

Inactivated Vaccine Trial of Mycoplasma gallisepticum in Ethiopia

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Abstract

The study and entire laboratory works were conducted from December 2014 to April 2015 in National Veterinary Institute, Bishoftu, Ethiopia. Formaldehyde inactivated Montanide ISA70 based Mycoplasma gallisepticum (MG) trial vaccine strain was confirmed the identity with known primer using PCR from locally isolates of National Veterinary Institute of Ethiopia. This study was aimed to develop formaldehyde inactivated Montanide ISA70 based MG vaccine in Ethiopia. It can help to device strategies in controlling the disease mainly through developing more effective vaccine which will replace the currently being imported vaccines by some farms. After culturing procedure, oil based inactivated MG trial vaccine was produced in suitable clean and secure accommodation. In this study, among different isolates, local isolate of Samuel farm in NVI was prepared and evaluated in chickens. The amount of immune antigen per 0.5 ml of the dose was 107 Colony forming units (CFU) of the bacteria. The trail vaccine was prepared and evaluated at the age of 16 weeks of chickens; the chickens were randomly divided into three groups (A, B and C), each having twenty birds (10 male and 10 female). Each of group B was vaccinated group of imported-live vaccine with 30 µl intraocularly for comparing with inactivated trial vaccine, 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 then challenge tests were performed. After challenge test, among non-vaccinated chickens (control or group A) 2 chickens were died (10%), 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 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 were observed (100%). It was concluded that oil based MG vaccine induces protective level of anti MG antibodies in chickens.

Keywords

Adjuvant, Chickens, Inactivated Vaccine, *Mycoplasma gallisepticum*, PCR

1. Introduction

The total poultry population in Ethiopia is estimated to be 51.35 million [1]. 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 is about 57 eggs and 2 kg per anum, respectively [2]. The sub sector is concerned with egg and meat production for income generation and home consumption [3] [4]. However, diseases among other factors are rampant and hindered the development of poultry production in the country and mortalities due to diseases which are estimated to be 20% - 50% but can go as high as 80% during times of epidemics [5].

Avian mycoplasmosis causes considerable economical losses to the poultry industry, especially in chickens and turkeys all over the world. 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 [6]. Vertical transmission of MG has been documented and it leads to infected progeny flocks [7]. It also causes respiratory disease including sinusitis and conjunctivitis in turkeys, pheasants, partridges, quail, ducks, geese and other avian species [6].

Mycoplasma gallisepticum is the most pathogenic avian *Mycoplasma*; however, considerable strain variability is manifested with respect of 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 [8]. *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 PCR 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 [9].

Mycoplasma gallisepticum infection is among one of the major economically important respiratory disease problems of both commercial and backyard poultry production systems [10]. 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 [11].

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. The current trial vaccine will prepare locally cheaper than imported live vaccine due to cheap labor in our country, will prepare with own production facility and imported vaccine transportation cost is nil.

2. Materials and Methods

2.1. Study Area

The study and entire laboratory work was conducted at National Veterinary Institute (NVI) of Ethiopia.

2.2. Study Animals

A total of sixty (60) chickens, white leghorns purchased from Alema farm of day old chickens, 30 male and 30 female non-vaccinated with MG vaccine and reared for two weeks in the facility of NVI (study area). The chickens were grouped in to three, 20 for inactivated vaccine, 20 for attenuated live vaccine (imported) and 20 for control. The animal use protocol has been approved from Addis Ababa university school of veterinary medicine.

2.3. Study Design

Among ten lyophilized isolates and molecularly identified strains at NVI, isolate of Samuel farm was selected to use as a seed for vaccine trial. The selection of the isolate was carried out based on history of the isolates and growth during renew, sample origin (farm type and place) and isolation year.

Media Preparation

Mycoplasma agar and broth was prepared as per OIE manual [9] and manufacture instruction for isolation of avian *Mycoplasma* [12]. The media was sterilized by autoclave and filtration according to their components and stored at 4°C until use.

3. Inactivated Mycoplasma gallicepticum Vaccine Trial

Two types of vaccines are available for the control of Mycoplasma gallisepticum.

These are mild to a virulent strains used as live vaccines, or inactivated oil-emulsion bacterins. Although there is an antigenic variability strains, it is thought that vaccination with a single strain is sufficient. The vaccine trial was a formalin in-activated oil base suspension of MG. The bacterin is used for the prevention of clinical signs associated with MG infection in chickens [13].

3.1. Characteristics of the Seed

Mycoplasma gallisepticum bacterin was prepared from a concentrated suspension of whole cells that is emulsified into an oil adjuvant.

3.2. Method of Culture for Vaccine Preparation

The identity was confirmed using PCR, local *Mycoplasma gallisepticum* isolate from Samuel farm (Isolate of NVI) used as a seed, in active form, was inoculated at 10 per cent v/v into Frey's broth and incubated at 37° C at 10 per cent CO₂ tension and observed daily for any growth. Sterility test for oil adjuvant and final product have been done. After 48 hrs of incubation, the cultured media tubes were taken out of incubator and observed for presence of turbidity.

3.3. 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 contamination of other products manufactured in the same facility.

