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ISOLATION, MOLECULAR IDENTIFICATION AND VACCINE TRIAL OF MYCOPLASMA
GALLISEPTICUM IN ETHIOPIA

MSc Thesis



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Microbiology, Immunology and Veterinary Public Health

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SIGNED DECLARATION SHEET

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the

requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate

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ABBREVIATIONS

APC	Antigen presenting cells
CFU	Colony forming unit
CO ₂	Carbon dioxide
CRD	Chronic respiratory disease
CSA	Central statistical agency
DNA	Deoxyribonucleic Acid
EARO	Ethiopian Agricultural Research Organization
ELISA	Enzyme – Linked immune sorbent assay
FAO	Food and agricultural organization
GI	Growth inhibition
GIS	Geographic information system
HA	Hemagglutination
HI	Hemagglutination inhibition
IB	Infectious Bronchitis
IFA	Indirect fluorescent antibody
I _g G	Immunoglobulin G
I _g M	Immunoglobulin M
IP	Immuno peroxidase
MAB	Monoclonal antibody
MBB	Mycoplasma broth base
MG	<i>Mycoplasma gallisepticum</i>
MS	<i>Mycoplasma synoviae</i>
NAD	Nicotinamide adenine dinucleotide
NCD	New castle disease
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PPLO	pleuropneumonia like organism
RAPD	Restriction amplified polymorphic DNA

RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic Acid
RSA	Rapid serum agglutination
SPA	Serum plate agglutination test
USA	United States of America

ABSTRACT

The study and entire laboratory works were conducted from December 2014 to April 2015 in National Veterinary Institute, Bishoftu, Ethiopia. A total of 120(20 chickens from four farms and 40 backyard chickens from commercial farms and local markets respectively) were used for the study. The chickens were slaughtered at national veterinary institute postmortem room and tracheal, air sac and lung samples were collected from slaughtered chickens. Isolation and molecular characterization of *Mycoplasma gallisepticum* strains was done based on standard recommended methods. And the objective of this study is to identify local strains and to undertake molecular characterization of *Mycoplasma gallisepticum* circulating in the chicken population of farms in Bishoftu. This can help to devise strategies in controlling the disease mainly through developing more effective vaccine which will replace the currently being imported *Mycoplasma gallisepticum* vaccines by some farmers. Then propagation was undertaken using appropriate culturing procedure. The mycoplasmal DNA was extracted and polymerase chain reaction was conducted for amplification of *Mycoplasma gallisepticum Mgc2 gene*. Amplified deoxyribonucleic acid fragments were analyzed by conventional 2% agarose gel electrophoresis incorporating appropriate size markers, followed by examination under Ultra violet light. The Polymerase chain reaction product for *Mycoplasma gallisepticum* was 185 bp. A number of 3 pooled (from 30 chickens) mycoplasma isolates were recovered from chickens in 2 farms and from back yard chickens in Bishoftu. Of these, 3 Mg isolates were identified using growth inhibition and rapid serum agglutination tests. Among six isolates (3 pooled and 3 lyophilized National veterinary institute isolates), (50%) or 3 samples were found strong positive and (50%) or 3 samples were weak positive to *Mycoplasma gallisepticum* as they gave 185 bp products, similar to the positive control when visualized electrophoretical analysis. Finally oil based Inactivated *Mycoplasma gallisepticum* vaccine was produced in suitable clean and secure accommodation, well separated from production facilities of National Veterinary institute. In this study, formaldehyde inactivated Montanide ISA70 based *Mycoplasma gallisepticum* vaccine from the Polymerase chain reaction confirmed positive from Samuel local isolate of National Veterinary Institute was prepared and evaluated in chickens. The amount of immune antigen per 0.5ml of the dose was 10^7 Colony forming units of the bacteria. At the age of 16 weeks, the chickens were randomly divided into three groups (A, B and C), each having twenty birds. Each bird of group B was vaccinated group of imported- live vaccine with 30 μ l dropped in the eye and each bird of group C was inoculated with 0.5 ml indigenous or trial vaccine subcutaneously at mid neck region and group A was used as a control.

After challenge test, among non-vaccinated chickens (control or group A) 2 chickens were died (10 %), thickened and cloudy appearance of the air sac showed 18 (90%), 2 chickens were not showed thickened and cloudy air sack (10%). Although among vaccinated group (inactivated vaccine or group C) all chickens did not show clinical signs or post mortem changes (100 %). From attenuated imported live vaccine (group B) no clinical signs or post mortem changes was observed (100 %). It was concluded that oil based *Mycoplasma gallisepticum* vaccine induces protective level of anti *Mycoplasma gallisepticum* antibodies in chickens.

Keywords: *Adjuvant, Chickens, Inactivated vaccine, Mycoplasma gallisepticum, and polymerase chain reaction.*

1. INTRODUCTION

The total poultry population in Ethiopia is estimated to be 51.35 million (CSA, 2013/14). In the country, poultry accounts for 15% of the total per capital meat consumption. It is also estimated that per capital egg and chicken meat consumption are about 57 eggs and 2kg per annum, respectively (FAO, 1993). The sub sector is concerned with egg and meat production for income generation and home consumption (EARO, 2000; Mohammed, 1998). However, diseases among other factors are rampant and hindered the development of poultry production in the country; and mortalities due to diseases are estimated to be 20% to 50% but can go as high as 80% during times of epidemics (Alemu, 1995).

Avian mycoplasmosis causes considerable economical losses to the poultry industry, especially in chickens and turkeys all over the world. *Mycoplasma gallisepticum* (MG) is responsible for what is called chronic respiratory disease in chickens and infectious sinusitis in turkeys. In broilers, it causes reduction in weight gain, decrease in feed conversion efficiency, and increase in mortality rate and carcass condemnations in slaughter houses. In breeders and layers, the disease causes a drop in egg production and an increase in embryo mortality (Ley, 2003). Vertical transmission of MG has been documented and it leads to infected progeny flocks (Bradbury, 2001). MG also causes respiratory disease including sinusitis and conjunctivitis in turkeys, pheasants, partridges, quail, ducks, geese and other avian species (Ley, 2003).

M. gallisepticum is the most pathogenic avian mycoplasma; however, considerable strain variability is manifested with respect host susceptibility, virulence, clinical presentation, and immunologic response. Integral membrane surface proteins (adhesins) that attach to receptors on host cells, allowing for colonization and infection, are important virulence factors involved in antigenic variation and immune evasion. (Linares, 2013)

Mycoplasma gallisepticum and *Mycoplasma synoviae* can be identified by immunological methods after isolation in mycoplasma media or by detection of their DNA in field samples or cultures. DNA detection methods based on the polymerase chain reaction are used in specialized laboratories. Once validated, they can be used on swab material or cultures. Several serological tests are used to detect MG or MS antibodies. The most commonly used are the rapid serum

agglutination (RSA) test, the enzyme-linked immunosorbent assay (ELISA) and the hemagglutination inhibition (HI) tests. Several commercial MG and MS antibody ELISA kits are available (OIE, 2012).

MG infection is among one of the major economically important respiratory disease problems of both commercial and backyard poultry production systems (Talha, 2003). In Ethiopia, although detailed studies are required to estimate losses as a result of prevailing diseases of poultry, it should not be underestimated as poultry are sensitive to different diseases and management problems. With the huge population of chickens and the industry being growing, major disease problems of commercial and backyard chickens, which impact the health and productivity, has not been well investigated in the country. There is no documented work as to the status and distribution of *Mycoplasma gallisepticum* infection although the agent was isolated at the National Veterinary Institute (Yasmin, 2007).

Despite the growing importance of MG vaccine in Ethiopia, it is being availed in by importation. In addition, as they are acquired from elsewhere via foreign currency and with higher transportation cost making them to be more costly. Therefore, this calls for development and production of effective MG vaccine locally after isolation and molecular characterization of MG strains for the local production.

The objectives are:

- Isolation, Molecular Identification of MG isolates circulating in Ethiopia.
- To develop inactivated MG vaccine.

2. LITERATURE REVIEW

2.1. Avian mycoplasmosis

Avian mycoplasmosis can be caused by several species of *Mycoplasma* (class Mollicutes, order *Mycoplasmatales*, family *Mycoplasmataceae*) (Quinn *et al.*; 2002) including *Mycoplasma gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae*. *M. gallisepticum* is the most important pathogen in poultry (Bradbury, 2001). It also causes disease in other avian species. *M. gallisepticum* infections are also known as chronic respiratory disease (CRD) of chickens, infectious sinusitis of turkeys and house finch conjunctivitis. They are egg transmitted and may spread laterally by direct or indirect contact (i.e. both horizontal and vertical transmission is possible) (Ley and Yoder, 1997)

M. meleagridis, found only in turkeys, causes respiratory disease in young turkeys and is involved in stunting, poor feathering and leg problems. MG is a cause of chronic respiratory disease (CRD) and egg production drops in chickens, turkeys and other avian species; and it is the most economically important of the avian *Mycoplasmosis* and the only one that causes an OIE notifiable disease (Ley and Yoder, 1997).

MG causes chronic respiratory disease of domestic poultry, especially in the presence of management stresses and/or other respiratory pathogens. Disease is characterized by coryza, conjunctivitis, sneezing, and by sinusitis, particularly in turkeys and game birds. It can result in loss of production and downgrading of meat-type birds, and loss of egg production. MS may cause respiratory disease, synovitis, or may result in a silent infection. MG and MS strains vary in infectivity and virulence, and infections may sometimes be unapparent (Charles and Graham, 1989; OIE, 2012).

The severity and appearance of the disease may be influenced by concomitant infection, other pathogens and/or by predisposing factors. Predisposing factors include nutritional deficiencies and intensive management with excessive ammonia and dust. Other pathogens include the viruses of New Castle disease (NCD) and infectious bronchitis (IB), including vaccine strains and pathogenic strains of *E. coli* and *Haemophilus paragallinarum* (Omuro *et al.*, 1971; Gross, 1990).

Experimentally infected poultry develop symptoms after 6 to 21 days. In natural infections, the incubation period is variable; infected birds may be asymptomatic for days or months until stressed. However, under field conditions, the incubation period is not established due to many variables, which influence the onset and the extent of the disease. Field outbreaks of MG near the onset of production (between the 26th and 38th weeks of age) may suggest a long incubation period (Bukhari, 2005).

Clinical manifestations are usually slow to develop and the disease has a long course (i.e. chronic). Economic losses from downgrading of carcasses, reduced feed conversion and egg production efficiency, and increased medication costs are additional factors that make MG the most costliest disease problem confronting the poultry industry. Moreover, conducting adequate prevention and control program is also expensive. The disease is of little or no public health significance (Calnek *et al.*, 1991; Talha, 2003).

2.2. Etiology

Avian mycoplasmosis can be caused by several species of *Mycoplasma*. Generally, Mollicutes (soft skin) are small prokaryotic organisms being devoid of cell wall and lacking the genetic capacity to synthesize one; but they have a single trilaminar membrane composed of protein, glycoprotein, glycolipid, and phospholipid (Bradbury, 2001).

Their genome size is small with 5×10^8 to 1×10^9 daltons, the base composition is poor in guanine and cytosine with mol % G + C of DNA ranging from 23% to 40% (Hirsh *et al.*, 1999; Quinn, 2002). Since they lack a cell wall, they are extremely pleomorphic; cell shape being spherical, pear shaped, spiral, and filamentous forms. The size (diameter) of spherical or coccoid form ranges from 0.25 to 0.45 μ m. Numerous species of avian *Mycoplasmas* are described (Table 1) with their biochemical reactions and usual hosts (Charles and Graham, 1989).

Table 1. Characteristics of Avian Mycoplasma.

