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EVALUATION OF SAFETY AND IMMUNOGENICITY OF INACTIVATED WHOLE CULTURE CCPP TRIAL VCCINE IN NATIONAL VETERINARY INSTITUTE, BISHOFTU, ETHIOPIA

MSc Thesis

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I declare that this thesis is my original work and has not been submitted to any other University for the award of masters' degree before. All sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for the degree of Masters of veterinary microbiology at Addis Ababa University, College of Veterinary Medicine and Agriculture.

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TABLE OF CONTENTS

CONTENTS PAGE

LIST OF ABBRIVATIONS

µl	Micro liter
BCA	Bicinconic acid
Bp	base pair
BSA	Bovine Serum Albumin
CBPP	Contagious Bovine pleuro pneumonia
CC	Conjugate control
CCPP	Contagious caprine pleuropneumonia
CCPPV	Contagious caprine pleuropneumonia vaccine
C-ELISA	Competitive Enzyme linked immunosorbent assay
CFT	Complement fixation test
Df	Degree of freedom
ELISA	Enzyme-Linked Immuno Sorbent Assay
G +C	Guanine +Cytosine
GIT	Growth inhibition test
HRPO	Horse reddish peroxidase
IgG	Immunoglobulin G
IHA	Haemagglutination inhibition
KC	Keratoconjunctivitis
LC	Large colony
M	Mastitis
MAKePS	Mastitis, Artheritis, keratitis, Pneumonia and Septicemia
MCCP	Mycoplasma Capricolum Subsp.capri Pneumoniae
NVI	National Veterinary Institute
OIE	Office international des Epizooties
PCR	Polymerase Chain Reaction
PI	Percent Inhibition
PPLO	Pleuropneumonia like organism
PPR	Pests des petites ruminants
rRNA	ribosomal RNA
SC	small colony

SDS	sodium dodecyl sulfate Polyacrylamide gel electrophoresis
SPC	Strong positive control
TMB	Tetra methyl benzoic Substrate
VFG	Viandefoie goat

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

ABSTRACT

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats occurring in many countries in Africa and Asia. It has been causing loss to Ethiopian goats contributing to the expansion of poverty of goat rearing society. In Ethiopia the inactivated CCPP vaccine has been produced and used. However, the vaccine produced by the institute could not satisfy the domestic demand let alone the foreign country demand. The nature of the seed strain, and the length of CCPP vaccine production process involving centrifugation and concentration which has time cost and involve expensive equipments has constrained the vaccine demand in NVI. As the result, the production technique which bypasses such lengthy production process and increase the vaccine yield per annual is mandatory. This study aimed to evaluate the safety and immunogenicity of whole culture CCPP trial vaccine which was produced by bypassing the lengthy centrifugation or concentration process. After the vaccine was produced its safety was confirmed and *Mccp* antibody free goats were grouped in to three and vaccinated and antibody level were evaluated by c-ELISA for eight weeks duration. The data obtained from safety and immunogenicity test results were analyzed by independent sample t-test and ANOVA by considering confidence interval of 95% and desired level of significance of 0.05. During comparison of safety tested and percent positive results, significance difference was not observed ($p>0.05$) but Comparison of the mean percent inhibition between whole culture vaccinated (61.52±2.92), positive control vaccinated (51.86±4.95) and negative control (40.65±2.84) showed presence of significant difference $p<0.05$ between the three groups. It was also seen that the serum antibody started to appear from seven days post-vaccination and continued for two months observation period. After 21 days the antibody level reached peak and remained peak until 8 weeks. The protectiveness of such peak antibody level should be checked by challenge test.

Key words: *Antibody, c-ELISA, Contagious caprine pleuropneumonia,* Goats, *Immunogenicity, NVI, whole culture CCPP trial vaccine*

1. INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats with high morbidity and mortality and occurring in many countries in Africa and Asia where the total goat population is more than 500 million (Acharya, 1992). A classical, acute CCPP is caused by *Mycoplasmas capricolum* subspp.*Capripneumoniae* (*Mccp*) (Leach *et al*., 1993; Mac martin *et al*., 1980), originally known as the F38 biotype. This organism was first isolated and shown to cause CCPP in Kenya (Mac Martin *et al*, 1980; Macowan and Minette, 1976). It has subsequently been isolated in the Sudan, Tunisia, Oman, Turkey, Chad, Uganda, Ethiopia, Niger, Tanzania, Eritrea and the United Arab Emirates (Srivastava *et al.,* 2010). CCPP was first reported in mainland of Europe in 2004, when outbreaks were confirmed in Thrace, Turkey, with losses of up to 25% of kids and adults in some herds. However, the exact distribution of CCPP is not known and it may be much more widespread than the zone represented by the countries where *Mccp* has been isolated as CCPP. This is because of the reasons that it often confused with other respiratory infections, and the isolation of the causative organism is difficult (Ozdemir *et al.,* 2005).

Confirmatory diagnosis is based on the isolation of *Mccp* from clinical samples of lung (Nicholas and Churchward, 2012). The ideal sample for *Mccp* isolation is pleural fluid obtained from a recently slaughtered or live infected goat (Thiaucourt *et al*., 1996). Unlike the true CCPP caused by *Mccp*, other *Mycoplasmas* infections can spread beyond the thoracic cavity (OIE 2008). In the laboratory, the major problem in *Mccp* isolation is its slow growth and frequent contamination of the culture by other *Mycoplasmas* (Thiaucourt *et al*., 1996; Nicholas and Churchward, 2012). Under an ordinary microscope, the organism has a branching filamentous morphology in exudates, impression smears or tissue sections, while other caprine *Mycoplasma*s usually appear as short filamentous organisms (OIE 2009)

The duration of the disease varies according to the environmental circumstances (OIE, 2009); however, the infected goat can survive for more than one month or even recover if placed in good rearing conditions coupled with proper treatment (Thiaucourt *et al*., 1996). A number of antibiotics and vaccines have been mentioned in the treatment and control of CCPP. In one case report, streptomycin-treated goats suffering from natural and experimental CCPP recovered on

the third day of treatment and became completely immune to reinfection with *Mccp* (Rurangirwa and McGuire, 2012). Commercially available vaccines such as Pulmovac and *Capri*doll (live) and CCPPV (killed) are produced in Turkey and Ethiopia, respectively. *Capri*vax is an inactivated CCPP vaccine prepared from *Mccp* strain by the Kenya Veterinary Vaccine Production Institute, Nairobi. The inactivated *Mycoplasmas* strain F38-saponin vaccine in natural CCPP cases showed 100% protection (Litamoi *et al*., 1989).

The presence of CCPP in Ethiopia had been suspected since 1983 and was confirmed later in1990 by isolation and identification of *Mccp* following outbreak of CCPP in Ogaden, eastern Ethiopia (Thiaucourt *et al*., 1992). Since then the disease has been known to be endemic in different regions of the country (Sharew *et al*., 2005). Outbreak of CCPP has been reported from almost all regions of the country especially from low land areas which are known in goat rearing regions (APHRD, 2010).Vaccine against CCPP is currently produced from *Mccp* (F 38 strain) which is Kenyan source by the National veterinary institute (NVI) in Ethiopia, which is inactivated by 0.05% formaldehyde and adjuvated with 0.3% saponin. This vaccine is extensively used for the control of disease in endemic area of the country (APHRD, 2010).

