was achieved. Sample from the grown culture was taken aseptically and observed for purity by Gram staining technique (OIE, 2021).

3.2.3. Saponin preparation

Saponin (product of Sigma, Germany), which was used both as inactivant and adjuvant, was prepared according to the manufacturer's instructions, and sterilized by filtration through 0.22 micron membranes. Its sterility was checked by culturing samples on quadruplicates of each of

the following sterility testing media: soya bean casein digest medium (SBCDM) agar, Sabouraud dextrose agar, 8 ml SBCDM broth, and 8 ml thioglycollate broth, with equal number of non-inoculated controls. The agar plates were streaked with 0.1 ml of the sample, while the broth media were inoculated with 1 ml. SBCDM agar, SBCDM broth, and thioglycollate broth tests, which were used to check for bacterial contaminants, were incubated at 37ºC for 14 days. On the other hand, Sabouraud dextrose agar, which was used to check for fungal contaminants, was placed at room temperature for the same length of time. The tests were checked regularly for the presence or absence of contaminant growth (OIE, 2018a).

3.2.4. Inactivation of the whole culture

Inactivation by saponin

According to Rurangirwa *et al.* (1987b) and OIE (2014, 2018b, 2021), saponin was used as an inactivating agent for Mccp and as an adjuvant for the vaccine. The culture proposed to be inactivated by saponin was made so by adding the prepared saponin in a proportion of 3 mg/ml and homogenizing by shaking. Then, the culture was incubated at 37ºC for 24 hours for the saponin to act.

Inactivation by heat

The culture proposed to be inactivated by heat treatment was subjected to moist heat at 56ºC for 45 minutes (Smith and Oliphant, 1981; Rodriguez *et al.*, 2004) using water bath (Wagtech). To make sure that the inactivation was due to the heat treatment, but not due to the saponin, inactivation test was performed prior to adjuvation with saponin.

Inactivation by 0.1% formaldehyde

Part of the prepared culture was inactivated by formaldehyde (37%; product of Loba Chemie, India) in a minor proportion (0.1%). The inactivation was carried out by applying the indicated amount of formaldehyde followed by homogenization and incubation at 37ºC for 16 hours for the better activity of the formaldehyde (Rodriguez *et al.*, 2004). Like the case of the heat inactivation described above, inactivation test was performed prior to adjuvation with saponin to make sure that the inactivation was due to the formaldehyde, but not due to the saponin.

3.2.5. Antigen estimation for the whole culture

The Mccp culture with adequate level of growth having optimum pH, turbidity, and purity was subjected to antigen estimation. 100 ml of Mccp culture was taken as a sample and centrifuged at 21,000 rpm for 30 minutes during which the supernatant was discarded and the pellet was washed three times to remove the remnant of the media. The amount of Mccp protein antigen was determined for the 100 ml sample, and was checked whether it had sufficient antigen (great or equal to 0.15 mg per ml of whole culture) by using BCA assay (OIE, 2021).

3.2.6. Vaccine formulation

The Mccp culture with a protein antigen content of greater or equal to 0.15 mg per ml of whole culture was subjected to vaccine formulation. The heat- and formaldehyde-inactivated culture was adjuvated by adding the prepared saponin in a proportion of 3 mg/ml, while the saponin-inactivated culture was considered adjuvated during the inactivation process (OIE, 2021).

3.2.7. Vaccine quality control

Identity test

The identity of the inactivated Mccp culture was confirmed by PCR using Mccp-specific primers. The PCR conditions consisted of an initial denaturation step of 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 15 seconds at 47°C, and 15 seconds at 72°C, and a final extension step of 5 minutes at 72°C. The expected amplified product was 316 base pairs long (OIE, 2021).