Mycoplasma Species	Usual Host	Glucose Fermentation	Arginine Hydrolysis	Phosphatase Activity
<i>M. gallisepticum</i>	Chicken, turkey	+	-	-
<i>M. synoviae</i>	Chicken, turkey	+	-	-
<i>M. meligradis</i>	Turkey	-	+	+
<i>M. iowae</i>	Turkey, chicken,	+	+	-
<i>M. gallopavonis</i>	Turkey	+	+	-
<i>M. cloacale</i>	Turkey	-	+	-
<i>M. gallinarum</i>	Chicken	-	+	-
<i>M. gallinaceum</i>	Chicken	+	-	-
<i>M. pullorum</i>	Chicken	+	-	-
<i>M. iners</i>	Chicken	-	+	-
<i>M. lipofaciens</i>	Chicken	+	+	-
<i>M. glycyphilum</i>	Chicken	+	-	+ or -
<i>M. columbinasale</i>	Pigeon	-	+	+
<i>M. columbinum</i>	Pigeon	-	+	-
<i>M. columborale</i>	Pigeon	+	-	-
<i>M. anatis</i>	Duck	+	-	+
<i>M. anseris</i>	Goose	-	+	-
<i>Acholeplasma laidlawi</i>	Various	+	-	+ or -
<i>Ureaplasma gallorale</i>	Chicken	-	-	
<i>Ureaplasma spp.</i>	Turkey	-	-	

Source: Calnek *et al.*, (1991)

Over 20 serotypes of MG have been discovered and the one to which chronic respiratory disease is attributable is known as S-6 serotype. It is found in chickens, turkeys, and ducks. The R-strain is widely used for the production of bactrins (inactivated vaccines) and it is the highly pathogenic (Virulent) strain; whereas the F, ts-11, and 6/85 strains are widely used for live vaccine production and have relatively poor pathogenicity (Talha, 2003; OIE, 2012).

2.3. Epidemiology

Mycoplasma gallisepticum causes disease in chickens, turkeys, and game birds including pheasants, chukar partridges, bobwhite quail, Japanese quail and peafowl. The organism has also been isolated from ducks and geese, as well as yellow-naped Amazon parrots, pigeons and greater flamingos. It has been found in wild peregrine falcons in Spain (Calnek *et al.*, 1991).

The severity and intensity of MG infection is variable. It is more severe and of longer duration in the cold months and it affects younger birds more severely than mature birds. Recovered birds may become carriers of MG (Bokhari, 2005). The disease commonly affects 4 to 10 weeks old birds especially the broilers. It may occasionally affect older birds as a complicated disease caused by other bacteria, *E. coli* and sometimes by respiratory viruses like NCD and IB viruses (Chauhan, 1993).

2.3.1. Distribution of the disease

MG infection has become an important flock problem in chickens and turkeys in all areas with worldwide distribution (Calnek *et al.*, 1991; Ley and Yoder, 1997). The prevalence of MG in commercial pullet and layer flocks in southern and central California, USA, in 1984 was estimated by testing serum and egg-yolk samples through ELISA. *M. gallisepticum* can be found worldwide. In the United States, this organism has been eradicated from most commercial chicken and turkey breeding flocks, but remains present in other poultry operations (Calnek *et al.*, 1991)

2.3.2. Transmission

M. gallisepticum is transmitted vertically within some eggs (transovarian) from infected breeders to progeny, and horizontally via infectious aerosols and through contamination of feed, water, and the environment, and by human activity on fomites (shoes, equipment, etc). Infection may be latent in some birds for days to months, but when birds are stressed horizontal transmission may occur rapidly via aerosols and the respiratory route, after which infection and clinical disease spread through the flock. Flock-to-flock transmission occurs readily by direct or indirect contact from the movement of birds, people, or fomites from infected to susceptible flocks (Ley and Yoder, 1997).

Some potential reservoirs of *M gallisepticum* in the USA are noncommercial (backyard) flocks, multiple-age layer flocks, and some free-ranging songbird species. Good management and biosecurity practices are necessary to ensure that *M gallisepticum* infections are not introduced to commercial poultry from these and other sources. In many outbreaks, the source of infection is unknown. Cold weather, poor air quality or crowding, concurrent infections, and some live virus vaccinations may facilitate infection, disease, and transmission (Ley, 1997).

This characteristic, where infection persists in the flock indefinitely, makes elimination of MG extremely difficult in multi-age breeder and laying complexes. As MG-clean pullets are raised in single-age farms and in isolation are brought onto the complex, they are often exposed to the organism at probably the worst possible time, at the onset of production. This cycle of spread continues in a complex with new flock introductions (Butcher, 2002a; Talha, 2003).

2.3.3. Predisposing factors

Mycoplasma gallisepticum may be associated with acute respiratory disease in chickens and turkeys, especially in young birds, with the turkey being more susceptible. The severity of the disease is greatly affected by the degree of secondary infection with viruses such as Newcastle disease and infectious bronchitis, and/or bacteria such as *Escherichia coli*. In turkeys there is synergism with avian pneumovirus infection. A more chronic form of the disease may occur and can cause reduced egg production in breeders and layers (OIE, 2012).

MG infection is highly influenced by stress because the organism seems to remain dormant in many flocks but when birds are stressed, it becomes active. As a result, more MG develops in cool or cold houses than in warm climate (Donald and Mack 1990). Once infected, birds remain carriers; clinical disease is severe under stress condition of poor management, crowding, cold weather, and concurrent infections (Talha, 2003; Bukhari, 2005).

2.4. Pathogenesis

It is presumed that MG enters the respiratory tract by inhalation, aerosol or via the conjunctiva but it is not clear how it surmounts the bird's natural defense mechanism (Bradbury, 2001). The respiratory tract and lungs are frequent sites of infection (Quinn *et al.*, 2002). *Mycoplasmas* tend to

adhere firmly to the host's mucous membranes and some species have been shown to affix to cells by specific attachment structures one of which is MG, which attaches firmly to mucosal cells by its well organized terminal organelles or blebs that govern motility, host-pathogen interactions, and ultimately pathogenicity (Talha, 2003). Generally, *Mycoplasmas* are capable of destroying the cilia of cells in the respiratory tract thus predisposing to secondary bacterial infection (Quinn *et al.*, 2002).

The organisms are extracellular and produce haemolysin, proteases, nucleases and other toxic factors that can lead to the death of host cells or to a chronic infection. Latency can occur and various stresses predispose to Mycoplasmal diseases (Quinn *et al.*, 2002). The main route of exit of MG from the host is via the respiratory tract (Bradbury, 2001) although infection of the genital tract may result excretion through egg or semen (Yoder and Hofstad, 1964). In vertical transmission, the highest rate of MG transmission occurs during the acute phase of the disease (Glisson and Kleven, 1984; Lin and Kleven, 1982). The presence of maternal antibodies to MG in embryonated eggs reduced the *in ovo* pathogenicity of infection and increased the probability of survival of the infected embryo (Levisohn *et al.*, 1995; Lin and Kleven, 1984).

2.5. Clinical Signs

M. gallisepticum infections vary from asymptomatic to severe, depending on the infecting strain and other factors. More severe infections are seen when the birds are infected concurrently with Newcastle disease virus, infectious bronchitis virus, *Escherichia coli* or other pathogens. Except for very young birds, the development of clinical disease may depend on the presence of other pathogens or stressors. Uncomplicated infections frequently cause no clinical signs or mortality in chickens (Bradbury, 2001). Flocks may have serologic evidence of infection with no obvious clinical signs (Ley and Yoder, 1997).

In natural infection, the most characteristic signs in adult flocks are tracheal rales, nasal discharge, and coughing. Feed consumption is reduced and birds lose weight (Ley and Yoder, 1997). There is reduced egg production in laying flocks, but is usually maintained a lowered level (Branton and Deaton, 2005; Mohammed *et al.*, 2005).

Experimentally, conventional and germ free chicks inoculated in the thoracic air sac with the S6 strain of MG developed severe respiratory disease characterized by sneezing and rales, decreased body weight gain, and significant mortality (Layton and Simkins, 1971). Cases of keratoconjunctivitis apparently caused by MG have been reported in layer chickens in Japan (Nunoya *et al.*, 1995). Chickens showed swelling of the facial skin, and the eyelids, increased lacrimation, congestion of conjunctival vessels, and respiratory rales. Sinusitis may result marked swelling of sinus infraorbitalis. Conjunctival inoculation of Australian field strains of MG combined with infectious bronchitis virus produced conjunctivitis in chickens (Soeripto *et al.*, 1989).

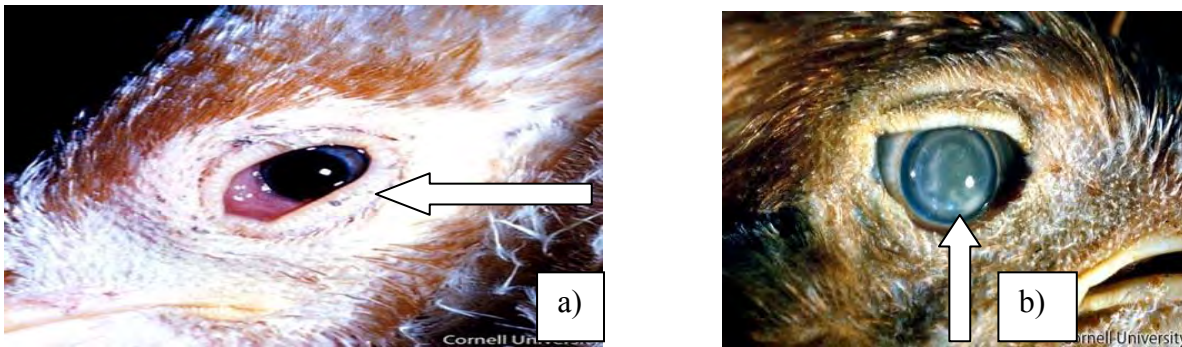


Figure 1. Conjunctivitis, edema (eyelid, periorbital) b) conjunctivitis, corneal opacity

Source: Atlas of Avian disease, Cornell University, 2002.

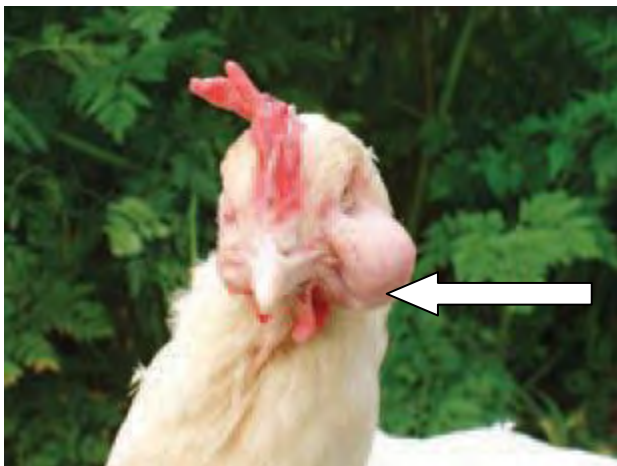


Figure. 2. Advanced case of infectious sinusitis after exudates in one sinus was removed (Calnek *et al.*, 2014)

In young chicks, there is rattling, sneezing and sniffing all indicative of respiratory difficulty. If

Complicated by other respiratory diseases, these symptoms are accentuated. In severe cases, mortality may go as high as 30% (Donald *et al.*, 1990). An outbreak is quickly followed by many secondary infections and it is these that do the damage. Except for very young birds, the development of clinical disease may depend on the presence of other pathogens or stressors; coliform organisms are particularly involved. Thus, visible identification of MG is often confused by symptoms of secondary invaders (Talha, 2003, Donald *et al.*, 1990).

2.6. Pathological 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, fibrinopurulent pericarditis and perihepatitis may be seen. 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. Trachea and conjunctiva may be congested and there may be pericarditis and fibrinopurulent perihepatitis along with massive air sacculitis. This occurs in severe cases or when a case is complicated with other pathogens such as *E. coli* (Gordon, 1979; Chauhan, 1993; Talha, 2003). Keratoconjunctivitis in layer chickens characterized by marked edema in the facial subcutis and eyelids with occasional corneal opacity may also develop (Talha, 2003, Gordon, 1979).

2.7. Diagnosis

History, clinical signs, and typical gross lesions may be suggestive of *M. gallisepticum*. Serology by agglutination and ELISA methods are commonly used for surveillance. Hemagglutination inhibition is used as a confirmatory test and molecular techniques namely, PCR, is suggestive. Polymerase chain reaction (PCR) test can be performed on clinical samples without the need for isolation. Its high sensitivity and fast turnaround time is making it the most frequently used test to monitor MG infection (Garcia *et al.*, 2005, Ley, 2013). Differentiation of isolates and tracing MG infections for epidemiological studies is usually done by molecular techniques such as random amplified polymorphic DNA (RAPD) (Fan *et al.*, 1995a; Geary *et al.*, 1994), restriction fragment

length polymorphism analysis (RFLP) (Fan *et al.*, 1995b), gene targeted sequencing (Ferguson *et al.*, 2005), or amplified fragment length polymorphism (AFLP) (Hong *et al.*, 2005), demonstrated that AFLP results correlated well with DNA sequence analysis and RAPD with AFLP analysis having a much higher discriminatory power and reproducibility. However, RAPD analysis is still widely accepted for strain differentiation of MG isolates (Fan *et al.*, 1995b).