Despite Ethiopia has been producing CCPP vaccine at the NVI, the currently used manufacturing system is unimproved method that follows concentration and centrifugation of the whole culture, which is time consuming and require expensive equipments like flex stand with hollow fiber. CCPP vaccine has got high demand domestically and abroad. However, NVI is currently not in a position to satisfy its customers with CCPP vaccine delivery. In some unpublished pilot studies it has been found that the protein content per milliliter of whole culture contains sufficient immunizing doses and maintains wastage of some protein during centrifugation. This study aimed to solve this issue by using whole culture CCPP vaccine with subsequent Adjuvation by 0.3% saponin.

2. OBJECTIVES

- To evaluate the safety of inactivated whole culture CCPP trial vaccine.
- To evaluate the immunogenicity of inactivated whole culture CCPP trial vaccine.

3. LITERATURE REVIEW

3.1.Economic importance of goats

Goats are among the major economically important livestock in Ethiopia. There are about 23.33 million goats in the country (IBC, 2004).They are playing an important role in the livelihood of resource for poor farmers. They provide their owners with a vast range of products and services such as meat, milk, skin, horns, bones, manure and urine for cash…etc. Goats are relatively cheap and are often the first asset acquired through purchase or customary means by a young family or by a poor family recovering from a disaster such as drought. Goats, once acquired, become a valuable asset providing security to the family as well as milk and dairy products (FAO, 2004).

3.2.Etiology

The causative agent of CCPP has recently been named *Mycoplasma capricolum subspp. Capripneumoniae* (Leach *et al*., 1993). Previously, this agent was known as *Mycoplasma spp*. type F38, as the taxonomic position had not been clearly determined, each isolate has been compared with one of the strains that had been isolated in Kenya: the F38 strain. The authors used the abbreviation F38 for these strains, in an attempt to link the new name with the former denomination. It is important to remember that *Mccp* was characterised only in 1976, meaning that all previous publications were to be analysed with care, since *Mycoplasma mycoides subspp. Capri (Mmc)* was considered responsible for CCPP before this finding (Edward, 1953; Gee, 1977; Jonas and Barber, 1969; Littlejohns and Cottew., 1977; Longley, 1951; Macowan and Minette, 1976 and Moulton, 1980). The etiological role of *Mccp* cannot be questioned now. Numerous writers have successfully reported the disease with the causative agent (Macowan and Minette, 1976 and Perreau *et al*., 1976). Since diagnostic tools have been improved, *Mccp* is regularly demonstrated in clinical cases in which there is a strong suspicion of CCPP (Bolske *et.al.*, 1996). Therefore, CCPP and *Mccp* fulfil all Koch postulates, as these have been revisited (Evans, 1976).

3.3.Epidemiology

In natural infections, the organisms are acquired by susceptible goats by inhalation of contaminated droplets from infected goats (MacOwan, 1984). The environment as a whole plays an important role in the appearance, evolution and severity of CCPP. Due to the high sensitivity of mycoplasmas to the external environment, close contact is essential between infected and naive animals for transmission to take place, and, overcrowding and confinement favours close contact and circulation of mycoplasmas, Stress factors due to malnutrition and movement over long distances can predispose the animal to disease, in Africa where extensive and traditional husbandry is practiced, pathogens spread when animals meet at watering points and grazing areas. Breed and sex appear not to affect the epidemiology of CCPP, but age is an important factor. Though all age groups are susceptible, mortality is higher among young animals than adults. Infective *M.capricolumsubsp Caprie pneumoniae* may persist in chronic, latent carriers, such as goats which have recovered from infection without becoming bacteriologic ally sterile, and are considered to be responsible for the perpetuation of the disease in a herd (Thiaucourt and Bšlske, 1996; Wesonga *et al*., 1998). This aspect of the epidemiology was described as early as 1881 in the case of CCPP in South Africa (McMartin *et al*., 1980, Lefëvre *et al*., 1987).

3.4.Genetic diversity

Mycoplasmas are the smallest free-living fastidious bacteria. They are about 300 nm in diameter, bound by a triple layered membrane and unlike conventional bacteria they don't have a rigid cell wall of murin (Robinson and Bebear, 1997). Their genome size is only one sixth to one third of that of *Escherichia coli* (Bascunana *et al.,* 1994). Mycoplasmas are phylogenetically related to gram positive bacteria with low G + C content (Razin *et al.,* 1983; Bascunana *et al*., 1994). The Mycoplasma mycoides cluster has two rRNA operons in which intra specific variations have been demonstrated (Heldtander *et al*., 2001). *Mccp* was once thought to be a homogenous taxon (Abu-Groun *et al*., 1994*;* Nicholas 2002; Manso-Silvan *et al*., 2007), but the discovery of two molecular markers showed some degree of heterogeneity among strains that opened a further channel for studies on the molecular epidemiology of CCPP (Manso-Silvan *et al*., 2011).

3.5.Transmission

Contagious caprine pleuropneumonia (CCPP) is transmitted directly by an aerogenic route through contaminated droplets (Thiaucourt *et al*., 1996). The outbreak of the disease follows the introduction of an infected animal into a group of susceptible goats (OIE, 2009). The disease is readily contagious and a short period of contact is enough for successful transmission through coughing (Thiaucourt and Bolske, 1996; OIE, 2009). No evidence of indirect contact has been shown as the organism is highly fragile in the environment *(*Thiaucourt and Bolske, 1996). It is quickly inactivated within 2 min at 60 °C but can survive for more than 10 years in frozen infected pleural fluid (OIE, 2009). Disease outbreak may occur after heavy rain, animal transportation over a long distance (OIE, 2009), poor climatic conditions and primary infections (Thiaucourt and Bolske, 1996). Formaldehyde can inactivate *Mccp* in 30 s at a concentration of 0.05%. A solution of 1.0% phenol can inactivate the organism within 3 min (OIE, 2009).

Incubation period of *Mycoplasma capricolum* subsp. *Capripneumoniae* is normally very short in the lungs (three to five days) but this may be prolonged (three to four weeks) depending on predisposing factors (Thiaucourt and Bolske, 1996). In primary infected goats, CCPP last for about two days with high mortality (McMartin *et al*., 1980)

3.6.Distribution

While the clinical disease has been reported in nearly 40 countries in Africa and Asia, *Mccp* has only been isolated in 13 countries, because few have the facilities for isolating and growing mycoplasmas (Nicholas, 2002). The presence of CCPP in Europe was suspected in 1920, when an outbreak occurred in Greece following the attack of goats from Turkey (Goncalves, 1982). However, a disease which was very closely resembled CCPP was reported in goats in Portugal in 1980. Classical CCPP, but from which *M.mycoids sub spp. mycoides LC* was isolated in 1996 was investigated in a goat herd in England containing some imported goats and which had suffered from severe respiratory disease resulting in many deaths (Nicholas, 2002).

3.7.Pathology

The gross pathological lesions are localized exclusively to lung and pleura and are often unilateral. Affected lungs can be totally hepatized, and have a port wine colour (Thiaucourt and Bošlske, 1996). A lung section shows a fine granular texture with various colours, but usually without any thickening of the interlobular septa. There is often an abundant pleural exudate and conspicuous pleuritis. The pleural exudates can solidify and form a gelatinous covering sometimes over the whole lung. In acute cases, the pleural cavity contains an excess of straw-coloured fluid with fibrin flocculations (Kaliner and MacOwan, 1976; Wesonga *et al*., 1993). In chronic cases there is a black discolouration of the lung tissue and sequestration of the necrotic lung areas. Adhesions between the lung and the pleura are very common and often very thick (MacOwan and Minette, 1977).

