Thesis Reference No.____________

ADAPTATION OF INACTIVATED *MYCOPLASMA GALLISEPTICUM* VACCINE IN ETHIOPIA

MVSc. RESEARCH

BY: ASHETU NEGEWO

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

MASTERS PROGRAM IN VETERINARY MICROBIOLOGY

JUNE, 2020

BISHOFTU, ETHIOPIA

ADAPTATION OF INACTIVATED *MYCOPLASMA GALLISEPTICUM* VACCINE IN ETHIOPIA

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 veterinary Science in Veterinary Microbiology

BY: ASHETU NEGEWO

JUNE, 2020

BISHOFTU, ETHIOPIA

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by: Ashetu Negewo entitled “Adaptation of inactivated *Mycoplasma gallisepticum* vaccine in Ethiopia" and recommend that it is accepted as fulfilling the thesis requirement for the degree of: Masters of veterinary Science in Veterinary Microbiology.

____ ___________ _____________

Chairman Signature Date

____ ___________ _____________

External Examiner Signature Date

____ ___________ _____________

Internal Examiner Signature Date

Advisors

1. Dr. Fufa Dawo (DVM, MVSc, PhD Associate prof.) ____________ ________
   Principal advisor Signature Date

2. Dr. Takele Abayneh (DVM, MVSc, PhD) ___________ ________
   Co- advisor Signature Date

Approval sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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Title: adaptation of inactivated *Mycoplasma gallisepticum* vaccine in Ethiopia.

Submitted by: Ashetu Negewo ________________ ____________

Name of student Signature Date

This thesis Approved for submittal to MVSc research thesis assessment committee.

1. Dr. Fufa Dawo (DVM, MVSc, PhD, Assoc. Prof)_____________ ___________

   Major Advisor Signature Date

2. Dr. Takele Abayneh (DVM, MVSc, PhD Senior researcher)__________ ______

   Co- advisor Signature Date

3. Department Head Name_______________ signature _______ Date______

STATEMENT OF AUTHOR

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Name: Ashetu Negewo Signature: ______________________

Addis Ababa University College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: **June 8, 2020**

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ACKNOWLEDGEMENTS

I would like to acknowledge all my family members with deep appreciation for their indispensable helps and encouragement. First, I would like to praise to the Lord for all his help in my life and my accomplishment in this study. I would like to acknowledge Dr. Marta Yami and the Management committee for giving me permission to attend further education.

I am also grateful to my advisor, Dr. Fufa Dawo, Dr. Liyuwork Tesfaw and Dr. Takele Abayneh for their advice guidance and correction of this manuscript.

I would like to thank the overall assistance of the National Veterinary Institute (NVI) by providing materials and space during the whole period of my research work. Full cooperation of NVI staff members Dr. Liyuwork Tesfaw, Ato Alebachew Belay, W/o Webet Woldemdehin, Ato Abinet Legesse, Ato Tulu Yadete, Ato Getachew Alemu and Ato Dereje G/yohanes by attending target animals and during laboratory work is unforgettable.

Finally, I would like to express my thanks and respect to my wife Ms Tigist Mengistu and my son Hundiket Ashetu.

ABBREVIATIONS

CFU	Colony forming unit
CRD	Chronic respiratory disease
CSA	Central statistical agency
HI	Hem agglutination inhibition
IB	Infectious bronchitis
LAMP	Loop-Mediated Isothermal Amplification
MG	*Mycoplasma gallisepticum*
MI	*Metabolic inhibition*
MM	*Mycoplasma meleagridis*
MS	*Mycoplasma synoviae*
NCD	New castel disease
OIE	Office International Des Epizootics
PPLO	Pleuropnemonia like organism
RPM	Rotation per minute
RSA	Rapid serum agglutination
RT-PCR	Real time polymerase chain reaction
SOP	Standard operation procedure
SPA	Serum plate agglutination test
SPF	Specific pathogen free

ABSTRACT

However Mycoplasmosis is economically very important disease in poultry farm, but still the disease is undermined in Ethiopia. There was no more study and adapted vaccine on this infectious disease. This experimental study was taken from December 2018 up to May 2020 at National Veterinary Institute, Bishoftu, Ethiopia. The objective of this study was to adapt inactivated *Mycoplasma gallisepticum* vaccine from National veterinary gene bank, to determine appropriate media which already used by national veterinary institute for production of mycoplasma vaccine of both contagious bovine pleuropnemonia and contagious caprine pleuropnemonia, for growth of *Mycoplasma gallisepticum* bacteria and to evaluate the adapted inactivated vaccine, safety by follow up of vaccinated chicken for seven days for any clinical sign and death. The media selection was by important three experiments which were optical density, power of hydrogen meter reading and titration. Identification of *Mycoplasma gallisepticum* vaccine strain was conducted by conventional polymerase chain reaction using species specific primers targeting *Mgc2* gene of *Mycoplasma gallisepticum* at 185bp before using for vaccine production. Polymerase chain reaction confirmed strain was used for the production of oil based formaldehyde inactivated *Mycoplasma gallisepticum* vaccine. In this study each activity was done according to national veterinary institute standard operation procedure. A total of 60 chickens were used for this experimental study. Chickens were grouped into three groups. All purchased chickens were screened by indirect Enzyme link immune sorbent assay test against *mycoplasma gallisepticum* anti body. The identity test of *mycoplasma gallisepticum* vaccine strain was positive at 185bp. The experiment indicates that contagious caprine pleuropnemonia growth media was proper media for growth of *Mycoplasma gallisepticum* vaccine. All chickens were free from *mycoplasma gallisepticum* anti body. The vaccine was considered to safe against *Mycoplasma gallisepticum* vaccine if the number of surviving vaccinated chickens that show no clinical sign and death at the end of the experiment.

Keywords: inactivated vaccine, *mycoplasma gallisepticum*, National veterinary institute and polymerase chain reaction.

1 INTRODUCTION

The total poultry population in Ethiopia was estimated to 56.53million. With regard to breed, 94.31%, 3.21% and 2.49% of the total poultry were reported to be indigenous, hybrid and exotic respectively. All poultry under rural holdings in this country are free ranging. Recently, commercial flocks have been emerged in urban and other urban areas in central parts of the country (CSA 2017).