Purity test

The inactivated culture was checked for the presence of general bacterial and fungal contaminants by culturing sample from the culture on quadruplicates of each of the following sterility testing media (media which generally do not support *Mycoplasma* growth): SBCDM agar, Sabouraud dextrose agar, 8 ml SBCDM broth, and 8 ml thioglycollate broth, with equal number of non-inoculated controls. The agar plates were streaked with 0.1 ml of the culture, while the broth media were inoculated with 1 ml. SBCDM agar, SBCDM broth, and thioglycollate broth tests, which were used to check for bacterial contaminants, were incubated at

37ºC for 14 days. On the other hand, Sabouraud dextrose agar, which was used to check for fungal contaminants, was placed at room temperature for the same length of time. The tests were checked regularly for the presence or absence of contaminant growth. The samples were also checked by Gram staining for bacterial contaminants (OIE, 2018a).

Inactivation test

For each of the inactivants applied, the inactivated culture was checked for its complete inactivation by culturing sample from it on quadruplicates of each of the media promoting Mccp growth: mycoplasma agar and 10 ml Hayflick broth, with equal number of positive controls (inoculated with the live aliquot of the culture) and negative (non-inoculated) controls in common. The agar plates were inoculated with 0.25 ml of culture, while the broth media were inoculated with 1 ml. Half of the plates were incubated at 37°C aerobically (in an atmosphere of air containing 10% CO_2 and adequate humidity) and the remaining half plates were incubated anaerobically in an anaerobic jar with a moisture source for 15 days (OIE, 2018a, 2021). On the other hand, all the test broth media were incubated aerobically at 37ºC for the same length of time (OIE, 2021). The tests were checked regularly for the presence or absence of Mccp growth. Each of the applied inactivation protocols was considered effective only when a negative growth (100% death/inactivation rate) was observed on each of the plates and tubes inoculated with the inactivated culture (Rodriguez *et al.*, 2004) along with only when Mccp, confirmed by identity test (PCR) for it, was found to show growth on each of the positive control plates and tubes (OIE, 2018a).

3.2.8. Experimental animals grouping and vaccination

An experimental study design was applied for the evaluation of different inactivants for F-38 strain whole culture CCPP vaccine. Mccp antibody-free goats were selected based on randomized complete block design methods for immunization and inactivant evaluation tests. Thirty goats were assigned and grouped into 5 experimental groups (A–E) each containing 6 goats: group A for the saponin-inactivated vaccine, group B for the heat-inactivated vaccine, group C for the 0.1% formaldehyde-inactivated vaccine, group D for positive control (vaccinated with 0.5% formaldehyde-inactivated CCPP vaccine of the same batch), and group F for negative control (left non-vaccinated, injected with 1 ml sterile Hayflick broth as a placebo). Allocation of

each of the vaccine preparations to the experimental animals was on a random basis to avoid any bias in the experiment.

3.2.9. Follow-up of the experimental goats

Safety test

Normally, no inflammatory reaction occurs due to Mccp (OIE, 2021), and virulence is not expected from an inactivated vaccine preparation. However, for the matter of OIE (2021) requirements, and due to the change in the way of inactivation, if it has an effect on the safety of the vaccine, safety test was considered and conducted.

The vaccinated goats were examined daily for the presence of injection site reactions and any abnormal local or systemic adverse reactions. Rectal temperature was checked twice daily (at 9:00 a.m. and 3:00 p.m.) for the first 14 consecutive days post-vaccination to check for any abnormal rise in the body temperature. Due to the presence of saponin in the vaccine, a localized swelling at the injection site as well as a transient feverish reaction was expected in the vaccinated animals (OIE, 2021).

Immunogenicity test

Five ml of blood was collected from each of the experimental goats once per week at days 7, 14, 21, and 28 post-vaccination (Peyraud *et al.*, 2014; OIE, 2021). Clear serum was extracted from the blood samples for the evaluation of seroconversion. ELISA, typically c-ELISA, was used to determine the Mccp-specific antibody that reacted with the Mccp antigen coated on microtiter plates. Sera collected from each group of goats immunized by vaccines inactivated separately by saponin, heat, and 0.1% formaldehyde were analyzed for measuring the antibody level.