Histological examination of the lung tissues may show acute serofibrinous to chronic fibrino-necrotic pleuropneumonia with infiltrates of serofibrinous fluid and inflammatory cells, mainly neutrophils, in the alveoli, bronchioles, interstitial septae and subpleural connective tissue. Intralobular oedema is more prominent but interlobular oedema has also been reported. Peribronchial and perbronchiolar lymphoid hyperplasia with mononuclear cell infiltration is also present (Kibor, 1990; Wesonga *et al*., 1998; Msami *et al*., 1998).

3.8.Immunology

Little is known of the immunology of CCPP despite a number of reported experimental infections. More recently monitored the humoral response of goats infected with a multipassaged *Mccp* strain 19/2 with several serological tests and PCR. While there was little evidence after infection of the infectious agent or clinical or pathological disease, apart from elevated temperatures and a transient cough in one goat, serological responses were detected by latex agglutination test and competitive ELISA. Immunoglobulin G (IgG) immunodominant bands of 23, 40 and 44 kDa were seen by immunoblotting in all experimentally infected animals as well as in some sera from a natural outbreak of CCPP in Eritrea which additionally showed bands of 62, 70 and 108 kDa (Muthomi *et.al*, 1983; Perreau *et.al*, 1984; March *et.al*, 2002)

3.9.Diagnosis

3.9.1. Clinical signs

All goats can be affected, whatever their age or sex (Thiaucourt *et al*., 1996). The incubation period generally lasts 10 days, but may vary between 2 and 28 days. Acute cases can be observed in regions where CCPP is introduced for the first time to naive populations (OIE, 2008). The first symptom to appear is a reluctance to walk, fever is extreme (41°C) but the animals continue to feed and ruminate, abortions are frequent in pregnant goats, gradually, the respiratory symptoms become prominent, respiration is accelerated and painful, and accompanied by violent coughing, in the terminal stages, the animals are unable to move, they stand with their legs wide apart, the neck is stiff and extended, saliva continuously drips from their mouth and their nose is obstructed by a mucopurulent discharge (Nicholas, 2002; OIE, 2009). The duration of the disease varies according to the environmental conditions. This duration may vary from a few days for animals that are subjected to harsh conditions, such as underfeeding, polyparasitism, the necessity to walk long distances for watering, bad climatic conditions...etc. However, animals can survive more than one month or even recover if they are placed in good condition. Subacute or chronic forms can be observed in regions where CCPP is enzootic. The symptoms are similar to those of the acute cases, but are not as strong. Coughing is irregular and usually follows a physical effort, and nasal discharge may be absent. In the absence of antibiotic treatment, mortality varies between 60% and 100% (Rurangirwa and McGuire, 2012; OIE, 2009).

3.9.2. Post-Mortem Examination

Pathological changes are confined to the chest cavity and consist of pleuropneumonia: unilateral hepatisation, an accumulation of straw-coloured pleural fluid and acute pleuritis of the pleura adjacent to the affected lung (Wesonga *et al*., 1993).

3.9.3. Isolation of Mycoplasmas

The growth of MccF38 usually takes four to five days, when first isolated, and the diameter of the colonies may be only 0.1 mm, these colonies can only be seen by close observation with a binocular microscope. In a liquid medium, the turbidity is very faint and should be compared with inoculated medium. The composition of the medium is very important and determines the rate of success as well as the size of the colonies. Some authors have recommended using fresh meat infusion to obtain good growth (Kibor, 1990); though this may not be necessary and commercial components may ensure more regular results. Horse serum permits good growth of MccF38. The serum should be added at a concentration of 20% to 30% by volume. Once again, the quality of this component must be tested, by comparing different batches from different manufacturers with dilutions of freeze-dried reference mycoplasma strains. Some authors have recommended the use of foetal calf serum (Jones and Wood, 1988) or donkey serum (Perreau, 1976).The latter may be interesting for developing countries, as it is very easy and cheap to obtain locally, fresh yeast extract is also an important component of the medium and the yeast extracts that are available commercially are not of the same quality. The other major difficulty lies in the presence of other bacterial and fungal contaminants in the samples. Usually, the classic bacteria are inhibited by the antibiotics, chiefly ampicillin, that is incorporated into the medium used for the primary culture. However, other mycoplasmas, such as *M. arginini* or *M. ovipneumoniae,* are often isolated. These two mycoplasma species are natural colonisers of the upper respiratory tract of goats and can be regularly isolated from healthy lungs (Bölske *et. al.,* 1989), as well as from diseased lungs (Gupta and Verma, 1984; Radwan *et.al.*1985). There is some indication that *M. ovipneumoniae* may have pathogenic potential but this is probably secondary. *M. ovipneumoniae* may play a role in lung lesions that have a multifactorial origin, particularly in association with viruses or other bacteria, such as the *Pasteurella* species (Nicholas, 2002).

In the case of CCPP lesions, *M.ovipneumoniae* is often isolated (Jones and wood, 1988). However, where MccF38 and *M. ovipneumoniae* co exist, the former is present in higher quantities, as has been demonstrated with a quantitative method, such as sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS). The isolation of *M.ovipneumoniae* from CCPP lesions

should therefore be interpreted with great caution and its aetiological role should be questioned. A highly pathogenic bacterium would presumably disseminate rapidly to numerous animals, hence all isolates from the same herd would be very homogeneous. That is not the case with *M. ovipneumoniae,* as numerous distinguishable strains of *M. ovipneumoniae* can be isolated from a single herd or even from a single animal (Ionas *et al*., 1991; Thirkell *et al*., 1990).

3.9.3.1. Selection of samples

One of the best samples is pleural fluid. Ten milliliters should be harvested aseptically from an animal that is in the acute phase of the disease. Dead animals should be avoided. The best method is to sacrifice at least one animal of the herd that did not receive any antibiotic treatment. Other samples may be gathered from live animals by lower intrathoracic puncture with broad diameter needles. Samples of hepatised lungs are also desirable. Square 3 cm sections should be taken from an area that borders normal lung tissue. There is no need to multiply the number of samples and all efforts should be directed towards obtaining samples of good quality. Samples can be kept at +4°C if transport to the laboratory does not take more than one or two days. Otherwise, they can be deep frozen at -20°C. In the latter case, samples can be stored for months without loss of mycoplasma viability. For storage longer than 10 months, it is recommended to keep the samples at -70°C. In the absence of cold chain, penicillin or ampicillin should be added to the sample to limit contaminant growth. It is important to point out some of the difficulties that can be encountered in isolating MccF38; two main difficulties can be described. First, MccF38 grows very poorly in vitro and, secondly**,** samples are often contaminated by other mycoplasmas (Thiaucourt et al., 1996; Nicholas and Churchward, 2012).

3.9.3.2. Treatment of samples

Swabs are suspended in 2–3 ml of culture medium. Tissue samples are best minced using scissors, and then shaken vigorously, or pulverised in medium using 1 gm of tissue to 9 ml of medium. Tissues should not be ground. The suspension is usually prepared with a mycoplasma medium, but if parallel bacteriological examination is to be carried out, a high quality

bacteriological medium, such as nutrient broth, may be used to provide a suspension suitable for both forms of examination. Pleural fluid, or a tissue suspension or swab, is serially diluted through at least three tenfold steps in the selected mycoplasmal medium and dilutions should also be placed on to solid medium (Thiaucourt and Lefevre., 1992).