In previous study there were no more report and adapted *Mycoplasma gallisepticum (Mg)* vaccine in Ethiopia, due to these the *Mg* vaccine was imported by foreign currency, which was difficult for both poultry farm owner and country.

However, the country is not benefitted from the sector. This is due to different contributing; Factors, among which are low genetic potential of the indigenous breeds, high prevalent of infectious diseases and traditional feeding practice (Dinka *et al.,2010).* A number of microbial diseases are the major health hazards being faced by poultry birds and mycoplasmosis is of paramount important. Among these species, *Mg* is the most important cause of chronic respiratory disease (CRD) in chickens. Although young birds are more susceptible than old birds to this disease but, the disease affects all age groups of birds. *Mg* is transmitted either horizontally from clinically infected or carrier birds by direct contact, or vertically from some carrier birds through trans ovarian transmission (Hassan and Khan 2014). Serious *Mg* infections in poultry farms are notifiable diseases for the World Organization for Animal Health (OIE, 2012).

In Ethiopia, although detailed studies are not available to estimate losses as a result of different prevailing diseases of poultry, plus avian mycoplasmosis, so these all under mined poultry disease needs detailed studies and vaccine as poultry are sensitive to different diseases and management problems. Current studies on avian mycoplasmosis revealed that the disease is prevalent in chicken in East Shewa, Ethiopia and variation in prevalence was observed among three commercial farms, selected from Bishoftu which

coded as A,B and C. the prevalence ranges from 4.25% to 46.8% (Jibril *et al*. 2018). Prevalence in local chickens at the level of some Woreda was also ranges from 47.5% to 72.7%.

Even though the *Mg* vaccine production capacity in Ethiopia, the country is using the vaccine of *Mg* (6/85 or ts-11) commercially present by importation which is via foreign currency and with higher transportation cost this making the present mycoplasma vaccine to be more costly for use at poultry farm, indicate there were no adapted vaccine of *Mg* in Ethiopia.

Therefore, the objective of this experimental study was adoption and production of *MG* vaccine from National veterinary institute gen bank and determining of appropriate media for production of vaccine.

OBJECTIVES:

- ✓ To adapt inactivated *MG* vaccine from NVI Gene bank of vaccine strain.
- ✓ To determine the appropriate media for *MG* trial vaccine growth through to time, titer and OD value.

2 LITERATURE REVIEW

Indigenous chickens are local breeds of chickens [*Gallus gallus domesticus*] reared in rural areas of most parts of the world. Commonly, no proper housing is provided and very little food supplementation is offered. They move freely, scavenging for food and water, nevertheless, these chickens provide eggs and meat to most rural and many urban consumers. In many Sub-Saharan African countries, the productivity of indigenous chicken is hampered by several infectious diseases.

Moreover, it is widely believed that indigenous chickens may act as potential reservoirs for important poultry diseases. Infectious disease such as avian mycoplasmosis is mentioned as a potential constraint to the health status and productivity of domestic chickens. The disease is mainly caused by two pathogens: *MG* and *MS.*

It causes- considerable reduction of weight gain and meat quality, increase in feed conversion rate in broilers, severe drop in egg production in layers, or increase in embryo mortality. Chickens may have not obvious symptoms or may exhibit coughing, sticky nasal discharge difficulty breathing, swelling of the face, sneezing, foamy secretion in the eyes, and a drop in body weight as well. Literature on the epidemiology of avian mycoplasmosis in backyard chickens in Africa is with few reports in Zimbabwe, Botswana, Benin, South Africa, and Ethiopia (Júnior *et al*., 2017).

2.1 **Etiology**

Taxonomy of mycoplasma

The *MG* is a domain of bacteria which belongs to Phylum: *Firmicutes,* Class: *Mollicutes,* Order: *M.tales,* Family: *M.taceae,* Genus: *Mycoplasma,* Species: *Mycoplasma gallisepticum.* Mycoplasma is belonging to the class Mollicutes (mollis=soft and Cutes=skin).

2.2 **Pathogenicity**

Mycoplasmas are the smallest free living eubacteria but differ from other bacteria not having cell wall. The complete genome sequence of *MG* strain reveal the presence of 996,422bp with an overall G+C content of 31% (Prajapa *et al*. 2018).

Some bacteria resistant to antimicrobial acts on bacterial cell wall such as penicillin, due to lack of cell wall. The economic significance of *MG* is much higher than *MS*. However, the economic impact of *MS* is now increasing worldwide by affecting egg shell quality and egg production with the recent emergence of arthropathic and amyloido-genic strains. Reduced body weight and poor feed conversion in broiler birds has long been reported in *MS* infected broilers (Muhammad *et al*. 2018).

Mycoplasmas are generally flask shaped with a narrowed tip structure that enables gliding motility and mediates tight attachment to host cell surface. LAMPs showed a more significant up-regulation of IL-1β expression. (Yu *et al*, 2018). Gliding motility allows mycoplasmas to escape mucocilary clearance, enabling them access the mucosal epithelial cells.

Therefore these properties across various species of mycoplasma play a significant role in virulence and disease pathogenesis upon infection.

2.3 **Epidemiology**

Host range

MG has been isolated from naturally occurring infections in chicken, turkey, pheasants, partridge pea fowl, bob white quail, and Japanese quail. In broilers it leads to reduce weight gain, reduced feed conversion efficiency, increased mortality, and increased death at the slaughter house. In breeders and laying hens, this disease causes decrease in egg production and an increase in embryonic mortality and decrease hatchability and quality of day-old chicks. In other birds like pheasants, partridges, quail, ducks, geese and other avian species, it causes sinusitis and conjunctivitis. Production losses between 10 and 20%

have been reported in layers. All age group of chickens and turkeys are susceptible to mycoplasmosis but young birds are more prone to infection than adults (Bharathi *et al*. 2018).

Distribution of the disease

MG infection has become an important flock problem in chickens and turkeys in all areas with worldwide distribution (Bharathi, Mahaprabhu, & Geetha 2018).

2.4 Transmission

Mycoplasmas are transmitted laterally by contact, inhalation and injection of contaminated feed and water. Also the bacteria can transmit through coughed and sneezed by infected birds,totally the disease can be transmitted horizontally and vertically.Veneral transmission is particularly important in the case of *MM*. *MS* infection can also be through the conjunctiva and upper respiratory tract. It has been reported by that *MG* and *M. gallinaceum* have been isolated from the oviduct of chickens. This suggests that egg transmission of this species is possible and, no clinical sign seen by infected birds for long time and still the infected birds stressed. (Okpara 2016).