2.7.1. Cultural isolation and identification

Samples are taken from live birds, fresh carcasses or the carcasses of birds that have been frozen when fresh. From live birds, swabs may be taken from the choanal cleft, oropharynx, oesophagus, trachea, eyes, cloaca and phallus. In the case of dead birds, samples may be taken from the nasal cavity, infraorbital sinus, trachea, or air sacs. Exudates can be aspirated from the infraorbital sinuses and joint cavities. Samples may also be collected from dead-in-shell embryos or chickens or poults that have broken the shell but failed to hatch. Samples can be taken from the inner surface of the vitelline membrane and from the oropharynx and air sacs of the embryo (Quinn *et al.*; 2002).

All samples should be examined as soon as possible after collection. If transportation is necessary, small pieces of tissue should be placed in mycoplasma broth, or swabs should be vigorously agitated in 1–2 ml of mycoplasma broth and then discarded. Alternatively, the swabs can be dipped in mycoplasma broth before the specimens are taken and then replaced in the swab holders for transportation. An ice pack or some other means of chilling should be included as MG and MS die rapidly at room temperature. Serial dilutions of specimens in mycoplasma broth may be of value because the presence of specific antibodies or antibiotics or inhibitory substances in tissues may inhibit mycoplasma growth unless they are diluted out (OIE, 2012).

Mycoplasmas are fastidious organisms and require specific growth factors, an isotonic medium and the absence of inhibitory substances, for growth. They require a protein based medium enriched (supplemented) with serum or serum factors, yeast extracts, glucose and bacterial and/or fungal inhibitors (Charles and Graham, 1989; Quinn *et al.*, 2002). Horse or swine serum (inactivated at 56 °C for 1 hour) should be used in media for the growth of MG. It is important that each new batch of medium should be tested with recently isolated MG cultures of low invitro passage because the

ability of each batch of medium, for some components especially serum and yeast extract, to support growth vary (Charles and Graham, 1989)

Yeast extracts, the other growth factor, may be beneficial and is usually supplied by commercial yeast autolysate or by fresh yeast extract. Glucose is fermented by *M. gallisepticum*, *M. synoviae* and several other species and is a common supplement. *Mycoplasmas* are resistant to penicillin (as they lack cell wall), which is added in the medium to inhibit (discourage) the growth of gram positive bacteria; and the component thallium acetate, for which *Mycoplasmas* are relatively resistant, helps to inhibit gram negative bacterial and fungal contamination (Gordon, 1979; Charles and Graham, 1989; OIE, 2012).

Media usually take the form of broth or solid agar, although semi solid agar and diphasic media (i.e. agar with broth overlay) are also used. Frey's medium or a modification of Frey's medium (Hay flick's medium) is commonly used for isolation and propagation of MG (Charles and Graham, 1989; Quinn *et al.*, 2002; Talha, 2003). However, no single medium formulation has been universally accepted as optimum for the growth of all avian *Mycoplasma* species. For example, *M. synoviae* needs nicotinamide adenine dinucleotide (NAD) as supplement in the media to support growth (Talha, 2003, Quinn *et al.*, 2002).

Solid media may help detection of slow growing *Mycoplasma* colonies, which can be over grown by saprophytes in broth. Some laboratories make serial dilutions of the inoculum in broth to avoid the inhibitory effects of a large inoculum where tissue enzymes tend to break down glucose and cause a drop in pH, or tissue inhibitors may be present. It is therefore, necessary to make serial dilutions of up to 10^{-3} for successful isolation (Charles and Graham, 1989; OIE, 2012).

Table 2. Formulations of two commonly used media for the isolation and propagation of avian Mycoplasmas.

Medium	Constituent	Amount
Frey's medium	Mycoplasma broth base (BBI, Cockeysville, MD)	22.5 g
	Glucose	3 g
	Swine serum	120 ml
	Fresh yeast extract	100 ml
	Cysteine hydrochloride*	0.1 g
	NAD*	0.1 g
	Phenol red (1%)	2.5 ml
	Thallium acetate (10%)	2.5 to 5 ml
	Penicillin G potassium	106 units
	Distilled water q.s.	1000 ml
	Adjust pH to 7.8 with 20% NaOH and filter sterilize	
PPLO broth	PPLO broth without crystal violet (Difco)	14.7 g
	Glucose	10 g
	Fresh yeast extract	100 ml
	Swine serum	150 ml
	Cysteine hydrochloride*	0.1 g
	NAD*	0.1 g
	Phenol red (1%)	2.5 ml
	Thallium acetate (10%)	2.5 to 5 ml
	Penicillin G potassium	106 units
	Distilled water q.s.	1000 ml
	Adjust pH to 7.8 with 20% NaOH and filter sterilize	

(Source: Charles and Graham, 1989)

* Note: Cysteine hydrochloride and NAD is added to support the growth of *M. synoviae*

Specimens are inoculated on to mycoplasma agar and into broth. Solid medium may help detection of slow growing Mycoplasma colonies, which can be overgrown by saprophytes in broth. It may be necessary to make serial dilutions up to 10^{-3} for successful isolation. Inoculated plates are

incubated at 37°C in sealed containers. Increased humidity and CO₂ tension in the atmosphere have been reported to enhance growth; these conditions may be obtained by the inclusion of damp paper or cotton wool, and by flushing the container with 5–10% CO₂ in nitrogen, by placing a lighted candle in the container, or by using a CO₂ incubator or suitable gas-generating system (OIE, 2012).

The caps of liquid medium containers should be tightly sealed before incubation at 37°C to avoid spurious changes in pH. For the first few days, the plates are examined daily for colonies with a stereoscopic microscope; after that they are examined less frequently. Cultures from field material should not be discarded as negative for at least 20 days. Broth medium should be examined daily for acidity, indicated by a change from red to orange or yellow in the indicator. (Quinn *et al.*, 2002)

Any observable growth is subcultured on to solid medium immediately. Even if no colour change occurs, subculture on to solid medium should be made after 7–10 days or earlier as the presence of an arginine-hydrolysing (alkali-producing) Mycoplasma species may mask the acid colour change produced by MG. Mycoplasma colonies on solid medium can usually be recognized, although they may not have the typical fried egg' appearance (Charles and Graham, 1989)

Bacterial colonies may appear on the first passage, but they are often more pigmented and fail to passage on mycoplasma media. Biochemical reactions (e.g. fermentation of glucose and failure to hydrolyse arginine) can assist in identification, but they are not specific for MG or MS and necessitate purification of the culture by cloning (OIE, 2012).



Figure 3. Colonies of *M. gallisepticum* on 20% chicken serum agar plate x 40. (47)

Immunological and DNA detection methods can be used to identify mycoplasma isolates. They include the indirect fluorescent antibody (IFA) and immunoperoxidase (IP) tests, both of which are simple, sensitive, specific and rapid to perform; growth inhibition (GI); and metabolism inhibition (MI). Purified (cloned) cultures are required for the GI and MI tests, but not for the IFA or IP test. IFA and IP can detect the presence of more than one species of mycoplasma, as the colonies specific for the antiserum will react while the others will not (Talha, 2003).

However, *M. imitans*, a Mycoplasma species that is serologically related to MG and that presents the same biochemical properties has been isolated from ducks, geese and sometimes from other nondomestic bird species in some countries. It may be distinguished from MG by use of a PCR-RFLP (polymerase chain reaction/restriction fragment length polymorphism), as described by Kempf. Alternatively, colonies of the isolate can be examined by immunofluorescence using serial dilutions of antisera to MG and *M. imitans* in parallel. The homologous antiserum should have a considerably higher titer. (OIE, 2012, Quinn *et al.*, 2002))

DNA detection methods for identifying MG or MS directly in tissues or for identifying laboratory isolates are discussed below and are usually based on the PCR (OIE, 2012). In certain circumstances where results of the above methods are not conclusive, inoculation of chick embryos or bioassays in live chicks may be appropriate. However these techniques are time-consuming and costly and tend to have been replaced by PCR technology, although they remain a useful research tool. (OIE, 2012)

2.7.2. Serological diagnostic tests

Serologic procedures are available to aid in the diagnosis of MG when infection is suspected and are also useful for flock monitoring in the control programs (Gordon, 1979; Talha, 2003). The serologic tests in common use may lack specificity and/or sensitivity. However, a positive serologic test together with history and symptoms typical of the disease would constitute a presumptive diagnosis pending isolation and identification of the organism (Gordon, 1979)

The most commonly used serologic tests are serum plate agglutination (SPA), hemagglutination inhibition (HI) and enzyme linked immunosorbent assay (ELISA); although several others have

been described such as radio immunoassay, micro Immunofluorescence and immunoperoxidase assay (IP) (Butcher, 2002b; Parker *et al.*, 2002; Talha, 2003).

2.7.2.1. Serum plate agglutination (SPA)

Sera are collected from a sample of the flock and, if not tested immediately, are stored at 4°C and not frozen. The test should be carried out at room temperature (20–25°C) within 72 hours of serum collection and the reagents should also be at room temperature. Prior centrifugation will reduce nonspecific reactions. (Talha, 2003)

SPA is probably the most commonly used test using commercially stained antigens, which vary in sensitivity and specificity from different manufacturers and batch to batch. The test is quick, relatively inexpensive and highly sensitive (Charles and Graham, 1989; Talha, 2003; OIE, 2012). Non specific reactors (false positive) however, may occur in some flocks infected with *M. synoviae*, or those recently been vaccinated with oil emulsion vaccines and/or tissue culture vaccines originated against various organisms (Talha, 2003, Abdulfath, 2004)).

Serum plate agglutination is a screening test and is prone to false positive reactions. It is possible to reduce false positive reactions by heating serum to 56°C for 30 minutes or by diluting serum or producing SPA antigen in medium containing liposomes instead of serum (Charles and Graham, 1989; Butcher, 2002b). There is no international standard for interpreting SPA test result, but a high proportion of positive sera in a flock (10% or more) indicate MG infection especially if confirmed by Hemagglutination inhibition (HI) or ELISA (Butcher, 2002b).

2.7.2.2. Hemagglutination inhibition (HI)

MG and MS are capable of haemagglutinating avian red blood cells (RBCs), and specific antibodies in sera cause inhibition. A strain should be selected that grows well and haemagglutinates reliably. The HI test requires a satisfactory haemagglutinating MG and MS antigen, washed fresh chicken or turkey RBCs, as appropriate, and the test sera. The antigen can be either a fresh broth culture or a concentrated washed suspension of the mycoplasma cells in PBS. It may be difficult to sustain a supply of high-titred broth culture antigen; however, the use of

concentrated antigen (usually containing 25–50% glycerol and stored at -70°C), increases the likelihood of nonspecific reactions. In the USA, MG and MS hemagglutination (HA) antigen can be purchased from the NVSL (OIE, 2012).

The hemagglutination inhibition test is used to confirm various rapid serum plate agglutination reactions. It is considered to be highly specific but less sensitive than the SPA test. Infected birds may not be test positive until three weeks or longer after infection as it detects IgG. In addition, there is antigenic variation among MG strains as measured by HI. Antigen prepared from one MG strain may not adequately detect HI antibodies in chickens infected with a different strain (Charles and Graham, 1989).

2.7.2.3. Enzyme Linked Immunosorbent Assay (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. One ELISA uses a MAb that recognizes an epitope on a 56 kDa polypeptide of MG. In this system, ELISA plates are coated with whole cell MG antigen and the sera under test are added as in the conventional indirect ELISA, but the reaction is assessed by the extent of blocking that occurs when the conjugated MAb is added. A similar 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, 2012; Aviakin and Ley, 1992; Yagihashi and Tajima, 1987.)

2.7.2.4. Molecular techniques

An alternative to conventional culture and identification is the use of specific DNA detection methods. MG or MS may be detected by hybridization with DNA probes, but now it is much more common to use the PCR to amplify specific portions of DNA in the test material. At least one commercial MG DNA test kit uses a PCR directly on material extracted from swabs. One commercial company produces a kit to detect MG field strains and one that identifies the vaccine F strain. Several 'in-house' PCR-based tests have also been published for MG including a multiplex

PCR, which is designed to detect all four avian *Mycoplasma* pathogens, but which has not been validated with clinical samples (OIE, 2012; Dohms *et al.*, 1993; Farnandez *et al.*, 1993).