3.9.3.3.Mycoplasma media

The medium used by MacOwan and Minette to culture *Mccp* organisms (Mohan *et. at*., 1990) is termed 'viandefoie goat' (VFG), and includes goat-meat, liver broth and goat serum. Alternative suitable media are modified Hayflick's, and modified Newing'stryptose broth (MacOwan and Monett, 1976). Media enriched with 0.2% (or up to 0.8%) sodium pyruvate perform considerably better, both for primary isolation and antigen production of *Mccp* (Balikci *et al*., 2008; Cetinkaya *et al*., 2009).

3.10. Identification of Mycoplasmas

3.10.1. Polymerase Chain Reaction (PCR)

This relatively new diagnostic method has radically improved the detection and identification of micro-organisms which do not grow easily *in vitro.* PCR is based on the amplification of specific DNA sequences with thermostable enzymes and nucleotidic primers that must be chosen with care (Mcpherson *et al*., 1992). This novel technique might improve dramatically the laboratory diagnosis of mycoplasma disease in developing countries, as PCR can be performed with dried material, hence removing the need for a constant 'cold chain' for samples. In the case of CCPP, the best sample may be pleural fluid that has been dried onto filter paper. The dried filter paper can then be wrapped in a plastic bag and sent to a reference laboratory (Bölske *et al*., 1996). The presence of antibiotics in the sample should not interfere with the reaction. However, it does jeopardize the success of isolation. Drying the sample reduces the size, thus facilitating transport and inactivating many pathogenic organisms. This technique has some limitations. PCR is not a reference technique as the reliability of the process may sometimes be questioned. It is still

necessary to isolate MccF38 to confirm without doubt. The isolation of strains is always needed to confirm periodically that the PCR technique used is specific. In addition, the isolation of strains gives access to the whole genome of the bacteria, whereas PCR amplifies only a portion. Obtaining the whole genome of a strain might be the only way to design improved diagnostic tools, based on different gene locations (Bölske *et al*., 1996).

Paradoxically, the high sensitivity of the method may sometimes prove to be a limitation, as the manipulation of amplified products may contaminate some reactions and could result in false positive results. The prevention of this risk should be a continual concern for technicians working with PCR. As a result, multiple negative controls must be included in all reaction sets. The PCR method which has been described for the detection of MccF38 (Bascuñana *et al*., 1994) is based on the amplification of a segment of the gene that codes for the 16S ribosomal RNA (rRNA). This gene is well conserved in bacteria but also possesses regions that are variable enough to ensure a distinction between species or subspecies. These variations are used to construct phylogenetic trees (Weisburg *et al*., 1989; Weisburg *et al*., 1991) and diagnostic tools (Johansson, 1993). In the case of CCPP, the primers were chosen specifically to amplify a fragment of this gene for the *mycoides* 'cluster'. The identification of MccF38 is made in a second step, by digestion of the amplified products with an endonuclease: pst I. Three fragments are obtained with MccF38 while only two are seen with other members of the *mycoides* 'cluster'. This difference indicates a single nucleotide substitution in one of the two genes that code for the 16S rRNA in the mycoplasmas (Bascunana *et al*., 1994).

3.10.2. Serological tests

Quite a few serological tests are available that are used in the field for the confirmatory diagnosis of CCPP. Indirect Haemagglutination (IHA) and complement fixation tests (CFT) are used to assay the antibody response of goat to *Mccp* (Massa *et al*., 1992). The CFT used for the detection of CCPP (Macowan 1976; Macowan and Minette 1977) is more specific, though less sensitive than the IHA (Muthomi and Rurangirwa 1983; Massa *et al*., 1992). The IHA specificity for the *Mycoplasma mycoides* cluster has been evaluated and the results were found to show cross-reactivity between these organisms (Jones and Wood 1988; Litamoi *et al*., 1989; Massa *et al*., 1992). The latex agglutination test which detects serum antibodies in CCPP-infected goats is

more sensitive than CFT and can be performed in field conditions using whole blood or undiluted serum with a prompt result (Cho *et al*. 1976). An indirect enzyme-linked immunosorbent assay (ELISA) has been developed to screen goat serum at a single dilution of antibody to *Mccp* (Wamwayi *et al*. 1989). The specificity and suitability of ELISA for large scale testing make it an appropriate tool for epidemiological investigation of CCPP (OIE, 2008). Direct antigen detection and blocking ELISA detects antibodies in the serum of naturally or artificially CCPP-infected goats (Wamwayi *et al*. 1989). Direct and indirect fluorescent antibody tests are the simple, reliable and rapid serological methods applied to clinical samples for the identification of most *Mycoplasma*s (Thiaucourt *et al*. 1996b). Among many, the indirect fluorescent antibody (IFA) test is the most commonly used and is applied to unfixed *Mycoplasma* colonies on agar (OIE, 2008).

The growth inhibition test (GIT) is the least sensitive and simplest of the tests available for CCPP diagnosis (OIE. 2008). It depends on the direct inhibition of *Mycoplasmas* growth on solid media by specific hyper immune serum, and detects primary surface antigens (Dighero *et al*., 1970; Rosendal and Black, 1972). The GIT is particularly useful in identifying *Mccp* because they appear to be serologically homogeneous, and antiserum to the type strain produces wide inhibition zones (OIE, 2008).

3.10.2.1. Competitive enzyme-linked immunosorbent assay (C-ELISA)

C-ELISA was developed and proved both specific and sensitive (Thiaucourt *et al*., 1994). This test has recently been reformatted as a kit containing pre-coated plates and ready-made reagents, including MAb 4/52. It is now a strict competition assay instead of a semi-blocking test as in the original publication. The new kit has been re-validated to establish its cut-off value, 55% inhibition (PI), to obtain a strict specificity of 99.9%. It allows the detection of positive sera in CCPP-infected herds, but its true sensitivity at the individual level has not yet been fully evaluated. As it is highly specific, it can be used to evaluate herd status using targeted sampling of recovered animals in the tested herds should greatly enhance the sensitivity without any specificity problem. In the OIE Reference Laboratory, the uncertainty of measurement for this c-ELISA has been evaluated at ±8 PI. This test can be used to evaluate the CCPP vaccine quality

as the seroconversion measured 1 and 2 months post-vaccination is proportional to the *Mccp* antigen or saponin content. However, the correlation between c-ELISA titre and protection has not yet been established (Peyraud *et al.*, 2014).

3.11. Control

Since the disease occurs in epidemics, antibiotic treatment, as the only control measure would be very uneconomical. Therefore, efforts have been directed towards controlling the disease by vaccination. The first immunization trial using *Mccp* was carried out by MacOwan and Minnette. They inoculated 20 goats intratracheally with a high-passage culture of *Mccp*. On contact challenge of the inoculated goats one month later, 11 of the 20 vaccinated goats were protected, whereas all 20 control goats contracted CCPP. The results provided an indication that goats could be protected against CCPP by using *Mccp* (MacOwan and Minnette, 1978).

4. MATERIALS AND METHODS

4.1. Description of the Study area

Experiments and laboratory diagnosis were done from September 2014 to April 2015 at National Veterinary Institute which is found in Bishoftu town. The town is situated 47 km south east of the capital city, Addis Ababa. It found at 9^0 N latitude and 4^0 E longitudes at an altitude of 1850 m above sea level in central highlands of Ethiopia (NMSA, 1999).