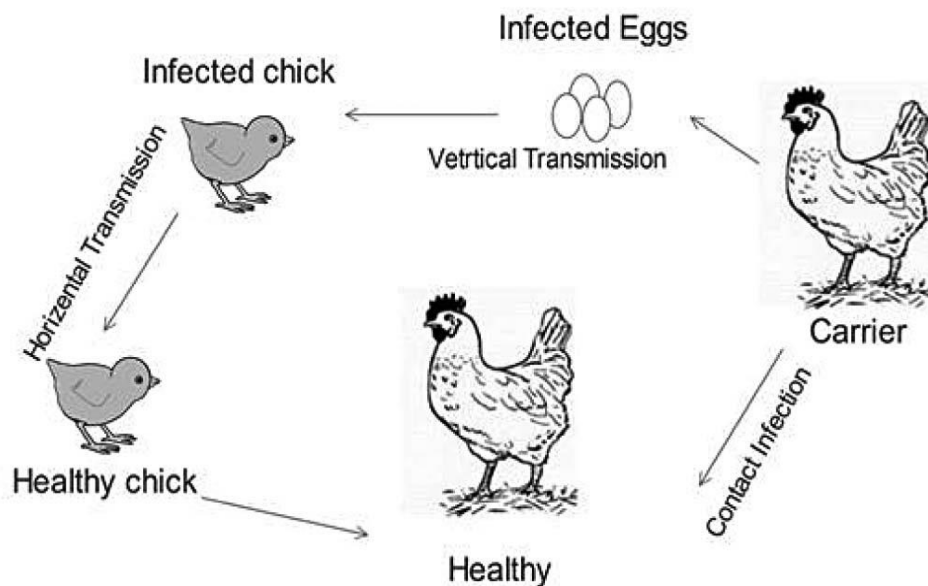

Figure 1: Transmission of *MG* (UMAR *et al*. 2016)

Predisposing factors

There are different explanatory variables which are risk factors for avian Mycoplas-lasmosis; among them Biosecurity, flock size, housing, management, season, Sex, age, route of infection, number of organisms and type or breeds of poultry (Islam1 *et al*. 2015).

The Mechanism of Disease Production

In the respiratory tract *MG* organisms have been observed on, in and between epithelial cells, this is due to it has initial an affinity for such cells. It is of interest, therefore, that observations made in chick embryo tracheal organ cultures infected with *MG* indicate that neither cyto adsorption nor hydrogen peroxide production are necessary for the impairment of cilial activity. Perhaps 2 main mechanisms of pathogenesis are involved; initially toxin production, especially when very large doses of organisms are given and later an immune response. The possibility that a toxin involved is supported by the following observations:

a) Similarity of the disease in turkeys with "rolling-disease" of mice,
b) The rapidity of onset,
c) Necessity for live organisms,
d) Protection afforded by anti-mycoplasma drugs even after the onset of signs,
e) Failure to influence the disease with immunosuppressive drugs,
f) The capillary endothelial reaction which is rapidly reversible and is
g) Unaccompanied by hyperplasia.

In the former, mycoplasma antigen without gammaglobulin is associated with the cardiac endothelium and inflammatory cells. This suggests that an inflammatory rather than an immune response is involved. This may be influenced by the relatively immature immune system of the embryo. In the production of osteodystrophy of poults caused by

M.meleagridis, interference with the nutritional supply rather than the blood supply to the growth plate seems to be significant since similar changes can be produced with deficiencies of choline, nicotinic acid and zinc, and furthermore the blood vessels appear normal. It has been suggested that no autoimmune mechanism is involved, but for all 3 mycoplasma a condition similar to biological imitation may play some part in pathogenesis

because it is generally much more difficult to produce growth inhibiting antibodies to these organisms in the chicken or turkey than in a species like the rabbit in which they are not pathogenic.

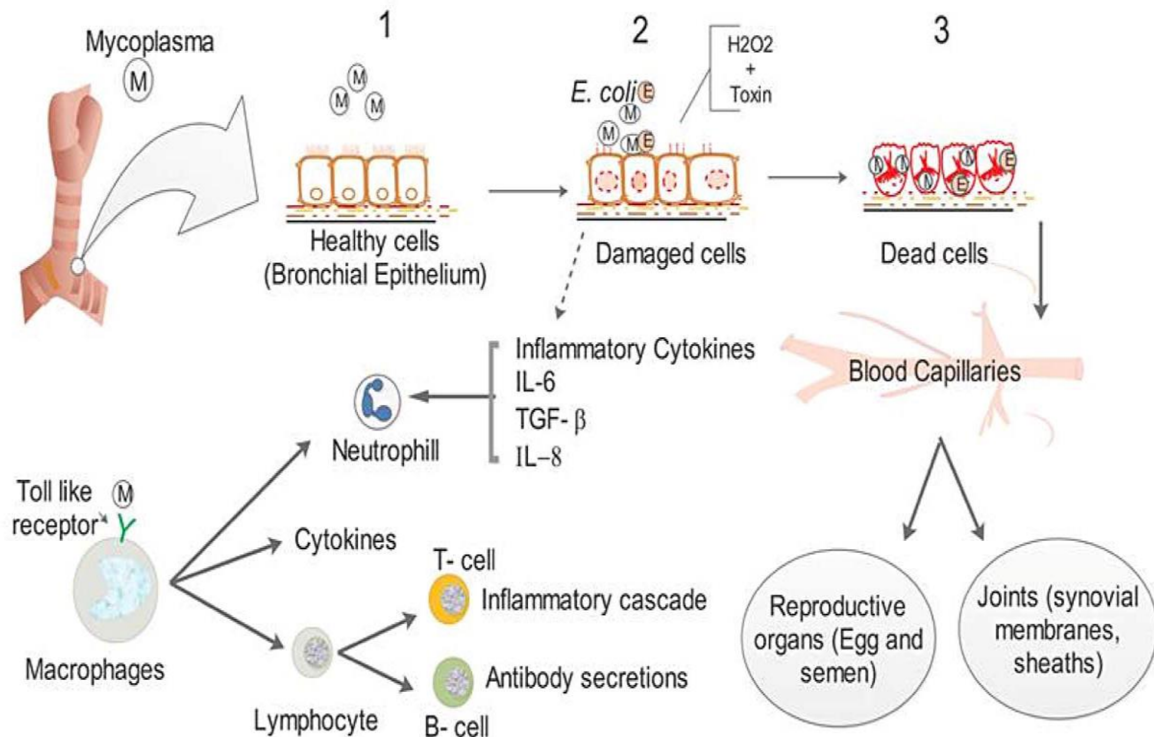

Figure 2: Mechanism of pathogenesis (UMAR, M, and S 2016).