Several methods are cited by Kempf and, in addition, a manual published by Lauerman contains a validated PCR assay for MG, MS, and other avian mycoplasmas based on unique sequences contained in the 16S rRNA gene. In the USA, a PCR based on the *mgc2* gene of MG or the *vlhA* gene of MS is becoming more widely used, because preliminary strain identification can be made by sequencing of the PCR product; it must be remembered that unrelated strains may occasionally share the same sequence (Dohms *et al.*, 1993; Farnandez *et al.*, 1993).

Rapid, sensitive and specific tests that detect nucleic acids from pathogenic *Mycoplasmas* are very attractive for the laboratory detection of infected flocks, and methods for direct detection of the three main pathogenic *Mycoplasmas* have been developed. In order to characterize isolates below the species level, PCR-based sub typing methods have been introduced. One of them, arbitrarily primed-PCR, results in strain-specific arrays of DNA fragments that can distinguish even closely related strains of a given species (Kempf, 1997).

2.8. Differential Diagnosis

In poultry, the differential diagnosis includes respiratory diseases such as Infectious bronchitis, mild Newcastle disease and avian influenza. *Haemophilus paragallinarum*, *Pasteurella multocida* and *Mycoplasma synoviae* infections should also be ruled out. In turkeys, other pathogens to consider include avian pneumovirus, *Pasteurella multocida*, and *Chlamydia* and *M. synoviae*. Mixed infections with *M. gallisepticum* and other organisms can occur. (Gross *et al.*, 1990, David H. Ley, 2013)

2.9. Economic Importance

Mycoplasma gallisepticum is the most economically significant mycoplasmal pathogen of poultry. *M. gallisepticum* infections can cause significant economic losses on poultry farms from chronic respiratory disease reduced feed efficiency, decreased growth and decreased egg production. The carcasses of birds sent to slaughter may also be downgraded. *M. gallisepticum* infections are

notifiable to the World Organization for Animal Health (OIE). This organism has been eradicated from most commercial chicken and turkey breeding flocks in the United States; however, it remains endemic in many other poultry operations. (Carpenter *et al.*, 1981; Ley and Yoder, 1997)

Mycoplasma gallisepticum is believed to cost the worldwide poultry industry over \$780 million every year. In the United States it is believed to cost over \$120 million on egg production alone. Infection can lead to the culling of an entire flock to prevent further spread (Ley and Yoder, 1997). Since the disease causes reduced feed and growth production, carcass condemnations, and retarded growth in juveniles, serious economic losses have occurred. Also, chickens have been documented to lose about 16 eggs over their laying cycle of 45 weeks. This adds up to be a loss of about \$140 million annually in the United States alone (Mohammed *et al.*, 1987).

2.10. Treatment

Wildlife rehabilitators should be careful to not misdiagnose *M. gallisepticum* infection with other diseases with similar clinical signs, such as avian influenza, chlamydiosis, Newcastle disease, infectious bronchitis, head trauma, and avian pox virus. *M. gallisepticum* can be treated with antibiotics such as tylosin, tetracycline, or oral enrofloxacin with ophthalmic gentamicin. These are given through food, water or injections. Especially tylosin gives good results in the feed. However, treated birds must be kept in captivity and isolation for a long time period because birds may become asymptomatic carriers. At this point, it is very difficult to verify if previously infected birds are still infected with *M. gallisepticum*. (Gordon, 1979; Chauhan, 1993)

Treatment and release is not wise for disease control in wild population, 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 of drinking water for 3 to 5 days (Gordon, 1979; Chauhan, 1993).

2.11. Control and Prevention

M. gallisepticum can be introduced into a flock by live birds or hatching eggs, as well as the movement of people and fomites. Sub-clinically infected small backyard flocks can be a source of infection for commercial poultry. In the US, lateral transmission is the most common means of introduction, as the disease has been eradicated from most primary and multiplier breeding flocks (Bradbury, 2001).

M. gallisepticum-negative breeding stock can be identified and maintained by serologic testing. Heat treatment or tylosin can eliminate egg transmission from valuable breeding animals. Biosecurity measures are important in preventing transmission on fomites. Wild or pet birds can also carry *M. gallisepticum*, and should be excluded from poultry operations (Ley and Yoder, 1997).

Infections can be eliminated from a farm by depopulation of the flock, followed by thorough cleaning and disinfection of the premises. Most commonly used disinfectants are thought to be effective for *M. gallisepticum*. Recommended disinfectants for buildings and equipment include phenolic or cresylic acid disinfectants, hypochlorite, and 0.1% glutaraldehyde (Bradbury, 2001).

Mycoplasmas are typically fragile and only survive in the environment for a few days; birds can be re-introduced after 2 weeks. Eradication from large, multiple-age commercial egg laying flocks is complicated by persistent infections and periodic shedding under stress. In these flocks, *M. gallisepticum* infections may be treated with antibiotics, which decrease the clinical signs but do not eliminate the infection. Other concurrent infections must also be treated. Several live *M. gallisepticum* vaccines are available for chickens. (Talha, 2003)

The failure to control MG infection on multiple production sites has led to the development of a number of vaccines although vaccination is recommended only where field exposure (outbreaks) is considered inevitable. Both live and killed vaccines are available and regardless of antigenic variation seen among MG strains seems to be effective (Bradbury, 2001, Talha, 2003).

R-strain is widely used for the production of bactrins (inactivated) and it is the highly pathogenic (virulent) strain; whereas the F, ts-11, and 6/85 strains are widely used for live vaccine production and have relatively poor pathogenicity (Talha, 2003).

The disease spreads from farm to farm predominantly by movement of people, contaminated equipments and vehicles. Thus, as a first step, initiate a self imposed quarantine or bio-security especially with people who handle birds and manure or people who have direct contact with birds, and visitors. Another potential means of transmission of MG that has not often been given much attention is the spread by wild birds and pet birds that may become infected and shed MG organism. Thus, wild-bird-proof poultry houses are needed wherever possible (Butcher, 2002a; Talha; 2003).

Therefore, strict isolation of flocks should be maintained to avoid introduction of the disease into a clean flock. Where excellent bio-security is practiced, there have been many instances where infection has not spread to adjacent houses within a complex. Moreover, strict bio-security is the best means of preventing introduction of MG to layer and breeder complexes (Butcher, 2002a; Talha; 2003; Bukhari, 2005). Current control programs are supported by the use of serologic testing as a means of monitoring the status of flocks; either found to be free from the disease or in which effort has been made to eliminate the infection. Thus, immunization and medication of breeding stock are mainly practiced with chickens (Gordon, 1979; Talha *et al.*, 2003; OIE, 2012).

3. MATERIALS AND METHODS

3.1. Study area

The study and entire laboratory works were conducted from December 2014 to April 2015 at National Veterinary Institute (NVI) Bishoftu, Ethiopia. The town is situated 47 km south east of the capital city of Addis Ababa. It lays 9° N latitude and 4° E longitudes at an altitude of 1850 m above sea level in central highlands of Ethiopia. The total poultry population of the country level is estimated to be about 51.35 million. In this report, poultry includes cocks, cockerels, pullets, laying hens, non-laying hens and chicks. With regard to breed, 96.83 percent, 2.37 percent and 0.8 percent of the total poultry were reported to be indigenous, hybrid and exotic, respectively(CSA, 2013/14).



Figure4.Map of Ethiopia indicating study site Bishoftu, East Shewa, prepared with GIS software.

The total poultry population at Oromia level is (19,313,887), cocks (2,207,650), cockerels (974,262), pullets (1,809,784), non-laying hens (626,388), chicks (6,788,323), laying hens (6,907,467) and at east shewa level (1,439,821), cocks (131,406), cockerels (87,765), pullets (148,214), non laying hens (66,555), chicks (509,499), laying hens (496,381). The estimate of total number of eggs produced of the country during the year is about 100.8 million (CSA, 2013/14).

In the commercial poultry production system, samples from commercial chickens (layers and broilers) were obtained from the four selected commercial poultry farms in Bishoftu, which were coded arbitrarily as farm-A, farm-B, farm-C and farm-D to keep their confidentiality. This area was selected based on the location accessibility and poultry farm population. Generally, the bio-security measures in all of the four farms were strict, with good management practice of proper ventilation and good hygienic status which is of standard one.

3.2. Study Animals

A total of 120, 20 chickens from each four farms and 40 backyard chickens were purchased from farms and local markets respectively. The chickens were slaughtered at NVI postmortem room and tracheal, air sac and lung samples were collected from slaughtered chickens.

3.3. Study Design

For isolation of MG from apparently healthy and sick chickens, four commercial farms were conveniently selected to buy twenty chickens from each farm and forty backyard chickens were purchased from local markets. The animals were slaughtered at NVI postmortem room and tracheal, air sac and lung samples were collected from slaughtered animals. And a total of sixty (60) chickens, 20 for inactivated vaccine, 20 for attenuated live vaccine (imported) and 20 for control were used.

Among ten lyophilized isolates, three lyophilized isolates was selected and processed for DNA extraction at Molecular laboratory of the institute. The extracted DNA was diagnosed using PCR for confirmation. The selection of the isolates was carried out based on growth during renew, sample origin (farm type and place) and isolation year.

3.4. Sample collection

Sampling and sample preparation were done according to the OIE of Diagnostic Tests and Vaccines for Terrestrial Animals (2012). For cultural isolation, post mortem examination was conducted on apparently healthy and clinically sick chickens brought from four commercial farms

and from local markets. The samples taken from ten chickens were pooled, chopped and mixed before inoculating on suitable mycoplasma media (12 pooled samples), In addition; lyophilized isolates of NVI were used for molecular characterization for identity and vaccine trial. Tissue samples of lung, air sacs, and trachea were collected also from gross post mortem lesions of sick chicken tissues for isolation of mycoplasma. Post mortem examination and cultural isolation were conducted at the National Veterinary Institute (NVI), Bacteriology laboratory, Bishoftu, Ethiopia.

3.5. Laboratory Investigation

3.5.1. Media preparation

Mycoplasma agar and broth was prepared as per OIE (2012) manual and manufacture instruction for isolation of avian mycoplasmas. Mycoplasma media generally contain a protein digest and a meat-infusion base supplemented with serum or a serum fraction, yeast factors, glucose and bacterial inhibitors (Charles and Graham, 1989, Kleven, 1994). The media was sterilized by autoclave and filtration according to their components and stored at 4 °C until use (Annex A).

3.5.2. Tissue sample processing and Isolation

Based on the standard cultural isolation procedures (Quinn, *et al*; 2002; OIE, 2012) and *Mycoplasma* isolation experience of the bacteriology laboratory section of NVI, isolation of the organism was conducted and Pooled samples from each farm (one pool for 10 chickens). Also the institute has been collected different local isolates of *Mycoplasma gallisepticum* of chickens from different commercial and backyard farms of the country for long period of time. A total of ten lyophilized samples were growth on suitable broth and agar media for this study and Were streaked on PPLO (pleuro-pneumonia-like organism) agar plates and broth; for this, tissue samples from gross post mortem lesions of lung, air sacs, and trachea were collected from clinically sick chickens of commercial poultry farms and from apparently healthy backyard chickens (Annex B).

Tissue samples of lung, air sacs, and trachea of each individual bird were crushed together, to increase the chance of isolation, using mortar and pistil with sterile sand added to facilitate tissue crushing. Then homogenous tissue suspension was passed through sterile sieve and filter paper, the filtrate was passed into sterile plastic test tubes and centrifuged at 5000 rpm for 20 minutes. Using sterile syringe (20 ml), the supernatant was passed though sterile bacterial membrane filter of 0.45µm (Annex 1B). Finally, the filtrate was dispensed (inoculated) on plates of Hayflick's agar

and broth media, excess fluid being sucked from the plates using sterile pasture pipette and incubated at under microarepphic condition(5% CO₂) .

The plates were examined daily for any visible colony growth under stereomicroscope (25×). Prominent and visible characteristic *Mycoplasma* (round colonies with central nipple) colonies were marked under stereomicroscope using lab marker pens. Then inside safety cabinet, marked colonies were incised including the agar with special scalpel meant for this purpose and sub-cultured on to Mycoplasma agar by pushing the block method and also the block sub-cultured in to an air tight (to prevent dehydration) glass tube of *Mycoplasma* broth. The agar was incubated at 37 °C in 5% CO₂ incubator and the broth at 37 °C normal incubator. After primary isolation and sub-culturing on to *Mycoplasma* agar and in to the broth, further sub culturing of four to six times was conducted from agar to agar, agar to broth and broth to broth (after passing a Millipore of 0.45µm membrane filter) to obtain pure colonies. After obtaining pure culture, colonies were tested for digitonin sensitivity. Digitonin is a compound which interfere protein and nucleic acid synthesis thus interfering the utilization of cholesterol, incorporated in the medium, for membrane synthesis and helps to distinguish *Mycoplasma* from *Acholeplasma*. *Mycoplasma* depends on cholesterol, which is supplied by serum added in the medium, whereas *Acholeplasma* do not depend on external cholesterol incorporated in the medium for membrane synthesis.