4.2. Experimental animals and husbandry

Experimental study was conducted at the NVI, Ethiopia. Twenty six male goats older than six months and of approximately equal age which had history of no previous exposure to CCPP and negative for *Mccp* specific antibodies were used for the experiment (figure1). The goats were purchased from North showa (Debre Brihan) live stock market, where farmers rearing cattle, sheep, goats and others live stocks together. Up on arrival to the experimental area, goats were treated by Albendazole and ox tetracycline and left for two weeks for adaptation and they were supplied with water ad libitum and appropriate feeds like wheat bran, maize and Alfalfa. During the trial, the goats were housed in separate areas from other livestock.

Figure 1 Picture of some of the goats used during the trial

4.3. **Experimental Design**

The goats were brought to NVI and kept for 14 days for any clinical sign of disease prior to period of actual trial. Serum was collected from these goats and checked for freedom from *Mccp* antibody by using c-ELISA. *Mccp* free goats were isolated and then randomly allocated into experimental groups as follows: five goats were used to evaluate the safety of the whole culture trial vaccine (n=5), seven goats were vaccinated with whole culture trial vaccine (n=7) and also seven goats were vaccinated with the existing vaccine (positive control) (n=7) and the other seven goats were kept as unvaccinated controls (n=7). Among the five goats three of them were injected with 2ml of the whole culture CCPP trial vaccine and two goats were used as a control, the trial vaccine and positive control were injected 1ml subcutaneously around thoracic area. The trial whole culture CCPP vaccine were produced by using the manufacturer's protocol in spinner bottle by excluding the concentration steps and the existing vaccine was taken from the quality assured ready for use and used as a positive control. The rectal temperatures were recorded for 14 days in goats used for safety and for 7 days in case of vaccinated and control groups. In all cases rectal temperature were measured twice daily at 9:am and 3:pm. Parallel to other tasks, About 5ml of blood samples were collected from each experimental goats once per week at days 7, 14, 21, 28, 35, 42,49 and 56 of post vaccination. Collected blood samples were allowed to stand for 3-6 hr at room temperature to enhance clotting and sera was further separated by centrifugation at 3000 rpm for 3 min to extract clear serum (OIE, 1992; Thiaucourt *et al*., 1994) and tested for sero conversion by c_ELISA at Research and Development laboratory of National veterinary institute.

4.4. **Preparation of the culture**

The whole culture was prepared from Kenyan isolate of *Mccp* seed obtained after 24 passages in vitro culture of the bacteria in Hyaflik medium. The positive control vaccine was obtained from commercially produced by NVI and quality assured, which was also prepared from the Kenyan isolate of *Mccp*. The media was inoculated with 20% horse serum and 20% *Mccp* inoculum according to the standard operating procedure of CCPP vaccine production of NVI and incubated at 37^{o}c in spinner bottle with slow agitation (see figure **2**). After 7 days, full turbidity was observed in spinner bottle and the culture was sampled and checked for PH (6.2) and sterility.

After the desired PH and sterility was obtained, the culture was inactivated by 0.05% formaldehyde, Sample was taken and centrifuged at 20000 rpm for 30 minute and washed three times by PBS**.**

Figure 2. Growing of *Mccp* in spinner bottle with slow agitation (90-100rpm)

4.4.1. *Protein estimation of Mccp in the whole culture*

The total protein of *Mccp* in the culture was determined by Buretic and Bradford methods using bovine serum albumin as a standard according to the description of OIE (2000). Each of the 50µl of known BSA standards and test samples (*Mccp pellet*) were separately mixed with the BCA reagent and Cupper sulphate in the proportion of 50:1 respectively and incubated at 37^{o}C for 1hr and the Optical density (OD) of the standard (BSA) and test samples were read under 550 nm wavelength (annex1) and the amount of protein content was estimated accordingly (OIE, 2000) and 0.958mg Ag was obtained per milliliter. Finally the culture was adjuvated by 0.3% saponin and converted to vaccine (see Table 1).

Table 1.Whole culture CCPP trial vaccine formulation protocol**.**

Total volume of inactivated culture	500ml
Initial concentration of Adjuvant(saponin)	20%
Final concentration of Adjuvant(saponin)	0.3%
Well homogenized Culture per vials	19.7ml
Total volume of vaccine per vials	20ml
Total vials	25

4.4.2. Whole culture CCPP trial Vaccine quality control

4.4.2.1. Purity and sterility test

As per the current CCPP vaccine production protocol of NVI, the formulated vaccine was checked for evidence of freedom from contaminants, its purity and sterility was seen by using Gram stain and sterility media such as SBCDM agar and broth, thioglycollate broth and sabouraud agar media.

4.4.2.2. Safety of the final whole culture CCPP trial vaccine on target animals

After the sterility was confirmed, the vaccine was injected and its safety was seen on goats. Safety of the trial vaccine was seen by injecting three goats with double dose (2ml) and leaving 2 goats as a control and a total of five goats. The rectal temperature were recorded two times daily for 14 days, local reaction at the site of injection and any external changes on the goats were observed and recorded as parameters to evaluate safety of the vaccine.

4.4.2.2. Inocuity test

Ten tubes marked from 1-10 and each containing 10ml of classical CCPP broth media were used and from inactivated whole culture 1ml was inoculated to four tubes, four tubes were inoculated with live *Mccp* inoculums as a positive control and the other two tubes were kept as un inoculated negative control. All the tubes were incubated at 37^{o}c for 10 days and observed for the presence or absence of the *Mccp* growth, and no growth was observed in tubes which inoculated with inactivated whole culture trial vaccine and negative control.

4.4.2.4. Immunogenicity test

The blood was collected on weekly bases and the sero-conversion was evaluated from 7th day post vaccination to 2nd month by c-ELISA (See Annex 2).

Table 2.Immunogenicity test schedule

Existing groups	**Adjuvation**	**No. of goats**	**Sera collection**
Trial vaccine	CCPP Whole culture vaccine adjuvated with 0.3% saponin	7	For two months post vaccination starting from day 7
Positive Control	Previously existing concentrated vaccine	7	For two months post vaccination starting from day 7
Negative Control	Non-vaccinated	7	For two months post vaccination starting from day 7
Total		21	

5. **DATA ANALYSIS**

All collected data regarding body temperature follow up were compared with the literature cutoff value to determine which animals were febrile and which ones were normal. The data on immunogenicity parameters were entered in to Microsoft office Excel computer program and then summarized first by using descriptive statistics. Statistical analysis was performed using statistical package for social science (SPSS)-version 20. The mean weekly percent positive was compared for positive control and inactivated whole culture trial vaccine by using independent sample t-test. The mean Percent inhibition of sero conversion of immunogenicity trial groups, and the mean weekly Percent inhibition of seroconversion were compared by using ANOVA. In all the analyses, a p-value less than 0.05 and a confidence interval of 95% were considered for significance.

6. **RESULT**

6.1. **Safety test**

During these all observation periods, the goats showed no abnormal situation except minor swelling at the injection site which subsided within a week. The body temperature recorded for safety test (see annex.3.) was evaluated and none of the goats were febrile during 14 days observation between the vaccinated and non-vaccinated, in both groups. Besides these five animals, the three groups tested for immunogenicity were also checked for their body temperature difference for the first 7 days observation and both in the morning and afternoon (see Table.3 and 4).