2.5 Diagnosis of Avian Mycoplasmosis

Clinical Signs

MG infections vary from asymptomatic to severe, depending on the infecting strain and other factors. Except for very young birds, the development of clinical disease may depend on the presence of other pathogens or stressors. In natural infection, the most characteristic signs in adult flocks are tracheal rales, nasal discharge, and coughing, etc.

Figure 3: Advanced case of infectious sinusitis Source (Bekele and Assefa 2018)

Post Mortem Lesions

In uncomplicated cases in chickens, the lesions typically include mild sinusitis, tracheitis and airsacculitis (Bradbury, 2001). If the chicken is infected concurrently with *E. coli*, thickening and turbidity of the air sacs, exudative accumulations, fibrino purulentpericard-itis and perihepatitis may be seen (see fig 3).

The most important gross pathological Lesions are cloudy appearance of one or more air sacs (Ley and Yoder, 1997). The gross lesions consist primarily of catarrhal exudates in the nasal and paranasal sinuses, trachea, bronchi, and air sacs. Usually, cases become complicated and the air sacs frequently contain cheesy (caseous) exudate.

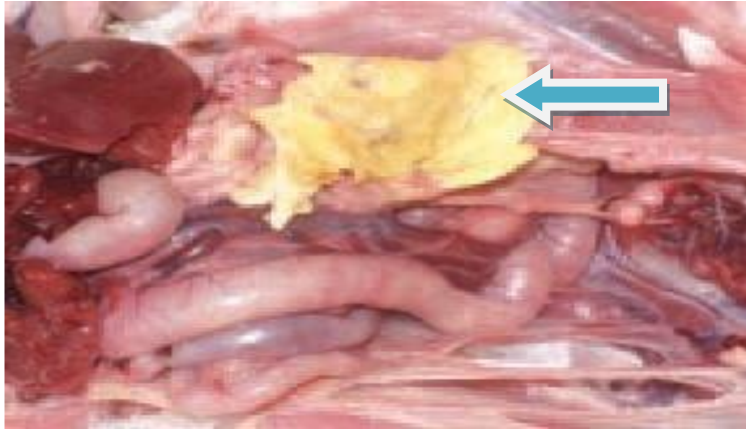

Figure 4: Aerosacculites (Calnek *et al*., 2014).

2.6 **Isolation and Identification**

MG and *MS* are relatively fastidious bacteria that require special nutrition.The gold standard for diagnosis of mycoplasmosis is isolation and identification of the causal organism. However, *Mycoplasmas* are slow growing organisms and it takes several days or even weeks for development of bacterial colonies. An early diagnosis of mycoplasmosis is critical in preventing the spread of infection.

Detection of *Mycoplasma* DNA in infected birds by PCR is a quick method of diagnosis. Commonly used media were PPLO broth, PPLO agar and Frey's agar medium which are some of the commercially available liquid and agar media used for *MG*/*MS* isolation; and after 72 hours of inoculation organisms appeared Fried egg-like colonies on these medium is typical of *MG*. Both *MG* and *MS* ferment glucose and form acid which can be detected by the phenol red indicator (Khatoon *et al*. 2018).

Serological Diagnostic Tests

Serological test like serum plate agglutination (SPA), hemagglutination inhibition (HI) and enzyme-linked immunosorbent assays (ELISA) are the mostly used for the diagnosis and to study the sero epidemiology of disease (Anna Sawicka *et al*. 2020).

Hemagglutinationinhibition

Test is based on inhibition of haemagglutinating capability of *M. gallisepticum* and *M. synoviae* to avian red blood cells by specific antibodies in sera. HI has high specificity but the disadvantages are low sensitivity.

Serum plate agglutination test

It is a simple, quick, and inexpensive test for the screening of *MG* and *MS* antibodies in serum. Positive sample agglutinate the colored antigen after mixing equal amounts of tested serum sample and stained *M. gallisepticum* or *M. synoviae* antigen. The test has efficient sensitivity but of low specificity.

ELISA

Several commercial *MG* and *MS* antibody ELISA kits are marketed. The sensitivity is determined to some extent by the manufacturer's recommendations for the cut-off levels for positive and suspicious reactions. Sensitivity may sometimes be 'damped down', to avoid the well-known cross-reaction between *MG* and *MS* (OIE terrestrial manual, 2018). Asimilar ELISA has also been marketed for *MS*. One advantage is that the system can be used for sera from any avian species without adaptation (OIE terrestrial manual, 2018).

Molecular techniques

Accuracy, time saving, and cost effectively of molecular techniques made them to complementary or even alternative for conventional diagnostic methods. PCR assays proved to be sensitive and specific for *MG* detection. Various PCR assay are described by different worker and used for diagnosis and screening of samples. Several PCR primers targeting different genes of *MG* were described previously. Besides selection of the mgc2 gene sequence has been used successfully for the differentiation of *MG* strains, including field and vaccine strains (Prajapati *et al*. 2018).

Differential Diagnosis

Since *MG* frequently complicated with other respiratory disease among others including NCD and infectious bronchitis, (Gross *et al*., 1990; Omuro *et al*., 1971; Soeripto *et al*., 1989), *MG* must be differentiated from these common respiratory diseases in chickens. NCD and IB or their antibodies may be present as separate entities or as part of the complicated CRD problem (Gross *et al*., 1990). Infectious coryza and fowl cholera can be identified by culture. *MS* infection may be identified by serologic test. Sometime supplicat

of both serologic and cultural tests are necessary to differentiate *MG* from other respiratory organisms. Other infections that should be considered in differential diagnosis include avian influenza, aspergillosis, and chlamydiosis.