Thus digitonin inhibits growth of *Mycoplasmas* (Hirsh, 1999; Quinn, 2002). For this sensitivity test, 1.5% digitonin impregnated paper discs were kept on agar plates which were inoculated with pure *Mycoplasma* colony and incubated at 37 °C in CO₂ incubator. One or two days later, the plates were examined under stereomicroscope (25× magnification) and there was no growth around the disc with inhibition of colony growth which confirms the pure colonies were to be *Mycoplasma*.

Then, biochemical tests such as fermentation of glucose and hydrolysis of arginine were done as preliminary biochemical identification of *Mycoplasma*. These biochemical tests were conducted in pure *Mycoplasma* broth culture tubes of about 5ml in to which 0.1ml of glucose was added to one of the tubes and 0.1ml of arginine to the other and incubated at 37 °C. Then the tubes were examined daily for any color change. Glucose fermentation was indicated by color change (red to

yellow) due to the pH indicator, phenol red. The tubes that showed color change were subcultured immediately onto agar plates and into broth tubes; otherwise the acid produced by the fermentation of glucose may kill the isolate.

Three isolated and lyophilized samples of NVI were reconstituted with 5 ml of sterile saline water. Solid medium was help detection of slow growing mycoplasma colonies, which was overgrown by saprophytes in broth (Kleven, 1994). Inoculated agar plate was incubated at 37°C in sealed containers and increased humidity and CO₂ tension in the atmosphere by using a CO₂ incubator.

The caps of liquid medium containers were tightly sealed before incubation at 37°C to avoid spurious changes in pH. For the first few days, the plates were examined daily for colonies with stereo microscope; after that they were examine less frequently (OIE, 2012). Broth medium was examined daily for acidity, indicated by a change from red to orange or yellow in the indicator. Any observable growth was subcultured on to solid medium immediately. Even if no colour change occurs, subculture on to solid medium was made after 7–10 days or earlier as the presence of an arginine-hydrolysing (alkali-producing) mycoplasma species may mask the acid colour change produced by MG(OIE, 2012).

3.5.3. Detection/identification of MG DNA by conventional PCR

Sampling and sample preparation were done according to the OIE of Diagnostic tests and Vaccines for Terrestrial Animals (2012). A total of six samples, three pooled isolate samples from farm C,D and one from back yard chickens, and three samples from Jemanesh (adama), Heromaya University, Samuael farm of lyophilized NVI isolates respectively were used for molecular analysis.

3.5.4. DNA Extraction

One micro liter broth culture of the respective MG isolates was used for DNA extraction.

The culture suspended in 1 ml of PCR-grade PBS in a 1.5 ml snap-cap Eppendorf tube. The suspension was centrifuged for 30 minutes at 14,000 rpm at 4°C. The supernatant was carefully removed with a Pasteur pipette and the pellet was suspended in 25 µl PCR grade water. The tube and the contents were boiled for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 *g* for 5 minutes. The supernatant was used as DNA template (OIE, 2012).

3.5.5. PCR Master Mix preparation

The master mix comprised, H₂O RNase free water -5µl, 10 × PCR Buffer - 5.00 µl, dNTP (10 mM) - 1.00 µl, F Primer MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT C-3 (5 pmole/µl) - 2 µl, MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3 R Primer (2 pmole/µl)- 2µl, Taq DNA polymerase (5 U/µl) – 0.50 µl, and MgCl₂ (25 mM) -1.50 µl ,Template (DNA)- 3 µl (ISO/IEC 17025:2005 NVI-QMS SOP).

3.5.6. DNA amplification (Polymerase chain reaction)

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).

3.5.7. Electrophoresis

Amplified PCR products were detected by conventional 2% agarose gel electrophoresis prepared and added 10mg/ml Ethidium bromide and dried for 20 mints. Added 1 µl 6X loading buffer in 5µl PCR product, then loading PCR product with loading to1X TAE buffer by incorporating 1Kb marker starting with 100bp, and Electrophoresis was run at 120V for 1:20 hours using (Biorad thermo EC2060.US system). Amplified PCR product was analyzed under UV light (Uvitec, Cambridge CB41QB. UK and Camera using Uvtec (UK) serial No 08200913) The PCR product band for *mgc2* gene was 185bp. Visualizations of the PCR products were carried out in a separate laboratory area (ISO/IEC 17025:2005 NVI-QMS SOP for gel documentation).

4. INACTIVATED MG VACCINE TRIAL

Two types of vaccines are available for the control of MG. These are mild to a virulent MG strains used as live vaccines, or inactivated oil-emulsion bacterins. Although there is antigenic variability among MG strains, it is thought that vaccination with a single strain is sufficient. The vaccine trial was a formalin inactivated oil base suspension of *Mycoplasma gallisepticum*. The bacterin is used for the prevention of clinical signs associated with *Mycoplasma gallisepticum* infection in chickens (Glisson, J. R., J. F. Dawe and S. H. Kleven (1984).

4.1. Characteristics of the seed

MG bacterin was prepared from a concentrated suspension of whole cells that is emulsified into an oil adjuvant. High antigen content is essential. For killed vaccines the most important characteristics are high yield and good antigenicity. It is assumed, but not proven, that virulent strains are desirable. The seed culture was free from all extraneous organisms (OIE; 2012).

4.2. Method of culture for vaccine preparation

PCR confirmed positive local *Mycoplasma gallisepticum* (MG) isolate from Samuel farm (Isolate of NVI) as a seed, in active form, was inoculated at 10 per cent v/v into Frey's broth and Agar, and incubated at 37 °C at 10 per cent CO₂ tension and observed daily for any growth. After 48 hrs of incubation, the cultured media tubes were taken out of incubator and observed for presence of rich growth of MG, similar to isolation of MG (Annex 1B).

4.3. Method of manufacture

The vaccine trial was manufactured in suitable clean and secure accommodation, well separated from production facilities of NVI. Special care was taken to avoid MG contamination of other products manufactured in the same facility.

Production of vaccine was on a seed-lot system, using a suitable MG species of known origin, passage history, and purity. The growth medium is similar to that given above (Annex 1A).

Broth medium was inoculated, with a rapidly growing inoculum, at a rate of approximately 5% (v/v) and incubation was at 37°C. For bacterin production, the antigen was concentrated by centrifugation and resuspended in a small volume of PBS before the emulsion was prepared. Bacterins were made as water-in-oil emulsions, typically Montanide ISA 70 (Bean and J.W. Lowenthal; 2002).

4.4. In-process control

4.4.1. Antigen content

At harvest, the titer was 10^8 CFU/ml. normally the antigen concentration of bacterins is difficult to standardize but in present study, it was based on packed cell volume, which was typically 1% (v/v) packed cells in the final product. Ten milliliter of growth suspension was transferred to graduate Hop kin's centrifuge tube. The tube was centrifuged at 3000xg (12000 rpm) for 20 min to estimate packed cell volume per ml of the media and results were recorded. The mass of MG antigen was adjusted to 1 per cent (1ml cell mass in to 100ml) in the Hopkins's tube using phosphate buffer saline (pH 7) as diluent(Hildebrand, D.G., D.E. Page and J.R. Berg (1983).

4.4.2. Inactivation of killed vaccine of MG

Inactivation was done with formaldehyde, the inactivating agent and the inactivation procedure was shown under the conditions of vaccine manufacture to inactivate the vaccine organism and potential contaminants (Christianson and F.L. Cole (1976).

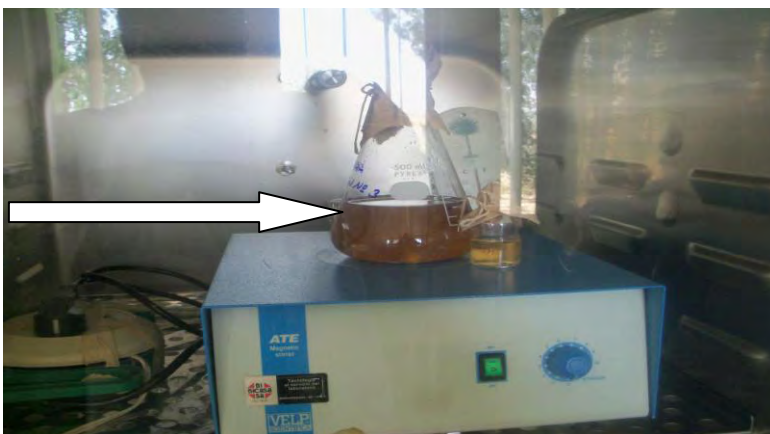


Figure.5. Inactivation of MG culture by formalin in Hayflic broth with magnetic stirrer.

For inactivation of growth of MG bacteria, 0.125 per cent formaldehyde was used and the tubes were incubated at 37 °C at 10 per cent CO₂ tension for providing 12 hrs of interaction time. To ascertain the completion of inactivation process, the inactivated broth was separately cultured on mycoplasma broth and agar, incubated at 37 °C at 10 per cent CO₂ tension and observed for seven days for appearance of any specific growth of Mycoplasma colonies or color change, respectively (Koski *et al.* 1976). After inactivation the final product was no evidence of growth of mycoplasma observed.

4.4.3. Sterility test for oil adjuvant and final product (Trial vaccine)

Oil adjuvant used in the vaccine was sterilized by heating at 160°C for 1 hour (OIE; 2012). Sterility tests were done with sterility media such as VF, Thioglycolat, Tryptic soy broth, Tryptose agar and Sabroud agar with incubation at 37 °c for 7 days. Also after confirmation of growth inactivation process, the montanide oil adjuvant was admixed at 4:1 ratio to properly emulsify the bacterial biomass. The inactivated culture was processed further to ensure the safety and sterility of the culture (Biro *et al.*, 2005).

4.5. Batch control

4.5.1. Rapid serum agglutination test

Sera were collected from all twenty control and forty chickens of vaccine test group and stored at 4°C until used. The test was carried out at room temperature (20–25°C) within 72 hours of serum collection and the reagents were also being at room temperature. To reduce nonspecific reactions centrifugation was done (Avakian, A. P., S. H. Kleven and J. R. Glisson (1988).

Test procedure

One volume (approximately 0.02 ml) of serum was dropped on to a clean white tile from each serum sample followed by one volume of stained antigen of MG. Care was taken to serum not to dry out before addition of the antigen. The antigen bottle was shaken vigorously and frequently during use to keep the correct amount of antigen in suspension and it was use a stirring rod to spread the mixture over a circular area of approximately 1.5 cm diameter. Then it was rocked the tile for 2 minutes. Agglutination was indicated by flocculation of the antigen within 2 minutes.

And included known positive and negative controls in the test (Figure 12), (Avakian, A. P., S. H. Kleven and J. R. Glisson (1988).

4.5.2. *Safety test*

After reconstitution, the trial vaccine was inoculated two vaccinal doses (1ml) subcutaneously at mid neck region (Hildebrand, D.G., D.E. Page and J.R. Berg (1983). into six chickens. None of the animals were dying within fourteen days, and the chickens were not showed signs of respiratory distress (dyspnea), mouth breathing, tracheal rales, nasal discharge, depression, and other adverse effects.

4.5.3. Evaluation of MG vaccine

Sixteen weeks old chickens were reared under standard management conditions in the experimental facility of NVI. The feed (Hi-Tech Feed,) and fresh water was provided *ad libitum* during whole period of experiment. Routine vaccination program for chicken was implemented. From All chickens, blood samples (1ml) were collected from wing vain for testing serum antibodies against MG at zero days before vaccination, at 21 days post vaccination and 10 days post challenge. The serum from each of the blood samples were separated, transferred to properly label serum vials and stored at -20 °C till further processing.

4.5.4. *Vaccination*

Routine vaccinations by trial vaccine for chickens were implemented on two groups (B and C). Prior to inoculation of both the oil based trial vaccine (indigenous) and imported- live vaccine at the age of 16 weeks, the chickens were randomly divided into three groups (A, B and C), each having twenty birds. Each bird of group A was as a control, Each bird of group B was as a vaccinated group of imported- live vaccine with 30µl dropped in the eye and each bird of group C was injected with 0.5 ml of indigenous trial-vaccine subcutaneously (s/c) at mid neck region (Hildebrand, D.G., D.E. Page and J.R. Berg (1983).