Table 3.Result of body temperature of safety tested goats for 14 days observation period

days	N	Whole culture		Positive control		Negative control	
		febrile	normal	febrile	normal	febrile	Normal
1	5	-	5	-	5	-	5
2	5	-	5	-	5	-	5
3	5	-	5	-	5	-	5
4	5	-	5	-	5	-	5
5	5	-	5	-	5	-	5
6	5	-	5	-	5	-	5
7	5	-	5	-	5	-	5
8	5	-	5	-	5	-	5
9	5	-	5	-	5	-	5
10	5	-	5	-	5	-	5
11	5	-	5	-	5	-	5
12	5	-	5	-	5	-	5
13	5	-	5	-	5	-	5
14	5	-	5	-	5	-	5

N is the number of goats used during safety test. Goats with rectal temperature range from 38.6 to 40^0c and plus or minus 0.5^0c were considered as normal and none of the goats were febrile in both safety and control groups. This indicates that the vaccine is safe to inject to the trial groups.

Table 4.Result of body temperature of immunogenicity tested goats for 7 days observation.

Day	N	Whole culture		Positive control		Negative control	
		febrile	normal	febrile	normal	febrile	Normal
1	21	-	7	-	7	-	7
2	21	-	7	-	7	-	7
3	21	-	7	-	7	-	7
4	21	-	7	-	7	-	7
5	21	-	7	-	7	-	7
6	21	-	7	-	7	-	7
7	21	-	7	-	7	-	7

N is number of goats used for immunogenicity test for 7 days observation of body temperature and goats used for inactivated whole culture vaccine, positive control and negative control were evaluated and none of the goats were febrile.

6.2. **Immunogenicity test**

For the period of eight weeks sero-conversion, ability of goats categorized under the three treatment groups were analyzed. Accordingly, the comparison of mean percent positive of sero-conversion for eight weeks duration of trial vaccine and positive control and also comparison of mean Percent inhibition for eight weeks in the three groups were given in tables 5 and 6 below.

Table 5.Descriptive statistic value of weekly mean percent positives of the three treatment groups and t-test analysis result of trial and positive control vaccines.

week	N	w1	w2	W3	w4	w5	w6	w7	w8	Mean	95% CI	Value	p-value
Whole culture	8	57.1	57.1	57.1	57.1	57.1	71.4	57.1	71.4	60.7	57.18-66.24	0.258	0.803
Positive control	8	85.7	71.4	42.9	85.7	42.9	42.9	57.1	42.9	58.9	42.76-74.96		
Negative control	8	0	0	0	0	0	0	0	0	0			

N=8 for all groups and is the number of weeks when goats were observed; CI-confidence interval; df =8.629. Note: the comparison of the mean difference of percentage positives between whole culture vaccinated (60.71%) and positive control vaccinated (58.86%) showed absence of significant difference between the two groups (P>0.05).

Table 6.Descriptive statistic value and Welch ANOVA analysis result of mean percentage inhibition of sera of the three treatment groups taken for eight weeks.

Groups	**Mean**	**STDv**	**SE**	**95% CI**	**Min**	**Max**	**F-cal**	**p-value**
Positive control	51.86	4.95	1.75	47.72-55.99	45.94	57.95	100.056	0.000
Whole culture	61.52	2.92	1.03	59.07-63.96	55.44	64.20		
Negative controls	40.65	2.84	1.00	38.28-43.02	36.66	44.63		

N=8 for all groups and is the number of weeks goats were observed; CI-confidence interval; df1=2, df2=13.459: As the above PI comparison showed presence of significant difference (P< 0.05) among the three treatment groups, to know between which groups significant difference exist, multiple comparison was made (in post hoc analysis of ANOVA) and the result is given as follows (Table7). It showed that mean PI of whole culture is the highest (61.52±2.92) and the mean PI of positive control (51.86±4.95) is greater than that of negative control (40.65±2.84).

Table 7.Multiple comparison of mean percent inhibition (PI) to see point of significance difference

Groups (I)	Groups (J)	Mean difference(I-J)	SE	p-value
Positive control	Whole culture	-9.661	2.03	0.001
	Negative control	11.206	2.02	0.000
Whole culture	Positive control	9.661	2.03	0.001
	Negative control	20.867	1.44	0.000
Negative control	Positive control	-11.206	2.02	0.000
	Whole culture	-20.867	1.44	0.000

NB: Mean difference is significant at level of < 0.05; SE is standard error. The above pair wise comparison by ANOVA indicated that the presence of significant difference among the mean PI value in the test groups. That is there was strong significant difference between whole culture vaccinated and positive control vaccinated where the PI of whole culture vaccinated was greater than the PI of positive control vaccinated and the PI of non-vaccinated controls was significantly less than both vaccinated groups. After the observation that means PI was significantly different among the three treatment groups analysis was also made to see if there is significance difference among the weekly PIs during the eight weeks observation (see table 8 below.

Table 8.Descriptive statistic of eight weeks observation of mean PIs on weekly basis

Week	N	w_1	w_2	W_3	w_4	w_5	w_6	w_7	w_8	Mea	95%CI	Max	min	t-cal	p-value
Whole culture	7	63.0	63.9	61.6	64.2	59.4	61.2	55.4	63.2	61.5	47.7-55.9	64.2	55.4	100.0	0.000
Positive control	7	57.4	57.7	48.6	57.9	45.9	49.7	48.6	48.7	51.8	59.0-63.9	45.9	57.9		
Negative control	7	43.3	44.6	41.8	40.1	40.1	36.6	36.8	41.5	40.6	38.2-43.0	36.6	44.6		

Note: PIW1, PIW2, PIW3, PIW4, PIW5, PIW6, PIW7, PIW8 indicates mean percentage inhibition of week 1 to week 8 respectively. NB: N is the number of goats used during the comparison. As it was indicated on above table the highest PI of seroconversion was seen on the 4^{th} week in both whole culture and positive control (64.2, 57.9) respectively and the lowest PI was recorded at W7 in whole culture and at W5 in positive control.

When the above mean PIs weekly observations are depicted graphically the mean PI of the whole culture was above both the mean PI of positive and negative controls in all weeks. In some cases, the mean PI of positive control failed below cut off value of c-ELISA (<50) but the mean PI of the negative control was as expected (below 50 throughout the week) (figure 3).