The test was performed by using Mccp-specific antibody detection kit (product of IDEXX Laboratories, USA) provided with the manufacturer's instructions. PI values were calculated from the respective optical density (OD) results using the following formula provided by the manufacturer.

$$PI = 100 \times (mAbC\bar{x} - SA\ (450)) \div (mAbC\bar{x} - CC\bar{x})$$

Where:

- mAbC$\bar{x}$ is the mAb control mean absorbance
- SA is sample absorbance
- 450 is the light wavelength (in nm)
- CC$\bar{x}$ is the conjugate control mean absorbance

According to the manufacturer's recommendation, and to obtain the 99.9% specificity (OIE, 2021), a PI value of greater or equal to 55 was interpreted as positive for anti-Mccp antibodies (seroconvert), while less than this cut-off value was considered negative.

3.3. Data analysis

The collected data was analyzed and summarized using Microsoft Excel (Office 2007). To determine whether there were any statistically significant differences among each of the inactivants, one-way analysis of variance (ANOVA) was used in R software (version R-4.2.0). Statements of statistical significance were based on p-value (p) ≤ 0.05.

3.4. Ethical approval

The experiment was carried out according to the ethics and guidelines of laboratory animal experimentation, as approved by the National Veterinary Institute Animal Research Ethics Committee.

4. RESULTS

4.1. Inactivating efficacy of the inactivants

For each of the inactivating agents applied, the inactivated culture was checked for its complete inactivation by culturing sample from it on media promoting Mccp growth. All of the applied inactivation protocols were found effective in inactivating the Mccp F-38 strain as no growth was observed on the media inoculated with the inactivated sample culture.

4.2. Safety of the vaccine preparations

During the follow-up periods, the vaccinated goats showed no abnormal conditions except, due to the saponin, a transient feverish reaction, which lasted only for the first two days post-vaccination, and a localized swelling at the injection site, which disappeared within a week. The body temperature of the goats, in general, remained within the range recommended for healthy goats (37.5 to 40.5°C) except during the first two days post-vaccination (as mentioned earlier), where the temperature exceeded the normal range (mean body temperature = 40.8°C), and there was no significant difference between the vaccinated and non-vaccinated groups as well as among the vaccinated groups ($p > 0.05$) (Tables 2 and 3).

Table 2: Mean morning body temperature of the experimental goats observed for 14 days

Group of	Mean	Standard deviation	*P*-value
Saponin-inactivated preparation	38.8	0.92	
Heat-inactivated preparation	38.9	1.05	
0.1% formaldehyde-inactivated preparation	38.9	1.03	0.115
Controls	38.5	0.69	

Table 3: Mean afternoon body temperature of the experimental goats observed for 14 days

Group of	Mean	Standard deviation	*P*-value
Saponin-inactivated preparation	39.5	0.98	
Heat-inactivated preparation	39.5	1.07	
0.1% formaldehyde-inactivated preparation	39.4	1.06	0.096
Controls	38.5	0.69	

4.3. Immunogenicity of the vaccine preparations

For the period of 4 weeks seroconversion, the status of the goats categorized under the 5 treatment groups were analyzed. Accordingly, percentage of sero-positivity (percent of the positive sera) and PI value of seroconversion for the 4 weeks duration of each of the 5 treatment groups are given in Table 4 and 5, respectively. Among the 4 vaccine preparations investigated, the 0.1% formaldehyde-inactivated one showed the highest percentage of sero-positivity (58.3%) and mean PI value (58.1).

Table 4: Percentage of sero-positivity for the 5 treatment groups observed for 4 weeks

Group	Number of tested sera	Sero-positivity (%)
Conventional vaccine	24	31.8
Saponin-inactivated preparation	24	33.3
Heat-inactivated preparation	24	50.0
0.1% formaldehyde-inactivated preparation	24	58.3
Negative control	24	0.0

Number of tested sera (24) = number of goats per group (6) × number of observation weeks (4)

Table 5: Mean OD and the respective mean PI value of sera for the 5 treatment groups observed for 4 weeks