2.7 Economic Importance

Due to *Mycoplasma gallisepticum* infection many poultry farms throughout the world are loss a million of US $ every year. This is a serious economic loss. Also, chickens have been documented to lose about 16 eggs over their laying cycle of 45 weeks (Peebles *et al*., 2012).

2.8 Control And Prevention

Test and slaughter are the most effective control measures for total eradication of *MG* infection, but in practice this is expensive and impossible, Due to its economic important (Hassan & Khan 2014).

Vaccines

Vaccination is an effective mean for controlling *MG* or *MS* in breeder and layer farm and it add to biosecurity measures of farm by enhancing the immunity of birds. Both killed and live vaccines are currently in commercial use. The commercial *MG* vaccine is used mainly in breeding flocks but also increasingly in laying flocks.

Killed Vaccines

MG killed vaccines (bacterins) protect young birds, egg layers from infection with virulent *MG*. Vaccines have been shown to reduce but not eliminate colonization by *MG* following infection (Prajapati *et al*., 2018).

Live vaccines

Among the live vaccine available for MG control, strrainst11 and 6/85 have long been applied in South Africa whereas the F-strain vaccine was not registered for use until the first quarter of 2015. Both ts11 and 6/85 are reported as avirrulent, safe and efficacious; which indicates that they are greater safe and low potential for transmission to unvaccinated flocks in comparison to F strain (Bwala *et al*. 2018).

Biosecurity

Biosecurity is important mean to prevent the entry and spread of disease in flock**.** It includes the acquisition of birds free from *MG* and *MS* antibodies and constant monitoring of breeder flocks. Proper hygiene in farm also play important role in control of avian mycoplasmosis. The *MG* infection mainly is transmitted vertically through ovaries hence the preferred method for control is to maintain disease free flocks ((Prajapati, Ogisharadhya, & Patil 2018).

Use of antimicrobials

Mycoplasma is susceptible to antibiotics such as macrolides and lincosamides (tylosin), tiamulin, and fluoroquinolones. Use of antibiotics like tylosin and tiamulin in dipping solution of hatcheries egg have effective role in prevention of vertical transmission of mycoplasma. A variety of drugs or antibiotics have been found useful in the treatment of clinically affected birds and in reducing but frequently not eliminating infection in birds and hatching eggs (Farran 2018).
MG organism is susceptible to certain antibiotics like streptomycin, oxytetracyclines, chlor tetracycline, erythromycin, spiramycin, tylosin, lincomycin, and spectinomycin. However, some strains of *MG* have been reported to be rather resistant to streptomycin, erythromycin, and spiramycin. When the disease occurs, 0.05% to 0.1 % tylosin in drinking water can be given for three to five days. Oxy-TTC (oxysteclin or terramycin) or

chlortetracycline can also be given at the rate of 200grams per ton of feed for several days which is commonly employed treatment and tend to provide favorable results. Tylosin has been injected subcutaneously at 3 to 5mg per kg of body weight or administered at 2 to 3gm per gallon (3.79liter) of drinking water for 3to 5 days. Antibiotic treatment or injections of infected breeding stock to control egg transmission have been used. Treatment may also be given for *E.coli*, the common complicating organism in the case of CRD. Egg dipping using tylosin or gentamycin solution is also used for treating hatching eggs.

2.9 **Status Of Avian Mycoplasmosis In Ethiopia**

According to (Jibril, *et al*.,2018) the prevalence of the *MG* in Ethiopia was investigated in different farm and Woreda. Higher prevalence of *MG* was reported in back yard chickens in Ethiopia (67.7%), (Júnior *et al.* 2017). In Ethiopia government poultry owned, non-governmental and private poultry owner still intensive management of small flocks up to farm of exotic breeds (Mammo, 2012). These holding numbers of flocks without healthy facility at urban and suburban facilitate the disease spread. As a result, more urban and suburban households now keep flocks of 50 to 1000 birds under semi-intensive management (Sambo *et al.,* 2014).

There is afew report of these diseases in Ethiopia; therefore, identification of circulating bacteria, from commercial farms and in village chickens from live markets both serologically and through culture and PCR (OIE, 2012) is important for appropriate vaccines production for control of the disease. In Ethiopia, CRD was identified as the major respiratory disease of chickens (Alamargot 1987), even though *MG* was not isolated. According to (Chanie *et al*., 2009), the occurrence of *MG* outbreaks in broiler chicken observed in three commercial poultry farms located at Bishoftu associated with Overcrowded, poor housing, sanitation and changes in environmental factors. Some reports have indicated the prevalent nature of infectious diseases (Lobago*et al*., 2003a, 2005; Ashenafi *et al*., 2004; Lobago and Woldemeskel 2004; Zeleke *et al*., 2005) in Ethiopia. Acording to Lobago and Woldemeskel (2004) study on sero-prevalence revealed that *MG* infection is widespread in village chickens. Natural infection with *MG* h

as been incriminated in average egg production loss in back yard chickens. There have been major changes in methods of keeping poultry where it becomes to be the most intensified of all branches of livestock farming.

However, health and production aspects associated with intensification should be considered, especially in developing countries like Ethiopia where intensive poultry production systems are emerging. Despite the growing importance of *MG* infection in Ethiopia, so far there is little information on epidemiology of *MG* infection in both commercial and backyard poultry production system. Therefore, in order to prevent and control *MG* infection in chicken of Ethiopia using vaccination, isolation and molecular characterization of *MG* strains circulating in Ethiopian chicken is important.

3 MATERIALS AND METHODS

3.1 Study Area

Enter of this experimental study and any experiments performed during this study were conducted at National Veterinary Institute from December 2018 to May 2020, located in Bishoftu town of Oromia regional state. The town is situated at 47 km south east of the capital city, Addis Ababa. It lays 9^{0} N latitude and 4^{0}E longitudes at an altitude of 1850 meter above sea level in central highlands of Ethiopia.