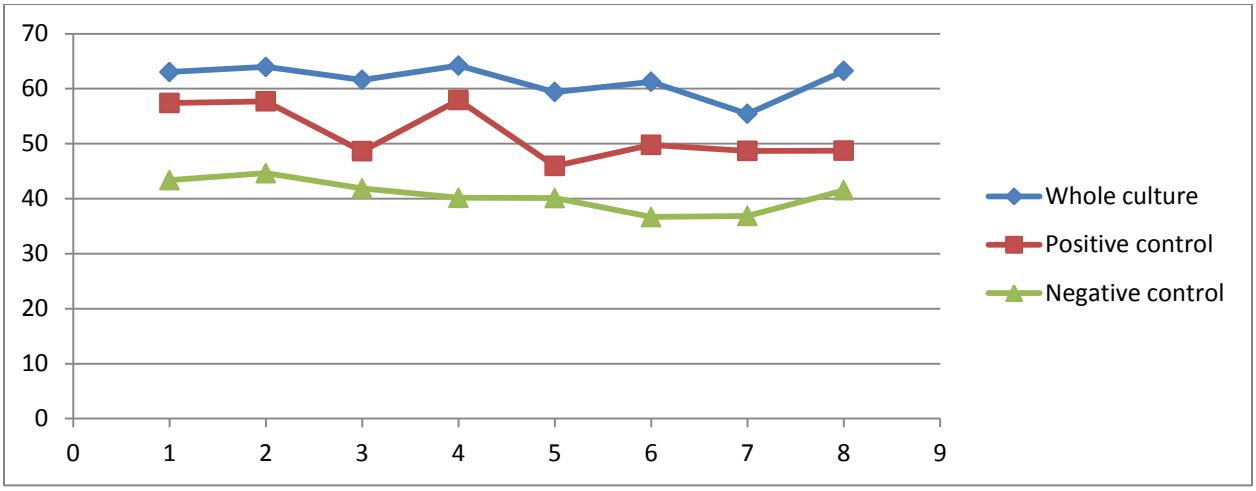

Figure 3.Mean percentage inhibition of week 1 to week 8

7. **DISCUSSION**

In this experimental trial inactivated whole culture CCPP trial vaccine was prepared based on standard operational procedure of CCPP vaccine production in NVI and the vaccine was prepared and checked for the quality control. The Safety test of this trial vaccine was done on goats prior to the commencement of the immunogenicity test. During analysis result of rectal temperature of safety tested goats which were recorded for 14 days on twice daily basis, none of the goats were showed abnormal body temperature. This was also seen on analysis result of rectal temperature records of immunogenicity tested goats, where the record showed absence of abnormality in whole culture vaccinated, positive control vaccinated and non-vaccinated controls. It has been recommended that for the vaccine to pass safety test, the animals showed no abnormal situation except minor swellings at the injection site which subsided within a week during observation not fewer than two weeks (OIE, 2014). So this trial vaccine was proved safe by this experiment.

The mean weekly sero-conversion (*Mccp* antibody positivity) of goats in three groups indicated that out of the whole culture vaccinated, 60.71% and out of the positive control vaccinated 58.86% were positive while non-vaccinated controls showed no sero-positivity throughout the trial of two months observation period. The sero-positivity between whole culture vaccinated and positive control vaccinated goats showed no significant difference between the two groups ($P>0.05$). This result indicated that the new trial vaccine has comparable merit to the existing NVI CCPP vaccine. It was impossible to compare this finding with other sources, as there was no previous works made on whole culture CCPP vaccine immunogenicity.

The mean percentage inhibitions of sera of three group goats were also compared to see the strength of sero-conversion during the experimental period. The result indicated that mean PI of whole culture is the highest (61.52±2.92) and the mean PI of positive control (51.86±4.95) is greater than that of negative control (40.65±2.84) and the difference was strongly statistically significant ($P=0.000$). The reason needs further investigation but it might be due to free lipopolysaccharides released to medium but which removed during concentration.

In this trial immunized animals were able to produce antibody during the first week of vaccination and has continued antibody production during the trial of eight weeks. As indicated on the literature after vaccination, antibody against *Mccp* has been known to reach peak level at 21 days and to protect goat for one year (OIE, 2014).

It is known that, challenge test is very important to check the protective level of a given vaccine beside other serological methods but due to lack of time, we did not conduct during the trial but, hope fully we will isolate the wild strain *Mccp* and we will do the challenge test.

In conclusion the newly designed whole culture CCPP trial vaccine is the better way for production of inactivated CCPP vaccine in large quantity and quality in order to satisfy customers inside the country and abroad beside the benefit of NVI. As it was shown on this experimental trial, the strength of antibody titer (as was seen by mean Percentage inhibition) of whole culture trial vaccine was higher than that of the former (old) CCPP vaccine based on serological test(c-ELISA).This could provide greater protection of goats that are vaccinated by whole culture CCPP vaccine than that vaccinated by existing vaccine but this needs further investigation by challenge study in the future.

8. CONCLUSION AND RECOMMENDATIONS

In the present study the trial whole culture CCPP vaccine was tested for its safety and immunogenicity in comparisons with existing vaccine and non-vaccinated controls. As it was shown in the result, the whole culture CCPP vaccine was found safe to vaccinated animals. In addition to this, the result also indicated that, the trial CCPP vaccine (whole culture) has equal merit to the existing old vaccine that the manufacturer, NVI is currently producing. On the other hand, even-though, significance difference was not observed in percentage positivity between the two groups of vaccine, the serum percentage inhibition strength for whole culture trial vaccine was higher than that of existing vaccine. It was also seen that the serum antibody started to appear from seven days post-vaccination and continued for two months observation period. After 21 days the antibody level reached peak and remained peak until 8 weeks. The protectiveness of such peak antibody level should be checked by challenge test.

From the obtained results of the present study, the following recommendations are forwarded:

- Inactivated Whole culture CCPP vaccine procedure SOP (standard operational procedure) should be developed and implemented.
- Challenge test should be conducted to confirm further protective level of whole culture CCPP vaccine by using wild strain *Mccp.*
- Field trial on safety and immunogenicity of inactivated whole culture CCPP trial vaccine should be conducted.

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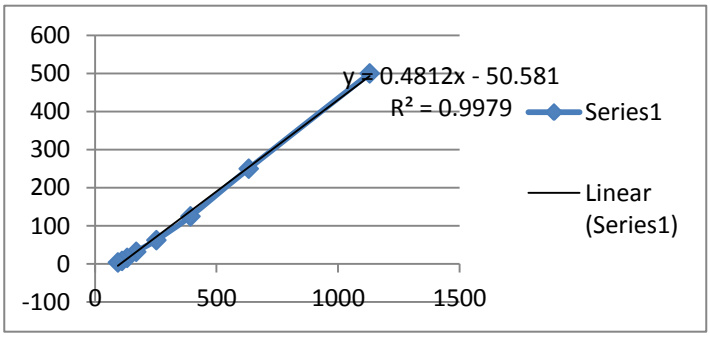
10. Annexes

Annex 1. Protein estimation of whole culture CCPP trial vaccine

Standard					Sample					
1119	1175	1096	1130	500	1145	1140	1145	1143.333	499.3633	998.7267
672	591	634	632.3333	250	686	686	733	701.6667	286.9217	1147.687
407	380	391	392.6667	125	428	414	428	423.3333	153.0433	1224.347
267	242	248	252.3333	62.5	253	250	255	252.6667	70.95267	1135.243
179	164	165	169.3333	32	131	165	171	155.6667	24.29567	777.4613
140	124	132	132	16	106	126	129	120.3333	7.300333	467.2213
117	116	100	111	8	94	103	107	101.3333	-1.83867	
104	91	87	94	4	85	94	94	91	-6.809	
									Ag in 1ml pellet	0.958mg
									20ml sample	64dose
									190ml whole culture	608dose



Annex 2. **C-ELISA protocol**

Controls may be dispensed anywhere on the micro plate.

Select the required number of coated micro plate and record the possession of each sample on a work sheet for each micro plate

1. Dispense samples, controls and Mab 4.52 into the prelate.

.Dispense 100µl of dilution buffer 24 in to each well of prep late.