Group	Mean OD	Mean PI	Standard deviation	95% CI
Conventional vaccine	0.39	51.6	13.1	46.08–57.03
Saponin-inactivated preparation	0.41	49.1	17.7	41.60–56.69
Heat-inactivated preparation	0.38	52.3	15.4	46.19–58.48
0.1% formaldehyde-inactivated preparation	0.35	58.1	12.7	53.02–63.22
Negative control	0.49	31.6	7.0	28.56–34.66

CI = confidence interval; the standard deviation and 95% CI are for the mean PI

To determine between which of the groups significant difference exists, multiple pair-wise comparison of the mean PI was made in post-hoc test. The result is given in Table 6. Unlike that of the rest vaccinated groups, the mean PI value of the saponin-inactivated one had no significant difference from such value of the negative control group ($p = 0.103$). Otherwise, the pair-wise comparison showed that there was no significant difference in mean PI value among the vaccinated groups ($p > 0.05$).

Table 6: Comparison of the mean PIs of the 5 treatment groups

Group (I)	Group (J)	Mean difference (I-J)	*P*-value
Conventional vaccine	Saponin-inactivated preparation	3.8	0.974
	Heat-inactivated preparation	-0.8	0.999
	0.1% formaldehyde-inactivated preparation	-6.6	0.838
	Negative control	20.2	0.029
Saponin-inactivated preparation	Conventional vaccine	-3.8	0.974
	Heat-inactivated preparation	-4.6	0.951
	0.1% formaldehyde-inactivated preparation	-10.3	0.495
	Negative control	16.5	0.103
Heat-inactivated preparation	Conventional vaccine	0.8	0.999
	Saponin-inactivated preparation	4.6	0.951
	0.1% formaldehyde-inactivated preparation	-5.8	0.890
	Negative control	21.0	0.022
0.1% formaldehyde-inactivated preparation	Conventional vaccine	6.6	0.838
	Saponin-inactivated preparation	10.3	0.495
	Heat-inactivated preparation	5.8	0.890
	Negative control	26.8	0.002
Negative control	Conventional vaccine	-20.2	0.029
	Saponin-inactivated preparation	-16.5	0.103
	Heat-inactivated preparation	-21.0	0.022
	0.1% formaldehyde-inactivated preparation	-26.8	0.002

Table 7 summarizes the weekly status of the seroconversion for the 5 treatment groups. As it is shown from the table, the saponin-inactivated preparation showed its highest mean PI value (62.7) during the second week of observation, after which, when compared to the rest of the vaccine preparations, the value declined significantly. On the other hand, the 0.1% formaldehyde-inactivated one had the most consistent mean PI value.

Table 7: Summary of seroconversion for the 5 treatment groups on weekly basis

PI	Group	Mean	Standard deviation	95% CI
PIW_1	Conventional vaccine	51.0	7.7	44.84–57.11
	Saponin-inactivated preparation	40.7	15.1	28.61–52.79
	Heat-inactivated preparation	49.6	12.3	39.75–59.37
	0.1% formaldehyde-inactivated preparation	53.3	7.2	47.59–59.07
	Negative control	32.7	6.6	27.38–37.94
PIW_2	Conventional vaccine	46.3	19.5	30.69–61.90
	Saponin-inactivated preparation	62.7	17.6	47.25–78.14
	Heat-inactivated preparation	48.0	11.8	38.53–57.44
	0.1% formaldehyde-inactivated preparation	60.8	10.9	52.03–69.51
	Negative control	30.4	4.9	26.49–34.29
PIW_3	Conventional vaccine	58.0	10.7	48.62–67.42
	Saponin-inactivated preparation	44.6	17.6	29.15–60.01
	Heat-inactivated preparation	59.0	18.9	43.93–74.14
	0.1% formaldehyde-inactivated preparation	60.1	16.0	47.31–72.85
	Negative control	38.2	8.2	30.98–45.39
PIW_4	Conventional vaccine	52.1	11.9	41.68–62.52
	Saponin-inactivated preparation	50.3	17.0	35.41–65.16
	Heat-inactivated preparation	52.8	18.8	37.75–67.78
	0.1% formaldehyde-inactivated preparation	58.3	16.7	44.90–71.69
	Negative control	24.8	8.9	14.75–34.92