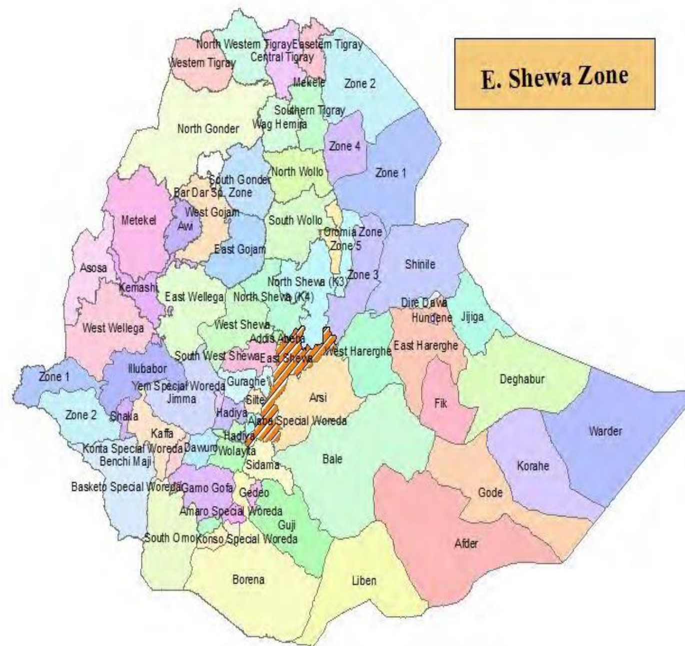

Figure5: Study area by Ashetu Negewo

The figure is sketched by Quantum Geographical System (QGIS 3.10.0) software map.

3.2 Study Population

A total of forty (40) chickens, white leghorns of the same sex, nine to ten weeks of age group and with good body condition purchased from Alema poultry farm and screened for the presence of *MG* antibody using indirect ELISA test by using ID vet kit (OIE, 2012)

according to the manufacturer procedure. Chickens were kept at NVI research and diagnostic laboratory experimental animal facility.

Figure 6: Experimental chickens, photo by Ashetu Negewo

3.3 **Study Design**

Chickens free or screened by indirect ELISA test, using ID vet kit against *MG* antibody were included in this trail. The chickens were grouped in to three groups of twenty chickens, according to this the first twenty chicken (G1) and G2 vaccinated with 1ml and 2ml oil adjuvated inactivated *mycoplasma gallisepticum* vaccine respectively for safety test of the vaccine and the third group (G3) was grouped as control. Chickens were randomly assigned to their treatment groups. Experiments were conducted according to the standard operation procedure SOP) for laboratory animal use at NVI.

3.4 **Media**

Two types of media, CCPP growth media (hayflick) and CBPP growth media (Gourlay) were prepared to compare the growth of *MG* against time and titer. Both media were prepared according to NVI media preparation guideline. Hay flicks' media containing mycoplasma broth 21.0g, glucose anhydrous 1gm, pyruvic acid 4g, fresh yeast extract 100ml, DNA (2%) 1ml, nystatin 50,000 IU, penicillin 200,000 IU and ingredients were reconstituted with distilled water of 700 ml and finally supplemented with 10% horse serum. Media PH was adjusted to 7.6 and sterilized through 0.2µm EKS filtration pad. Gourlay media containing tryptose 20gm, glucose anhydrous 5gm, NaCl 5gm, $NaH_2PO_4$2.5gm, glycerol 3.5gm, yeast extract 1gm, penicillin 100,000IU, were reconstituted with 1000ml distilled water and supplemented with 10% horse serum. PH was adjusted to 7.6 and sterilized through 0.2µm EKS filtration pad.

3.4.1 Evaluation of growth

Two media types were used to evaluate the growth of inactivated *Mg* vaccine preparation, which were already used at NVI for the production other Mycoplasma vaccines. *Mg* was estimated for growth with the standard values of OD, PH and titer at the expected growth time. Growth was checked at different time intervals. First sterile media was measured and then cultured media was measured at 12, 24 and 72hrs.

3.5 **Candidate Vaccine Strain**

Identification of *MG* DNA was done by conventional PCR using species specific primer forward 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT C-3', and reverse 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC -3' targeting *Mgc2* gene of *MG* at 185bp. The origin of vaccine strain which used for vaccine production was from NVI gene bank.

3.5.1 DNA Extraction

One microliter broth culture of the respective MG isolates was used for DNA extraction. The culture suspended in 1 ml of PCR-grade PBS was centrifuged for 30 minutes at 14,000 rpm at 4°C. The supernatant was removed and the pellet was again suspended in 25µl PCR grade water and boiled for 10 minutes, then placed on ice for 10 minutes before centrifugation at 14,000 g for 5 minutes. The supernatant of suspended pellet after centrifugation was used as DNA template (OIE, 2012).

3.5.2 DNA amplification (Polymerase chain reaction)

Primers targeting *mgc2* genes of *MG* expected band size of 185bp was used for amplification. Primers used: forward 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT C-3', reverse 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC -3' (OIE, 2012) for PCR. Amplification of the *mgc2* gene was performed using Positive and negative controls and 45 µl volume of the reaction mixture was dispensed into each PCR tube. The tubes were then taken to another clean area where the appropriate DNA sample (5 µl) was added to each tube. Positive and negative controls were used in each run. The tubes were then placed in a thermal cycler (Applied Biosystem2720) for the following cycles: initial denaturation at 94°C/5mints for one cycle, 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and stored 4°C (OIE, 2012; ISO/IEC 17025:2005 NVI-QMS SOP).

3.5.3 Agarose gel Electrophoresis

2% w/v, agarosegel was prepared in 0.5xTris borate EDTA buffer and dried for 20 minutes. 1µl 6x loading buffer in 5µl PCR product and loaded into separate well of the pre-prepared gel. 1kb plus DNA molecular marker (10 µl of 100bp) was added into the first and last lane and run at 120 volt for 80 minutes using (Biorad thermo EC 2060, US system). Amplified PCR product was analyzed under UV light visualization of desired size of DNA bands in the gel red stained agarosegel under gel documentation system by

comparing with molecular marker (Uvitec, Cambridge CB41QB UK and Camera using Uvtec (UK).

3.6 Preparation of Inactivated *Mycoplasma gallisepticum* Vaccine.

Lyophilized two vials of PCR confirmed (*Mg*) strain obtained from NVI gene bank were reconstituted with five ml (5ml) of hayflick media were inoculated in 7.6 PH of hayflick media, and lyophilized two vials of *Mg* strain from NVI gene bank were reconstituted with 5ml of gourlay media were inoculated in 7.6 gourlay media which were prepared as NVI standard operation (SOP) and incubated at 37 ^{0}C at 10% CO_2 with slow agitation of 250 rotations per minute (**250RPM**) for 72hrs. The growth of culture after incubation was checked by PH meter reading and antigen concentration. PH meter was calibrated by buffer solution with PH7 and PH4 before used for growth check of culture. This PH meter reading value principles were with comparison of uninoculated media as control.