.Dispense controls in to appropriate wells of prep late:

a) Dispense 110µl of dilution buffer 24 in to two appropriate wells (conjugate control wells:

Note: Total volume of dilution buffer 24 in CC wells: 210µl

b) Dispense 11 μl of undiluted strong positive control in four appropriate wells (strong positive control wells: SPC). NB: two SPC can be replaced by your own internal reference controls.

c) Dispense 11 μl of undiluted positive control in two or four appropriate wells (positive control wells: PC).

d) Dispense 11 μl of undiluted Negative control in two appropriate wells (Negative control wells: NC)

. Dispense 11 μl of undiluted sample per well in to remaining wells of prep late.

.Dispense110 μl of diluted Mab 4.52 in to each wells of prep late except in CC (no Mab).

.Homogenize contents of the wells of prep late.

2. Transfer 100 μl from each wells of the prep late to appropriate wells of coated micro plate.

3. Cover the micro plate (with a lid, almunium foil or adhesive plate cover) and incubate for 1hrs (±5 min.) at +37^{o}c (±3^{o}c) with a gentle agitation.

4. Wash each well with appropriately 300μl of washing solution two times. Aspirate or discard the liquid content of all well after each wash. Following the final aspiration, firmly tap residual wash fluid from each micro plate on to absorbent materials. Avoid micro plate drying between wash and prior to addition of the next reagent.

5. Dispense 100 μl of diluted anti mouse IgG HRPO conjugate in to each well.

6. Cover the micro plate (with a lid, almunium foil or adhesive plate cover) and incubate for 1hrs (±3 min.) at +37^{o}c (±3^{o}c) with a gentle agitation.

7. Repeat step 4 but this time wash thee times.

8. Dispense100 μl TMB substrate 9 in to each well.

9. Incubate 20 min at +37^{o}c (±3^{o}c) in a dark place.

10. Dispense 100 μl stop solution 3 in to each well. Shake the micro plate by gentle tapping. Wipe carefully the underside of the micro plate.

11. Measure and record the optical densities values of sample and controls at 450nm.

12. Calculate the result.

Note: when using robotics, incubation of micro plates in an incubation chamber allows working without plate cover. Use of robotics is also not compatible with gentle microplate tapping or wiping.

Plates can be held up to 1hr in the dark prior to reading.

The duration of substrate incubation can be adjusted to yield and OD of 1.00 in the MabC wells.

Interpretation of the result

.For this test, the positivity of threshold is set at 50% of percentage of inhibition

.Samples with percentage of inhibition greater than or equal to 50% is considered positive or presence of Mccp antibodies.

.However, every measurement has an uncertainty which depends from the kit itself and of the capacities of the testing laboratory. It is advisable to perform this ELISA testing under quality assurance, whenever possible with an acceleration (i.e. ISO17025), and determine this uncertainty of measurement around the cut off value.

Annex 3. **Temperature record**

Temperature record for safety of whole culture CCPP trial vaccine										
controls					Vaccinated for safety					
Day	c-04		C-25		c-34		C-22		C-06	
	Mor	aft	Mor	aft	Mor	Aft	Mor	aft	Mor	Aft
1	37.9	40.7	39.5	41.4	38.8	39.6	40.5	41.5	38.8	39.9
2	38.3	40.1	39	40.6	38.3	40.1	38.7	40.3	37.8	39.7
3	37.2	40	36.5	40.2	37.4	39.8	38.1	38.9	37.2	40.1
4	36.3	39	37	39.1	37.9	39.1	37.9	39	35.9	40.1
5	38.3	40.2	38.2	40	38.4	39.8	38.4	40.2	36.6	40.1
6	37.1	39.2	38.1	40.1	38.5	39.2	38.8	40.1	38.2	39.9
7	37.9	39.8	38.1	39.6	38.1	39.1	38.1	39.6	38.6	40.2
8	37.7	39.2	38.3	39.2	38.3	39.7	38.2	39.4	38.4	39.7
9	37.6	39.3	38.4	39.3	38.8	39.6	38.4	39.7	38.6	40
10	37.8	39.2	38.6	39.8	38.4	39.8	38.2	39.4	38.5	39.4
11	37.6	39.4	38.4	39.6	38.9	39.4	38.6	39.4	38.6	39.7
12	37.8	39.3	38.3	39.7	38.6	39.7	38.3	39.4	38	40
13	37.6	39	38.6	39.3	38.6	39.7	38.6	39.7	38.7	39.4
14	38.1	39.2	38.2	39.6	38.2	39.6	39	39.7	37.9	39

Temperature record for whole culture trial vaccine group														
day	571		580		581		587		C-31		C-33		c-17	
	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft
1	38.4	38.5	40.2	40.4	39.1	40.5	39.1	40	38.9	40.3	38.8	40.2	39.4	40.2
2	38.2	39.4	39	40.1	37.2	39.6	37.4	39.6	38.9	40.2	39.9	39.6	38.3	39.3
3	38.1	39.4	39.2	39.9	37.1	39.1	37.1	40	38.7	39.7	39.5	39.3	38.1	39.1
4	38.4	39.9	38.2	39.8	37.8	39.9	38.1	39.5	39	39.5	39.1	39.2	39	39.2
5	38.4	39.4	38.2	39.1	38	39.3	38.7	37.3	38.9	39.5	37.7	38.2	38.3	39.1
6	38.1	39	38.4	39.1	39.9	40	38.4	39.2	40.2	40.3	38.7	39.6	38.7	39.7
7	38.2	39	38	39.1	38.4	39	38.2	39.1	38	39.2	39	39	38.4	39.3

Temperature record for positive control group														
day	21		19		574		577		583		588		15	
	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft
1	37.9	40	39.2	40.1	37.8	38.1	38.8	38.1	38.6	39.2	38.8	40.4	39.1	40.4
2	36.8	39.1	39.9	39.8	39.1	40.1	38.6	40.2	38.1	39.5	37.8	40	39.5	40.9
3	37.3	39.2	39.8	39	38.2	39.2	38.3	39.1	38.2	39.2	37.9	40.1	39.2	40.2
4	37.1	39.1	39.8	39.7	38.1	39.9	39.1	40.1	39.1	39.2	37.1	40.1	39.1	39
5	37.1	38.1	38.2	40.1	38.9	39.8	38.7	38.8	38.7	39.5	37.9	39.9	38.8	39.6
6	37.1	38	39.1	39.7	38.7	39.4	38.6	39.5	38.8	39.7	38.5	39.7	39.5	40
7	38.	39	38.4	39.1	38.2	38.5	37.8	39	38.2	39.1	37.6	38.8	38.4	39.2

Temperature record for negative control group														
Day	24		26		575		586		14		5		4	
	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft	Mor	Aft
1	38.5	39.7	38	39.9	38.5	39.3	38.9	39.6	39.8	39.2	38.7	40.2	38.4	39.5
2	38.6	39.8	37.4	40	38.3	40.6	38.6	40.1	38.2	39.2	38.2	40.2	38.9	39.7
3	38	39.9	37.9	40.1	39	40.1	39	40.1	38.1	39.3	38.4	40.1	39	39.7
4	38	39.1	38	40.1	39.1	39.7	38.1	39.2	38.1	39.1	37.9	40.2	39.4	39.7
5	37.9	39.2	37.9	39	37.8	39.3	38.5	39.8	38.4	39.8	38.7	39.9	38	39.7
6	38.3	39.9	38.1	39.7	38.4	39.7	39.1	39.5	38.2	39.6	38.4	39.8	38.5	39.6
7	38.2	39.6	37.8	39.8	38.1	38.8	37.5	38.9	37.7	39.2	38.1	39.3	37.5	38.7