PIW_1–PIW_4 = mean PI of week 1 to 4, respectively; CI = confidence interval

The above weekly observation is depicted graphically below (Figure 4) in the way that illustrates the kinetics of Mccp-specific antibodies in each of the 4 weeks of observation. When compared to the rest of the vaccine preparations, the 0.1% formaldehyde-inactivated one found to be the best in that, except for the first week of observation, its mean PI value remained above the cut-off value (55) of the c-ELISA employed.

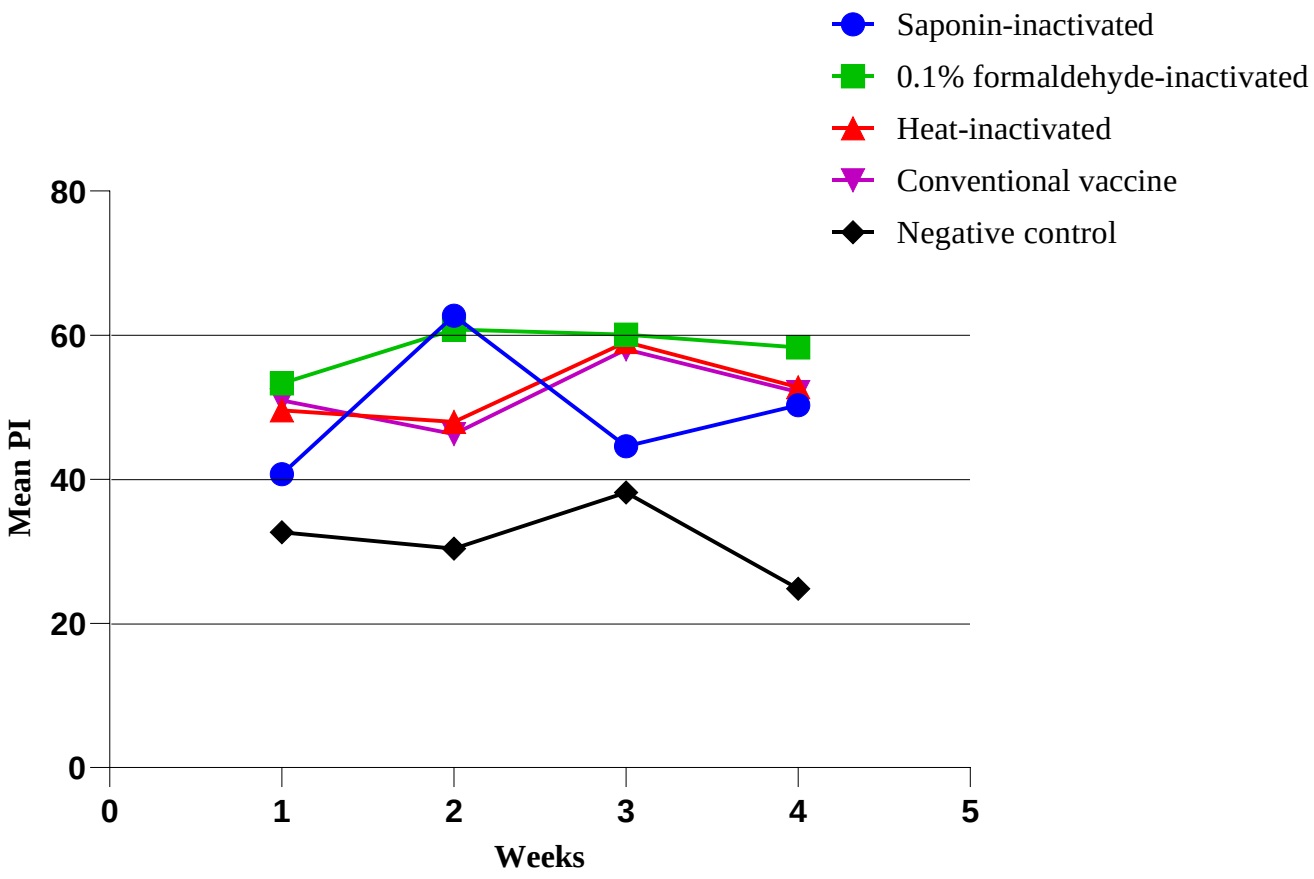

Figure 4: Line graph showing the weekly mean PI value of the 5 treatment groups

5. DISCUSSION

In this study, saponin (3 mg/ml), formaldehyde (in a proportion of 0.1%), and heat treatment (56°C for 45 minutes) were used to inactivate the CCPP whole culture vaccine produced in NVI with an objective to replace the existing formaldehyde inactivation, or otherwise to make use of a minor amount of formaldehyde in the inactivation process of the vaccine. All of them were found to be effective in inactivating the Mccp F-38 strain. Regarding the inactivation by saponin, the result is in agreement with the phenomenon described by OIE (2014, 2018b, 2021). Rodriguez *et al.* (2004) also inactivated *Mycoplasma agalactiae* and *M. putrefaciens* using the indicated concentration of saponin. However, these authors reported that it was ineffective to inactivate *M. capricolum* (strain Mur 2). This can be attributed to the difference in the lability among the subspecies and strains of *M. capricolum* to saponin. The result of inactivation by 0.1% proportion of formaldehyde, a minor concentration than generally used, is in accordance with that obtained by Rodriguez *et al.* (2004). Even though these authors, on the other hand, reported that a heat treatment at 56°C failed to kill *M. capricolum*, the current study showed the effectiveness of the treatment. Smith and Oliphant (1981) and Rodriguez *et al.* (2004) also reported the effectiveness of the treatment for the inactivation of *M. mycoides* and *M. putrefaciens*, respectively. The disagreement may be due to a difference in the resistance to heat at the subspecies and strain levels; they examined strain Mur 2, while strain F-38 of the subspecies *capripneumoniae* was investigated in this study.