The trail vaccine was manufactured in suitable clean and well separated room to avoid the product contamination with other organisms and with other product manufactured in the same room by the same staff. Purity of the product was evaluated by gram staining; only slide background seen.

Figure7: Day one culture of hayflick and gourlay media at incubator, photo by Ashetu Negewo.

3.6.1 Standardizing and optimization of antigen Titer

Since high yield and good antigenicity was desirable in case of killed vaccines, at harvest the titer was adjusted to $10^{8-}10^{9}$ CFU/ml. This titer was determined by pour plate method with tenfold serial dilution using 0.5ml of culture inoculum per petridis of Mycoplasma agar based media.

3.6.2 Inactivation of trail vaccine and sterility test

Inactivation of *Mg* broth culture was done with the addition of formaldehyde at a rate of 0.125 % followed by incubation at 37^{0}C at 10 percent CO_2 tension to provide 12 hrs of interaction time, the inactivated broth was separately cultured on mycoplasma broth and agar, incubated at 37 ^{0}C at 10% CO_2 tension and observed for seven days for appearance of any specific growth of Mycoplasma colonies or color change, this is to ascertain the completion of inactivation process. Since there was no evidence of growth from inactivated culture, it was further processed.

3.7 **Adjuvant preparation**

The inactivated vaccine was prepared using Oil adjuvant (Montanide ISA70).Oil adjuvant was sterilized by heating at 160°c for one hour by steam autoclave. Sterility of the adjuvants was tested using test media such as, thioglycolate, tryptic soya broth tryptose agar, sabroude agar into which the oil and alum adjuvants were inoculated and incubated at +37°c for 48hrs. The media were followed-up for any growth of microorganisms.

Sterilized oil adjuvant was mixed with the trial vaccine separately in 1:1(one part trail vaccine and one part adjuvant) ratio. A sample of the adjuvated trial vaccine was tested for identity using species-specific PCR assay and the inactivated vaccine was stored at +4°C protected from light for subsequent evaluation of its Safety.

3.8 **Safety test of trial vaccine**

The safety of the killed (inactivated) trail vaccine of *MG* was evaluated in small group of chickens (eighteen chickens) inoculated in two different doses (1ml, 2ml) six of them with 1ml and six of them with 2ml subcutaneously at mid neck region. Six chickens were as control. Then the birds were under close follow-up 2 weeks for any post vaccinal adverse reactions.

4 RESULTS

4.1 Media Selection

Table 1: determining of media for good growth of *MG*

Time	Media type					
	Gourlay			hayflicks		
	OD	PH	Titer	OD	PH	Titer
0hrs	0.123	7.6	-	0.123	7.6	-
12hrs	0.253	7.12	10^2	0.256	7.03	10^4
24hrs	0.261	6.98	10^5	0.272	6.81	10^6
72hrs	0.261	6.72	10^6	0.481	6.67	10^9

PH, potential hydrogen OD, optical density

The above table revealed that, the growth of *MG* was evaluated in both Gourlay and hyphilic media. In comparison an optimal growth was observed in hyphilic media rather than Gourlay. The growth of *MG* measured as per criteria set at 72 hrs resulted OD = 0.481, this tells us the total density of both dead and live bacteria in broth culture PH = 6.67, this indicates that media changed from alkaline to acidic due to growth of bacteria in broth culture and Titer = 10^9CFU/ml. This result indicated that growth was in conformity with the standard 10^8-10^9 (OIE, 2012) in hayflicks media.

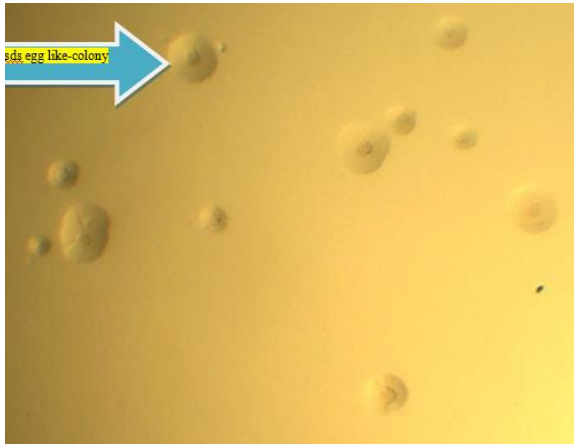

Figure 8: *Mycoplasma gallicepticum* colony, by stereomicroscope with camera and photo, by Ashetu Negewo

4.2 Candidate vaccine strain

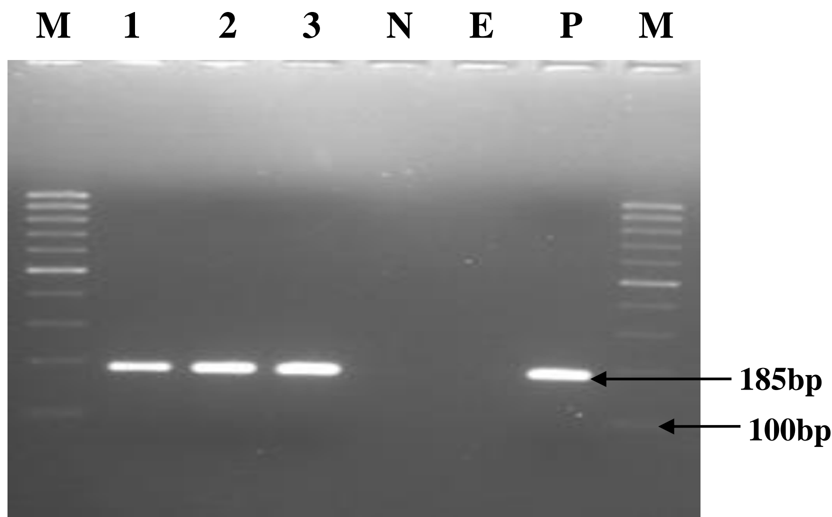

Figure 9: *MG* trail vaccine strain identity test

M- Molecular ladder started at 100bp. the same *MG* vaccine strain culture was used for identity test, as three samples for selection of good gel-electrophoresis picture. Lane1 was *MG* vaccine strain culture sample, Lane2 was *MG* vaccine strain culture sample and Lane3 was *MG* vaccine strain culture sample. N- Negative control without template- negative. E

– Extraction control (RNase free water) - negative. P- Known positive control – positive (around 185 bp).