4.5.5. Challenge test

All chickens were challenged with a 24- hour broth culture (OIE; 2012), of a wild strain of MG (Samuel farm isolate of NVI) 3 weeks post-vaccination. A simple challenge method is inoculation of 0.1 ml of the challenge culture into the posterior thoracic air sac. All birds were necropsied at 10 days post-challenge, and air sac lesions were scored.



Figure.6. Inactivated MG vaccine prepared for trial

5. RESULT

5.1. Post mortem examination

Clinically sick chickens of commercial poultry farms were showing signs of respiratory distress (dyspnea), mouth breathing, tracheal rales, nasal discharge, depression, facial swelling and with history of reduced appetite (Figure 7). The most frequently encountered gross post mortem lesions were congested and hepatized lung, cloudy and thickened air sacs, and hyperemic and mucoid trachea were observed.

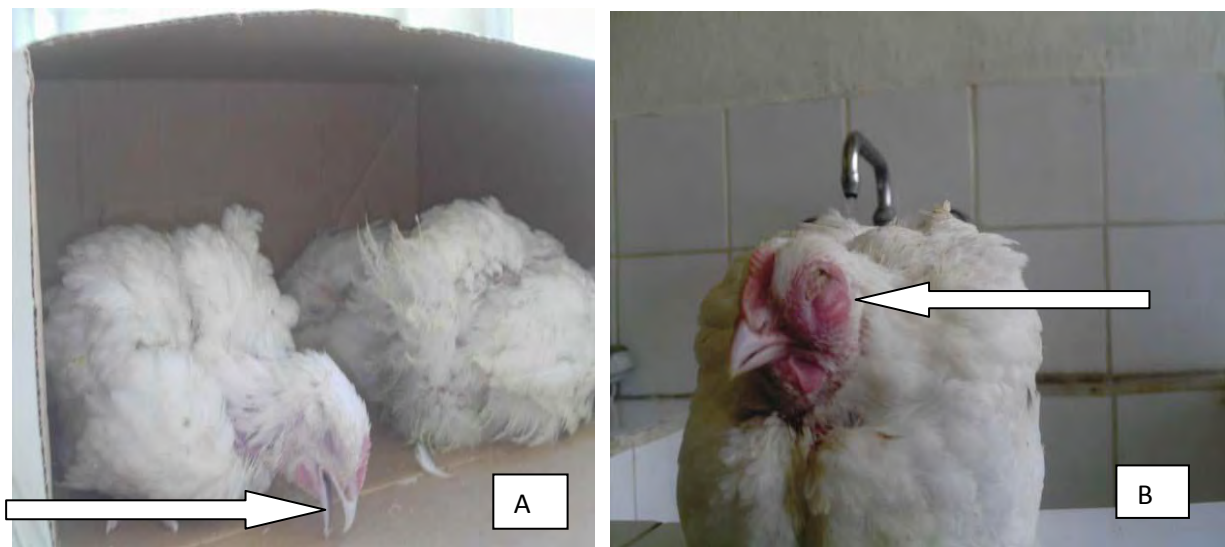


Figure.7. Clinically sick chickens.

Post mortem examination was conducted on a total of 40 backyard chickens and 80 clinically sick commercial chickens. During the examination, attention was given to respiratory organs and samples were collected from gross post mortem lesions from lung, trachea, and air sacs. Out of the post mortem examinations conducted, a total of 3 and 20 gross lesions were observed on respiratory organs (lung, trachea and air sacs) from backyard chickens and commercial chickens, respectively (Table 3).

5.2. Isolation

M. gallisepticum was isolated from clinically sick chickens of commercial poultry farm C, D and from one pooled samples of back yard chickens. Primary isolates with prominent *Mycoplasma* fried egg like micro colonies were observed under stereomicroscope (25× magnification) after three to four days of incubation. Pure *Mycoplasma* micro colonies were obtained after five to six subsequent sub culturing was conducted onto agar plates (Figure 8).

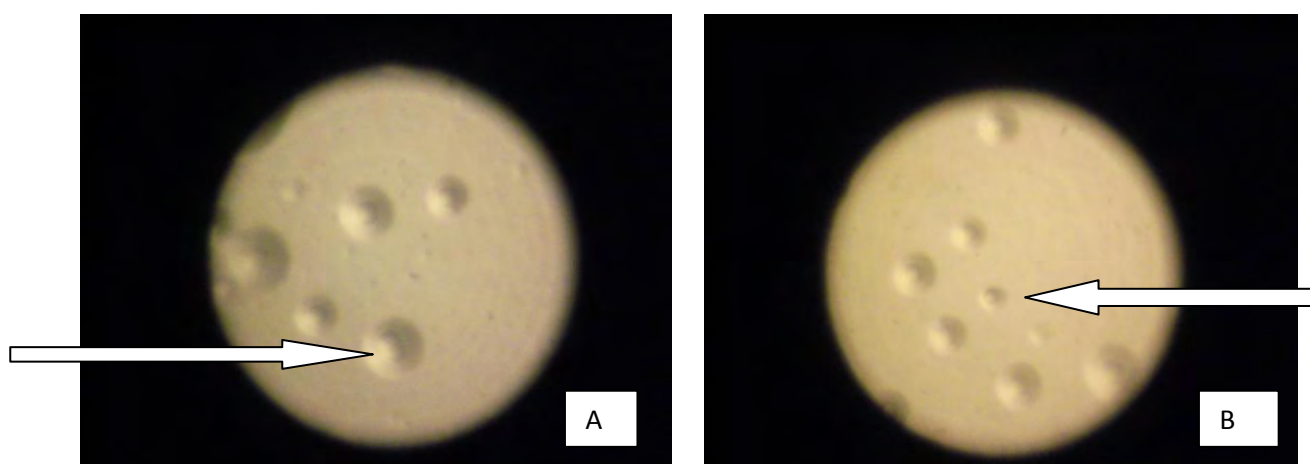


Figure.8. Pure *Mycoplasma* micro colonies on PPLO agar with characteristic round and central nipple (25× under stereomicroscope).

Table 3. *Mycoplasma* isolated from tissue samples of lung, trachea, and air sacs

Character	No. of post mortem Conducted	No. of gross lesions	No. isolated	Remark
Commercial Chickens	80	20	2 pooled	All fermented glucose & Sensitive to digitonin
Backyard Chickens	40	3	1 pooled	All fermented glucose & Sensitive to digitonin
Overall	120	23	3 pooled	Suspected isolates were <i>M. gallisepticum</i>

From 120 chickens from backyard and commercial farms only 3 pooled (25%) from farm C, D and one from back yard chickens were positive to mycoplasma micro colonies and sensitive to digitonine and ferment glucose.

All the isolated pure colonies were sensitive to digitonin (1.5% impregnated discs) which was indicated by growth inhibition around the disc kept on plates. Thus, the pure isolates were *Mycoplasma* as they were sensitive to this compound. From the preliminary biochemical tests such as fermentation of glucose and hydrolysis of arginine conducted, all the isolates fermented glucose which was indicated by color change from red to yellow but no hydrolysis of arginine was observed (i.e. no color change to arginine added broth tubes) (Figure 9).



Figure.9. Preliminary biochemical tests with fermentation of glucose which was indicated by color change (red to yellow) due to pH indicator, phenol red.

Table 4. In vitro cultivation of *Mycoplasma gallisepticum* and confirmation through growth inhibition test

No. Organs	No pooled Samples	culture isolation		growth inhibition test	
		Positive Samples	percentage	positive samples	percentage
1. Pooled samples. (Air sac, lung, trachea	12	3 pooled	25	3 pooled	25

5.3. Results of DNA Amplification

Among six isolates, (50%) or 3 samples were found strong positive and (50%) or 3 samples were weak positive to Mg as they gave 185 bp products, similar to the positive control when visualized electrophoretically (figure 10).

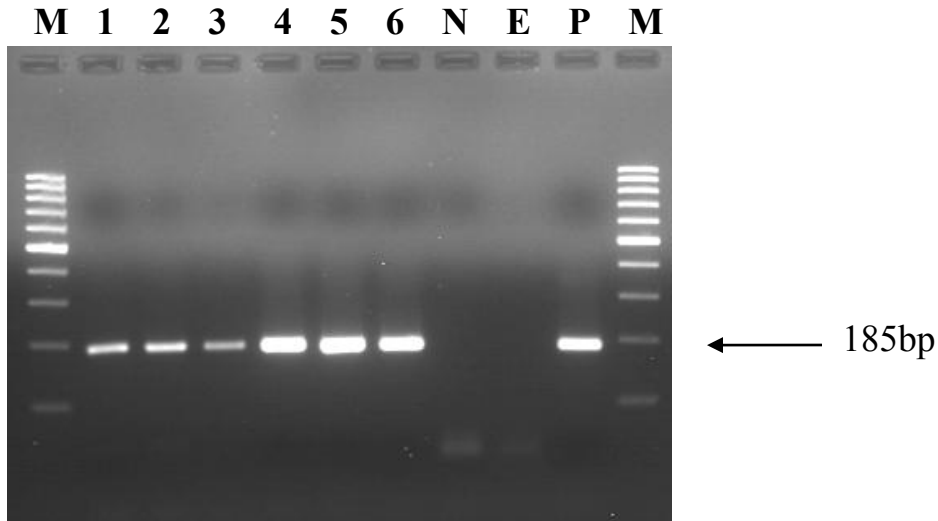


Figure.10.*Mycoplasma gallisepticum* identity test samples reference no MB722/15 classical PCR test result picture
Date-18/03/2015

Electrophoresis profile of MG DNA was obtained from six samples by PCR (185bp) on to 2% agarose gel. Lane M- Molecular marker started 100bp (Fermentas), lane 1- Jemanesh farm-weak positive , lane 2- back yard poled sample – weak positive , lane 3- Haromaya University 05-2011-weak positive , lane 4-Farm D isolate–strong positive, lane 5- farm C isolate – strong positive, lane 6- MG Samuael farm –strong positive , lane N- Negative control without template – No amplification, lane E – RNase free water extraction control- No amplification, and lane P- positive control –strong positive around 185 bp.

Table 5. Molecular identification of *Mycoplasma gallisepticum*.

No.	types of Samples	No samples	PCR result		culture result	
			positive	percentage	positive	percentage
1.	culture of isolates	3	2(++++)	66.66	3	100
			1(++)	33.33	-	-
2.	lyophilized samples	3	1(++++)	33.33	3	100
			2(++)	60	-	-

NB. (++++) strong positive, (++) weak positive

5.4. Evaluation of MG vaccine

Chickens in all groups were monitored daily up to 21 days of experiment and observations were recorded for their general conditions, clinical signs of disease or any mortality.



Figure.11. Chickens prepared for vaccine trial and challenge test

5.5. RSA test result

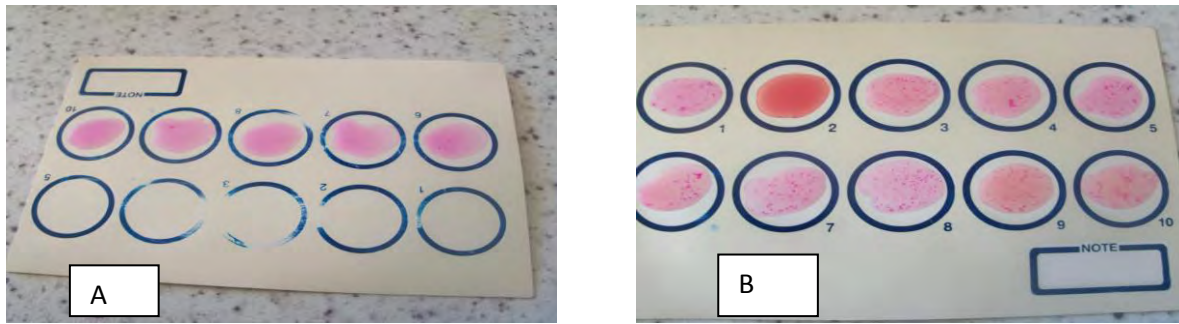


Figure.12. A) Pre vaccination RSA test for MG all were negative and B) positive control for MG

All control and vaccine groups were negative to MG infection and maternal antibodies and the vaccination and challenge test were continued.