The safety test result showed that all of the three vaccine preparations were safe as the vaccinated goats showed no any abnormal conditions like severe injection site reactions and local or systemic adverse reactions except a local edematous reaction at the injection site, which disappeared within a week. As stated by OIE (2021), the localized swelling, however, is attributed to the pro-inflammatory effect of the saponin, a characteristic for its immune-stimulating ability, and is regarded as normal (expected); otherwise, no inflammatory reaction occurs normally due to Mccp. A similar finding was also reported by Ayelet *et al.* (2007b) and Tesgera *et al.* (2017). On the other hand, except for the first two days, the body temperature of the vaccinated goats recorded for the 14 days was within the range of the normal goat body temperature specified by Radosits *et al.* (2007) (37.5–40.5°C), and had no significant difference from that of the control (non-vaccinated) ones ($p > 0.05$). The febrile condition during the first

two days (mean body temperature = 40.8°C) was due to the pyrogenic (inflammatory) nature of the saponin, and, according to OIE (2021), this is regarded as normal (expected). The finding reported by Ayelet *et al.* (2007b) also supports this phenomenon.

When compared to the non-vaccinated group, all of the vaccine preparations showed immunogenicity though, like the case in the study conducted by Tesgera *et al.* (2017), some of the vaccinated goats in each group did not show sero-positivity. This could be attributed to factors such as the level of nutrition and stress. Even though the difference in seroconversion among the vaccine preparations is shown to be not significant, the 0.1% formaldehyde-inactivated one apparently exhibited the highest percentage of sero-positivity (58.3%) and mean PI value (58.1). Rodriguez *et al.* (2004) also reported that, as examined by immunoblotting, inactivation by 0.1% formaldehyde proportion greatly conserves the antigenicity of the killed pathogen. The fixative nature of formaldehyde described by Cromey (2012), which is used to preserve chemical structures in a state and to increase their mechanical strength and stability, might have took the advantage by preserving the important epitopes. This phenomenon can also lead to the concern that high formaldehyde concentration affects the immunogenicity of the vaccine through reduction of the antigenic potential of the pathogen by degrading and destructing important epitopes (Nunnally *et al.*, 2015).