The above figure showed that all *MG* candidate vaccine strains were positive around 185bp.

4.3 *MG* Trail Vaccine Identity Test

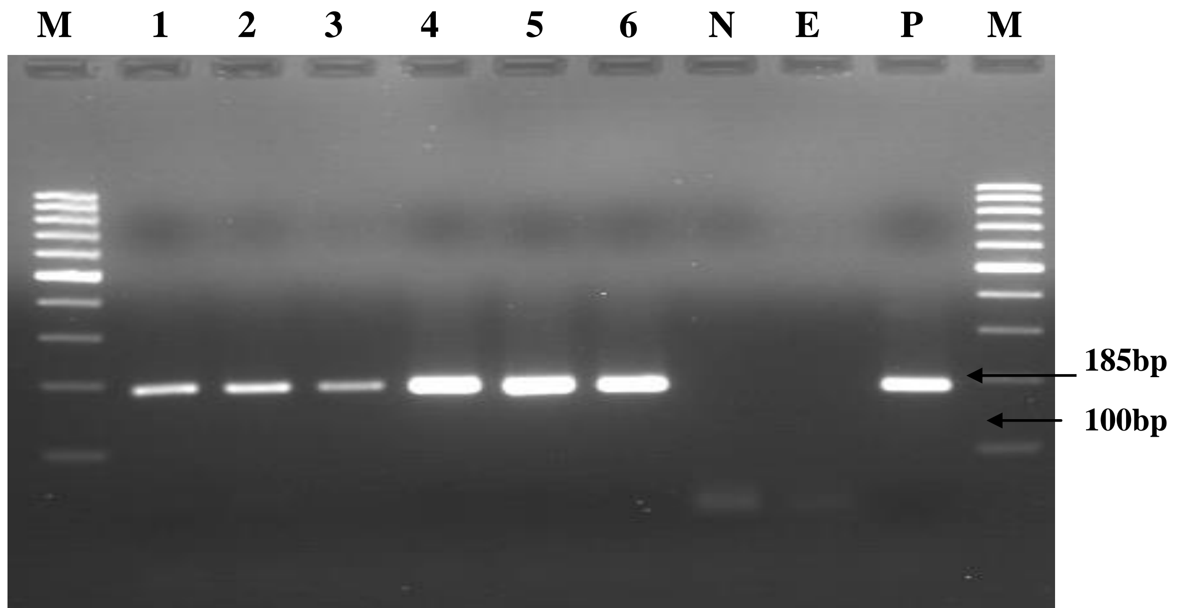

Figure10: Vaccine identity test

M-Molecular ladder was started 100bp. 1- represent *Mg* vaccine (original), 2-represent.*Mg* vaccine (1/10), 3 indicate.*Mg* vaccine (1/20), 4-shows *Mg* vaccine (1/40), 5-indicate.*Mg* vaccine (1/80), 6-reperesent.*Mg* vaccine (1/160), N was Negative control without template, E was Extraction control (RNase free water), P was Known positive control (around 185 bp).The above figure reveled that, *Mg* trail vaccine titer was positive around 185bp.

4.4 **Safety Test**

Double doses (1 ml) of reconstituted trail vaccine of *MG* was inoculated, subcutaneously at mid neck region (OIE, 2012) into six chickens and 2ml into six chicken. The injected chickens were followed for fourteen days, None of the chickens were dying and the chickens were not showed signs of respiratory distress, and other adverse effects related to injection of *mycoplasma gallisepticum* trail vaccine with double dose, the result indicates that the vaccine was safe.

5 DISCUSSION

According to (Júnior, Taunde, & Zandamela 2017), the prevalence and *Mycoplasma gallisepticum* infection in chickens represent a major problem for commercial poultry farms. The previous study demonstrated that *Mycoplasma gallisepticum* infections prevalent up to village (backyard) chickens. The study also indicates that some common clinical signs of *Mycoplasma gallisepticum* infection such as, nasal discharge, dyspnea, and mouth breathing, tracheal rales, facial swelling, gross post mortem lesions of overcrowded and hepatized lung, hyperemic and mucoid trachea, cloudy and thickened air sacs. *Mycoplasma gallisepticum* strain from NVI gene bank was grew well in Hayflicks broth media than gourlay broth media within 72 hours at 37˚C with 10% CO_2 and colony count result was 10^9 in colony forming units (CFU) per 1ml of the medium.

The adjuvant used for this trial vaccine was oil adjuvant. High yield and good safety of killed vaccines determined by first used seed as vaccine strain. Identity test was done for the seed before used it as starting working seed bank for trail vaccine production, and result indicates seed was free from all extraneous organisms. In this experimental study *MG* trail vaccine inactivated by, formaldehyde with concentration of 0.125% from NVI gene bank and identity confirmed with known primer using PCR was prepared.

Therefore, by this study it is concluded that Oil adjuvant based *MG* bacterin (killed *Mycoplasma gallisepticum* trial vaccine) is adopted for protection of infection from *Mycoplasma gallisepticum* in Ethiopia.

6 CONCLUSION AND RECOMMENDATION

Whatever there are no more prevalence studies in Ethiopia on *Mg* infection, some previous prevalent studies indicates the infection present both at backyard and commercial chickens.

For the future all poultry farms present in Ethiopia will hopefully uses this oil based inactivated *Mycoplasma gallisepticum* trial vaccine adapted at National Veterinary Institute, the vaccine also grant full for economic loss of poultry farms deuto this infection disease. The vaccines not only approve the farmer problems but, also approve the country's foreign currency, by exporting the vaccine. This is the first report on adapting Oil adjuvated (Montanide ISA 70) inactivated *Mycoplasma gallisepticum* vaccine in Ethiopia and the vaccine production department of National Veterinary Institute is potentially useful in the control of *Mycoplasma gallisepticum* and hence further work in validating and immunogenicity of the vaccine production should be considered.

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