5.6. Challenge test result

Among non-vaccinated chickens (control or group A) 2 chickens were died (10 %) and thicken and cloudy appearance of the air sac showed 18 (90%), 2 chickens were not showed thickened and cloudy air sack (10%). Although among vaccinated group (inactivated trial vaccine or group C) all chickens were not showed clinical signs or post mortem changes (100 %) and also from attenuated imported live vaccine (group B) no clinical signs or post mortem changes observed (100 %).

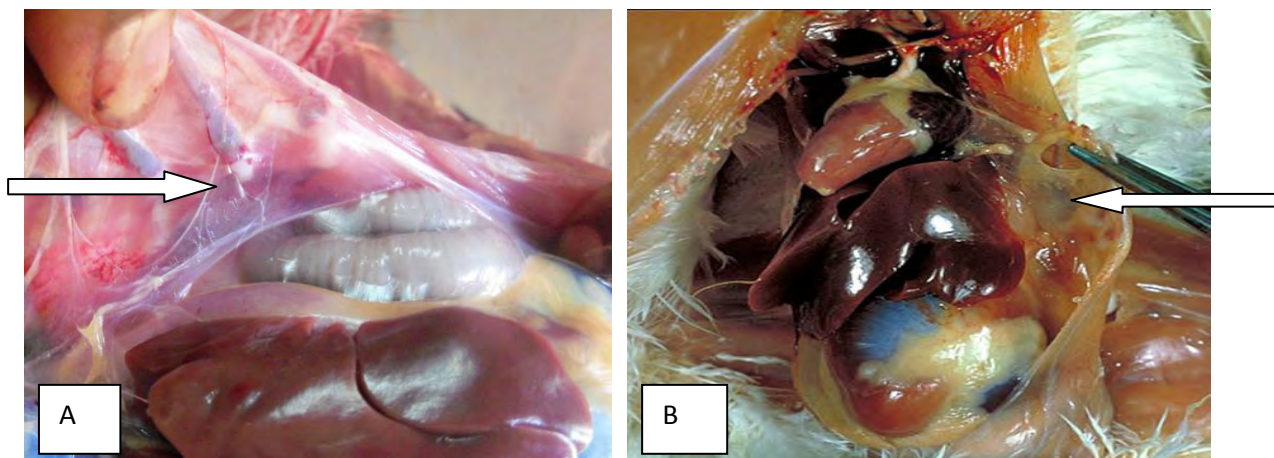


Figure.13. A) Air sac lesion negative, from vaccinated group after challenge test and B) Air sac lesion positive after challenge test from control group.

Table6. Challenge test response elicited by *Mycoplasma gallisepticum* at 10 days after challenge

Group	No. of chickens Per Group	Air sac lesion observed	No. of death	% (percent/group)
A (control)	20	18	2	90
B (live vaccinated)	20	0	0	0
C (vaccinated with Bacterins)	20	0	0	0

6. DISCUSSION

The previous studies on the prevalence of MG in commercial layer and broiler chickens showed that MG infection represents a major problem for chickens reared in commercial poultry farms (Pradhan *et al.*, 2000; Saleque *et al.*, 2003). The study of Talha (2003) demonstrated that MG infections are not only prevalent in commercial layer and broiler chickens but also widespread in village (backyard) chickens. Clinical signs of nasal discharge, dyspnea, and mouth breathing, tracheal rales, facial swelling; gross post mortem lesions of congested and hepatized lung, hyperemic and mucoid trachea, cloudy and thickened air sacs were seen.

Clinical disease was prominent in commercial chickens as they are high producing birds with reduced disease resistance whereas local chickens from which the organism was isolated were apparently healthy indicating local chickens are resistant, although lower in productivity, to the clinical disease of MG infection and in apparently carrying the organism.

DNA was extracted from pure culture of MG following the manufacturer's instruction in the facilities of the molecular biology laboratory of the National Veterinary Institute and amplification of the *mgc2* gene was performed using primers , MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3' and MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3'.

Amplified DNA fragments were analyzed by conventional 2% agarose gel electrophoresis. Among six isolates, No 1, 2,3,4,5, and 6 (50%) or 3 samples were found strong positive and (50%) or 3 samples were weak positive to Mg as they gave 185 bp products, similar to the positive control when visualized electrophonically (Figure 10).

Due to lack of differentiation in colony characteristics of different *Mycoplasma* species and time consuming nature of previous techniques, PCR was applied for diagnosis of MG using specific primers. However, in these study both isolation and molecular identification was done and the local isolate of NVI (*Mycoplasma gallisepticum*) from Samuael farm of Bishoftu grew well in PPLO broth within 24 hours at 37⁰ C with 10% CO₂ and showed 10⁸ colony forming units (CFU) per ml of the medium that gave 1.5% packed cell volume (PCV) in the Hopkins tube. *Mycoplasma gallisepticum* is wall less bacteria and does not produce endo or exotoxin, so the culture was washed and purified to get rid of the growth medium.

In the previous study formaldehyde inactivated Montanide ISA70 based MG vaccine from the PCR confirmed positive local isolate was prepared and evaluated in broilers. An amount of immune gen per 0.3ml of the dose was 107 Colony forming units of the bacteria. At the age of 14 days, the broilers were randomly divided into three groups (A, B and C), each having twenty birds. Each bird of group A, B and C was inoculated with 0.3 ml of sterile Frey's broth (negative control), indigenous vaccine (IN-VAC) and imported (IM-VAC; VaxFact-USA) subcutaneously at mid neck region, respectively. The birds of group A (Control group) showed 28.78 ± 2.53 , 21.50 ± 2.23 , 14.55 ± 1.17 , 7.18 ± 0.64 , 4.67 ± 0.37 mean anti-MG ELISA titer on 0, 15, 30, 45 and 60 days of age, respectively. However, the birds of group B showed 29.38 ± 2.82 , 153.26 ± 8.00 , 332.67 ± 9.05 , 475.78 ± 27.69 , 500.03 ± 25.38 serum mean anti-MG ELISA titer on 0, 15, 30, 45 and 60 days of age, respectively and the birds of group C showed 28.57 ± 2.25 , 106.77 ± 7.11 , 286.13 ± 10.04 , 353.45 ± 10.13 , 489.89 ± 18.76 serum mean anti-MG ELISA titer on 0, 15, 30, 45 and 60 days of age respectively. It is concluded that oil based MG vaccine induces protective level of anti-MG-ELISA antibodies in broilers that persist for more than 45 days post priming.

In the present study formaldehyde inactivated Montanide ISA70 based MG vaccine from the PCR confirmed positive local isolate was prepared and evaluated in broilers using air sack score method and all birds were necropsied at 10 days post-challenge, and air sac lesions were scored. Among non-vaccinated chickens (control or group A) 2 chickens were died (10 %) and thicken and cloudy appearance of the air sac showed 18 (90%), 2 chickens were not showed thickened and cloudy air sack (10%). Although among vaccinated group (inactivated trial vaccine or group C) all chickens were not showed clinical signs or post mortem changes (100 %) and also from attenuated imported live vaccine (group B) no clinical signs or post mortem changes observed (100 %). This study concludes that Oil based MG bacterin (killed MG vaccine) protect infection from MG.

7. CONCLUSION AND RECOMANDATION

The previous prevalent studies and this study revealed that *M. gallisepticum* infection is widespread in both commercial and backyard chickens. The overall result recorded in the present study and isolation and molecular characterization of *M. gallisepticum* from clinically sick commercial chickens of poultry farms and apparently healthy backyard chickens indicates the organism is circulating among the population of chickens in the study area.

The diagnosis of Mg and Ms in Ethiopia was made by the demonstration of antibodies against both of them by the serum agglutination test and then by indirect ELISA test. In this research, PCR technique is applied for the detection of Mg in different samples for the first time which is considered as sensitive and rapid tool. The cost of PCR can be reduced by pooling samples. PCR method can improve the diagnosis of Mycoplasma in Ethiopia. In this study we conclude that Oil based MG bacterin (killed MG vaccine) protect infection from MG.

To the best of our knowledge, this is the first report on molecular identification of Mg in Ethiopia. The researcher will be send for further serotyping of these isolates, using modern technique such as sequencing for strain identification near feature at international laboratories.

In view of the above conclusion, the following points are forwarded:

- The huge population of chickens in the country which are kept under scavenging system of production may serve as a source of infection for commercial chickens, so there should be strict isolation of commercial chickens, as the industry is growing, to avoid contact with backyard chickens.
- Control and prevention of MG should be based on maintaining MG free flocks in single age production sites. However this is difficult in multi age production sites where infection is inevitable. Therefore, appropriate use of live and killed vaccines in multi age sites especially in breeder and layer flocks should be there.
- Oil adjuvated (Montanide ISA 70) inactivated MG vaccine was developed in this study and the vaccine production department of NVI is potentially useful in the control of MG and hence further work in validating the vaccine production should be considered.

8. REFERENCE

- Abbas, A.K., A. H. Lichtman and S. Pillai (2007) Cells and tissues of the adaptive immune system. *In: Cellular and molecular immunology*, 6th Ed. Saunders Elsevier, Philadelphia, pp- 56.
- Alamargot, J. (1987): Avian pathology of industrial poultry farms in Ethiopia. In: IAR (ed.) First National Livestock Improvement Conference, Addis Ababa Agricultural Research. Pp. 114- 117.
- Alemu, Y. (1995): Poultry production in Ethiopia. *World Poultry Science Journal*, **51**(2): 197-201.
- Aviakin, A. P., Ley, D. H., and McBride, M. T. (1992): Humoral immunity response of turkeys to strain S-6 and a variant MG studied by immunoblotting. *Avian Diseases*, **36**: 69-77.
- Avakian, A. P., S. H. Kleven and J. R. Glisson (1988) Evaluation of the specificity and sensitivity of two commercial ELISA kits; the serum plate agglutination test and the haemagglutination test for *Mycoplasma gallisepticum*. *Avian Dis.* **32**: 262-272.
- Boehm U, TK Lamp, M Groot and JC Howard (1997) Cellular responses to interferon gamma. *Ann. Rev. Immunol.* **15**: 749-95
- Bokhari, A. S. (2005): *M. gallisepticum*, a recent cooperative extension article. University of California, Colorado State. <http://www.state.co.us/don.html>.
- Bradbury, J. M. (2001): Avian Mycoplasmosis, In: Frank Jordan *et al* (eds.) *Poultry Diseases*. 5th edn. W.B.Saunders Company, Iowa. Pp. 178-193.
- Butcher, G. D. (2002a): *M. gallisepticum*, a continuing problem in commercial poultry. IFAS extension, University of Florida, Gainesville. <http://www.edis.ifas.ufl.edu>.
- Butcher, G. D. (2002b): Factors to consider in serologic testing for *M. gallisepticum* (MG) and *Mycoplasma synoviae* (MS).IFAS Extension, University of Florida, Gainesville, <http://www.edis.ifas.ufl.edu>.
- Calnek, B. W., Barnes, H. J., Beard, C. W., Reid, W. M., and Yoder, H. W. (2014): Mycoplasmosis. In: *Diseases of poultry*, 9th edition, Iowa State University press, Iowa. Pp. 196-230.
- Carpenter, T. E., Mallinson, E. T., Miller, K. F., Gentry, R. F., and Schwartz, L. D. (1981): Vaccination with F-strain *M. gallisepticum* to reduce production losses in layer chickens. *Avian Diseases*, **25**: 404-409.