Some reports show that inactivation by heat fixation is generally unsatisfactory, because it results in extensive damage (denaturation) to the surface proteins that could act as antigenic determinants (Rodriguez *et al.*, 2004; Dai *et al.*, 2019). In this study, however, the Mccp preparation inactivated by a heat treatment at 56°C for 45 minutes was found significantly immunogenic. This might be due to that the inactivation temperature and time applied was relatively low as compared to that applied in other bacterial preparations mentioned, for instance, by Tola *et al.* (1999) and Rodriguez *et al.* (2004).

According to OIE (2021), naïve goats vaccinated with saponin-inactivated CCPP vaccine must demonstrate a persistent, specific, and high-titre seroconversion to Mccp antigen. The present study is in disagreement with this phenomenon in that such preparation showed no significantly different seroconversion from the negative control group ($p = 0.103$). It showed a mean PI value of above the cut-off value only during the second week of observation (62.7), and this value declined (fell below the cut-off value) during the next weeks. This deviation might be attributed

to the issue that unlike formaldehyde and heat, saponin may lack the ability to fix (preserve) the important antigenic structures and to increase their stability. The difference that the concentrated (the case of the mentioned source) and whole (the case of the present study) nature of the vaccine might have also led to this disagreement. However, as described by Rurangirwa *et al.* (1987b) and OIE (2014, 2018b, 2021), saponin was shown to have dual effects: inactivation and adjuvation.

Even though the correlation between post-vaccination seroconversion (ELISA titre) and protection efficacy is not established, the demonstration of a significant seroconversion ensures that the vaccine preparation contains the appropriate antigen, and that it can induce an immune response in vaccinated animals (OIE, 2021). In addition, exhibiting seroconversion values equivalent to that of the conventional vaccine, which is thought to be protective, can signify that the new preparation is appropriate to be used as a vaccine. This phenomenon describes the 0.1% formaldehyde- and heat-inactivated preparations evaluated in the present study.

Regarding the immunogenicity test for CCPP vaccine, OIE (2021) recommends follow-up of seroconversion mainly for 2 months post-vaccination. In the current study, however, due to the inconvenience of laboratory facilities and limitation of time and as it was considered that 1 month of observation is significant to see the pattern of seroconversion in comparison to that of the conventional vaccine, as well as the mentioned source also suggests for 1 month in some circumstances, the seroconversion was evaluated only for 4 weeks. The remaining 4-week seroconversion is going to be investigated up next as per the collected sera.

6. CONCLUSIONS AND RECOMMENDATIONS

The present study revealed that in addition to the conventional inactivation by 0.5% proportion of formaldehyde, the whole culture CCPP vaccine can also be inactivated alternatively by heat and minor proportion of formaldehyde (0.1%) without affecting its safety and immunogenicity. When occupational safety is considered due to the toxic nature of formaldehyde, heat inactivation, which is relatively safer, can be employed. Inactivation by minor formaldehyde proportion (0.1%) can be chosen to maximize the immunogenic potential of the vaccine, as well as to minimize the amount of residual formaldehyde in the vaccine and the exposure level of the involved personnel to formaldehyde. On the other hand, even though saponin was found effective in inactivating the CCPP vaccine, the preparation did not show satisfactory seroconversion results, leaving the immunogenicity issue under question. Therefore, the current study concluded that saponin is not appropriate for the inactivation of CCPP whole culture vaccine.

The current study recommends the following remarks.

- Since the serological tests that are used for CCPP have not been evaluated for a correlation between post-vaccination seroconversion and protection, the protective efficacy of the vaccine preparations should be investigated further by challenge study.
- Evaluation under field conditions can also be conducted to ensure the quality of the vaccine.
- Inactivation of CCPP vaccine by UV radiation, phenol, BEI, and sonication should also be investigated for further possible improvement of the potency of the vaccine.

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