- Charles, H. D., and Graham, H. P. (1989): Mycoplasmosis. A laboratory manual for the isolation and identification of avian pathogens, 3rd edition, Kendall/Hunt publishing com. University of Pennsylvania. Pp. 57-62.
- Chauhan, H. V. S. (1993): Poultry disease diagnosis and treatment, Wiley Eastern limited.
- CSA (2013/14): livestock production in Ethiopia, Agricultural sample survey, volume II, statistical bulletin 573, August 2014, Addis Ababa.
- David H. Ley Mycoplasma gallisepticum Infection in Poultry (2013).
- Donald, D. B., and Mack, O. N. (1990): Commercial chicken production manual, 4th edition, Chapman and Hall, New York. Pp. 777-802.
- EARO (2000): Animal Science Research Strategy Directorate, Poultry Research Strategy. Addis Ababa, Ethiopia. Pp. 1-33.
- FAO (1993): Ethiopian livestock sector development project report No. 24/93 CP-ETH 45SR 18, Feb, 1993. Vol I or II. Rome: FAO. Pp. 8-17.
- Garcia, M., Liu T., Levisohn, S., Yogev, D., and Kleven, S. H. (2005): Molecular variability of the adhesins-encoding gene PVPA among *M. gallisepticum* strains and its application in diagnosis. *Journal of Clinical Microbiology*, **39**(5): 1882-1888.
- Gesbert, F., and Kempf (1997): Comparison of serological tests for the detection of *M. gallisepticum* antibodies in eggs and chicks hatched from experimentally infected hens. *Veterinary Microbiology Science Direct*, **60**(2-4): 207-213.
- Glisson, J. R., and Kleven, S. H. (1984): *M. gallisepticum* vaccination: Effects on egg transmission and egg production. *Avian Diseases*, **28**: 406-415.
- Glisson, J. R., J. F. Dawe and S. H. Kleven (1984). The effect of oil-emulsion vaccines on the occurrence of nonspecific plate agglutination reactions for *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. *Av. Dis.* 28 (2): 397-405.
- Gordon, R. F. (1979): Avian Mycoplasmosis. In: Poultry disease, ELBS edition, Baillere Tindal, UK. Pp. 42-49.
- Hirsh, C. D., Zee, Y. C. (1999): The Mollicutes. In: Veterinary Microbiology. Blackwell scienceLtd. Abingdon. Pp.165–172.
- Hildebrand, D.G., D.E. Page and J.R. Berg (1983). MG laboratory and field studies evaluating the safety and efficacy of an inactivated MG bacterin. *Avian Dis.* 27(3): 792-802.

- Hilton, L.S., A.G.D. Bean and J.W. Lowenthal (2002). The emerging role of avian cytokines as immune therapeutics and vaccine adjuvants. *Vet. Immunol. Immunopathol.*85: 119-128.
- ISO/IEC 17025:2005 NVI-QMS SOP gel documentation.
- Karaca, K. and K.M. Lam (1987). An inactivated temperature sensitive *Mycoplasma gallisepticum* mutant for protection against airsacculitis. *Avian Pathol.* 16:513-519
- Kita, M., Y. Ohmoto, Y. Hiari, N, Yamaguchi and J. Imanishi (1992). Induction of cytokines in human peripheral blood mononuclear cells by mycoplasmas. *Microbial. Immunol.*36: 507-516.
- Koski, T.A., G.G. Christianson and F.L. Cole (1976). Inactivation of mycoplasma by the use of phenol, formalin and beta-propiolactone. *J. Biol. Stand.*151-154.
- Levisohn, S., Glisson, J. R., and Kleven, S. H. (1985): In ovo pathogenicity of *M. gallisepticum* strains in the presence and absence of maternal antibody. *Avian Diseases*, **29**: 188-97.
- Ley, D.H., and Yoder, H.W. (1997): Mycoplasmosis/ *M. gallisepticum* infection/. In: Calnek B.W. (1991), *Diseases of Poultry*. 9th edi. Iowa State University Press, Ames. Pp. 194-207.
- Ley, H.D (2003). *Mycoplasma gallisepticum* infection. In: Saif, Y.M. (Ed.), *Diseases of Poultry*. 11th Ed. Iowa State University Press, Ames, Iowa. 722-744
- Lin, M. Y., and Kleven, S. H. (1982): Pathogenicity of two strains of *M. gallisepticum* in turkeys. *Avian Diseases*, **26**: 360-364.
- Mohammed, H. O., Carpenter, T. E., and Yamamoto, R. (1987): Economic impact of *M. gallisepticum* and *M. synoviae* in commercial layer flocks. *Avian Diseases*, **31**: 477-482.
- Muir W. I., W. L. Bryden, AJ Husband (2000). Immunity, vaccination and the avian intestinal tract. *Develop. Compar. Immunol.*24: 325-342.
- Nascimento, E.R., V.L.A. Pereira, M.G.F. Nascimento and M.L. Barreto (2005). Avian mycoplasmosis update. *Brazilian J. Poult. Sci.*7: 01-09.
- OIE (2012): Avian Mycoplasmosis, In: *Manual of diagnostic tests and vaccines for terrestrial animals*, OIE, France.
- Quinn, P. J., Carter, M. E., Markey, B., and Carter, G. R. (2002): *The Mycoplasmas*, In: *Clinical Veterinary Microbiology*, Mosby, Virginia Tech, and Blacksburg, USA. Pp. 320-326.
- Rimler, R.B., R.B. Davis, R.K. Page and S.H. Kleven (1987). Infectious coryza, preventing complicated coryza with *Haemophilus gallinarum* and *Mycoplasma gallisepticum* bacterins. *Avian Dis.* 22:140-150.

- Sasipreeyajan, J., D.A. Halvorson and J.A. Newman (1985). Bacterin to control the vertical transmission of *Mycoplasma gallisepticum* in chickens. Avian Dis. 29:1256-1259.
- Sen, G.C. and P. Lengyel (1992). The interferon system. J. Biol. Chemist. 267: 5017-5020.
- Talha, A. F. S. M. (2003): Investigation on the prevalence and significance of *M. gallisepticum* in village chickens and possibility of establishing *M. gallisepticum* free flocks and significance of *M. gallisepticum* on different production parameters in layer chickens in Bangladesh. MSc Thesis. The Royal Veterinary and Agricultural University, Denmark and Bangladesh Agricultural University, Mymensingh,.
- Tizard, I.R. (2004). *In: Veterinary immunology, an introduction.* 7th Ed. Saund. Pub.105-12
- Whithear, K.G., K.E. Harrigan and S.H. Kleven (1996). Standardized method of aerosol challenge for testing the efficacy of *Mycoplasma gallisepticum* vaccines. Avian Dis. 40 (3): 654-660.
- Yagihashi, T., T. Nunoya and M. Tajima (1987). Immunity induced with an aluminum hydroxide-adsorbed *Mycoplasma gallisepticum* bacterin in chickens. Avian Dis. 31:149-155
- Yasmin, (2007): Isolation and sero prevalence study on *Mycoplasma gallisepticum* infection.
- Yoder, H.W.J (1991). *Mycoplasma gallisepticum* infection. *In: Hofstad, M. S.; Baren, H. J.; Calnek, B.W.; Reid, W.M.; Yoder, H.W. Jr. Editors. Diseases of Poultry. 9th Ed. Ames: Iowa State University Press.*198-212.

9. ANNEX

Annex 1. Cultural isolation of *M. gallisepticum*

A) Mycoplasma media preparation

* **Basic medium**

Modified Hay flick's media and/or PPLO (pleuropneumonia like organism) broth base (Difco) without genital violet (GV) preparation:

- 14.7 gram was measured using beam balance and added to 700 ml of distilled water.

-Autoclaved at 121oC for 15 minutes.

-Then cooled at 56 oC in water bath and stored at -20 oC until used

* **Supplements:** as Mycoplasma are fastidious (slow growing) organism, protein enriched media mainly serum is required to support growth. The other growth factors include glucose, DNA, and yeast extract. To avoid contaminants like bacteria and fungi, inhibitory factors such as penicillin and thallium acetate (nystatin) was also added

in the media. Supplement preparation include: - 150 ml of horse serum (pre heated at 56oC for 1hour). - 25% fresh yeast extract (100ml): Add 250gm into 1000ml dis. H₂O and heated to boiling point (100oC) Cooled and centrifuged at 3000 rpm for 20 min. then the supernatant decanted and pH adjusted to 7.8 by adding 20% NaOH. Clarified by centrifugation and filter sterilized and stored at -20o C until used - 10% glucose (10ml): add 10gm to 100ml dis. H₂O and pH adjusted to 7.8 – 8.0 with 20% NaOH. Then sterilized by filtration and stored at + 4oC. - 5ml of penicillin (200,000 IU): add 106 IU benzyl penicillin in to 5 ml of dis. H₂O and stored at + 4oC (shelf life of one week) - 10ml of thallium acetate (5%) : add 5gm to 100ml of dis. H₂O. Then filter sterilized and stored at -20oC. - 20ml of 0.1% phenol red: add 0.1gm to ground in 2.8ml (0.1M NaOH) then add to 100ml of dis. H₂O. Autoclave at 115oC at 1 atmosphere for 30 minutes and stored at + 4oC. - Finally, the supplements can be mixed together and stored at -20oC until used.

Finally, to dispense the basic media and supplement into each agar plates, first remove basic media and supplement from the -20oC refrigerator. Boil the basic media in water bath until the temperature drops up to 60oC and keep the supplement at room temperature. Then pour 20ml basic media and 5ml supplement into each plates and mixed gently

Formulations of two commonly used media for the isolation and propagation of avian Mycoplasmas.

Medium	Constituent	Amount
Frey's medium	Mycoplasma broth base (BBI, Cockeysville, MD)	22.5 g
	Glucose	3 g
	Swine serum	120 ml
	Fresh yeast extract	100 ml
	Cysteine hydrochloride*	0.1 g
	NAD*	0.1 g
	Phenol red (1%)	2.5 ml
	Thallium acetate (10%)	2.5 to 5 ml
	Penicillin G potassium	106 units
	Distilled water q.s.	1000 ml
	Adjust pH to 7.8 with 20% NaOH and filter sterilize	
PPLO broth	PPLO broth without crystal violet (Difco)	14.7 g
	Glucose	10 g
	Fresh yeast extract	100 ml
	Swine serum	150 ml
	Cysteine hydrochloride*	0.1 g
	NAD*	0.1 g
	Phenol red (1%)	2.5 ml
	Thallium acetate (10%)	2.5 to 5 ml
	Penicillin G potassium	106 units
	Distilled water q.s.	1000 ml
	Adjust pH to 7.8 with 20% NaOH and filter sterilize	

(Source: Charles and Graham, 1989)

* Note: Cysteine hydrochloride and NAD is added to support the growth of *M. synoviae*

B) Laboratory procedures for isolation

Tissue sample processing and inoculation of agar plates - Gross post mortem lesions of lung, trachea, and air sacs of individual birds were taken from clinically sick chickens of commercial poultry farms and from those backyard chickens bought sampling purpose. The samples were recorded individually. - To maximize the chance of isolating the organism, tissue samples of lung,

trachea, and air sacs were crushed together using mortar and piston. Crushing was done with the help of sterile sand and PBS to facilitate tissue crushing. - Homogenous tissue suspension was then passed through sieve and then filter paper. The supernatant poured into plastic tubes and centrifuged at 5000 rpm for 20 minutes. - Again, inside the safety cabinet, the supernatant decanted into sterile plate and sucked into sterile syringe (20ml). Then passed through sterile bacterial membrane filter of 0.45µm Millipore. -The final filtrate dispensed, distributed on to the surface of agar plates and the excess fluid sucked from the surface of the plate using Pasteur pipette. -

The inoculated agar plates were incubated at 37°C with 5% CO₂, gas generating incubator- The next few days, plates were examined frequently for any observable micro colony growth under stereomicroscope (× 25 magnification) on daily bases- Primary isolates with prominent micro colonies were selected under stereomicroscope, blocks of agar containing micro colonies were incised using sterile scalpel meant for this purpose; and subcultured on to agar plate by “pushing block” method and also inoculated the block into an air tight broth tubes and incubated - Further subculture could be made either from agar to agar, agar to broth, or broth to broth. But broth to broth subculture may need to filter the broth culture into sterile membrane filter of 0.45 µm Millipore before inoculated in to another broth tube.

C) DNA extraction procedure

- 1. Cultured cells:** centrifuged a maximum of 5×10^6 cells for 5 min at 300 x g (190 rpm). Resuspend in 200µl PBS. Add 20 µl proteinase K. proceed to step 2.
2. Add 200 µl Buffer AL. Mix thoroughly .incubate blood samples at 56⁰c for 10 min.
3. Add 200 µl ethanol (96- 100%). Mix thoroughly by vortexing.
4. Pipet the mixture in to DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g. Discard the flow-through and collection tube.
5. Place the spine column in a new 2 ml collection tube. Add 500 µl Buffer AW2; Centrifuge for 1 min at ≥ 600 xg. discarded the flow-through and collection tube.
6. place the spine column in a new 2 ml collection tube, add 500 µl Buffer AW2 , and centrifuge for 3 min at 20,000 x g (14000rpm).discard the flow-through and collection tube.
7. Transfer the spin column to a new 1.5ml or 2 ml microcentrifuge tube.
8. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15- 25⁰c). Centrifuge for 1 min at ≥ 6000 x g.
9. Optional: repeat step 8 for increased DNA yield.