Production of vaccine was on a seed-lot system. For the bacterin production, the antigen was concentrated by centrifugation and resuspended in a small volume of phosphate buffer saline (PBS) before the emulsion was prepared. Bacterins were made with Montanide ISA 70 [14].

3.4. In-Process Control

3.4.1. 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 $3000 \times$ g 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 (1 ml cell mass in to 100 ml) in the Hopkins's tube using phosphate buffer saline (pH 7) as diluent [15].

3.4.2. Inactivation of Killed Vaccine

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 [16].

For inactivation of growth of MG bacteria, 0.125% formaldehyde was used and the culture was incubated at 37° C at 10% CO₂ tension for providing 12 hrs of interaction time. After inactivation process the bacteria were washed with sterile buffer saline water to remove protein contamination. To ascertain the completion of inactivation process, the inactivated broth was separately cultured on *Mycoplasma* broth and agar, incubated at 37° C at 10% CO₂ tension and observed for seven days for appearance of any specific growth of *Mycoplasma* colonies or color change, respectively [17]. After inactivating the final product, there was no evidence of growth of *Mycoplasma* observed.

3.4.3. Sterility Test for Oil Adjuvant and Final Product (Trial Vaccine)

Oil adjuvant (Montanide ISA 70) used in the vaccine was sterilized by heating at 160°C for 1 hour in dray autoclave [9]. Sterility tests were done with sterility media such as VF, Thioglycolat, Triptic 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 (4 parts of oil adjuvant to 1 part of bacterial biomass) to properly emulsify the bacterial biomass. The inactivated culture was processed further to ensure the safety and sterility of the culture [18].

3.5. Evaluation of Mycoplasma gallisepticum 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. From All chickens, blood samples (1ml) were collected from wing vein for testing serum antibodies against *Mycoplasma gallisepticum* 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 for further processing.

3.5.1. Rapid Serum Agglutination Test

Sera were collected from all twenty controls and forty chickens for trial vaccine test group and stored at 4°C until used and the RSA test is done on the same day of collection. The test was carried out at room temperature (20°C - 25°C) within 72 hours of serum collection and the reagents were also being at room temperature. To reduce nonspecific reactions centrifugation was done [19].

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 *Mycoplasma gallisepticum (Lot No:* 01143 *Mycoplasma gallisepticum Ag pro-duced by Salsbury laboratories).* 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 [19]. And included known positive and negative controls in the test (**Figure 1**).



Figure 1. (a) Pre vaccination RSA tests for MG all were negative and (b) positive control for MG (Photo by Legesse Bekele, 2015).

3.5.2. Safety Test

After reconstitution, the trial vaccine was inoculated double doses (1 ml) subcutaneously at mid neck region [15] into six chickens. None of the chickens 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.

3.5.3. Vaccination

At the age of 16 weeks (**Figure 1**), the chickens were randomly divided into three groups (A, B and C), each having twenty birds. 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 grouped as each bird of group A was as a control, each bird of group B was as a vaccinated group of imported-live vaccine from Ji Lin ZhengYe Biological products co,. LTD, China and with 30 µl dropped in the eye and each bird of group C was injected with 0.5 ml of indigenous prepared trial-vaccine (**Figure 2**) subcutaneously (s/c; at mid neck region) [15].

3.5.4. Challenge Test

All chickens (**Figure 3**) were challenged with a 24 hour broth culture [9] of a wild strain of *Mycoplasma gallisepticum* 3 weeks post-vaccination. A simple challenge method was inoculation of 0.1 ml of the challenge culture into the posterior thoracic air sac.

4. Result

4.1. RSA Test Result

All control and vaccine groups were negative to *Mycoplasma gallisepticum* infection and maternal antibodies (Figure 1) and the vaccination and challenge test were continued.

4.2. Challenge Test Result

All birds were necropsied at 10 days post-challenge, and air sac lesions were scored showing the presence of turbidity of the air sac. 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%), (Figure 4(b), Table 1). Although among vaccinated group by (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 4(a), Table 1). This implies that our killed *Mycoplasma gallisepticum* trial vaccine can potentially protects infection from *Mycoplasma gallisepticum* and shows a promising and potent result.



Figure 2. Inactivated MG vaccine prepared for trial (Photo by Legesse Bekele, 2015).



Figure 3. Chickens prepared for vaccine trial and challenge test (Photo by Legesse Bekele, 2015).



(a)



(b)

Figure 4. (a) Air sac lesion negative, from vaccinated group after challenge test; and (b) Air sac lesion positive after challenge test from control group (Photo by Legesse Bekele, 2015).

Table 1. Challenge test response elicited by *Mycoplasma gallisepticum* at 10 days afterchallenge test.

Group	Number of chickens per group	Air sac lesion observed	Number of death	Percentage
A (control)	20	18	2	90
B (live MG vaccinated)	20	0	0	0
C (Vaccinated with trial bacterin, MG)	20	0	0	0

5. Discussion

The previous studies on the prevalence of *Mycoplasma gallisepticum* in commercial layer and broiler chickens showed that *Mycoplasma gallisepticum* infection represent a major problem for chickens reared in commercial poultry farms [20] [21]. The study of [10] demonstrated that *Mycoplasma gallisepticum* 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 in those studies.

The selected local isolate of NVI (*Mycoplasma gallisepticum*) from Samuael farm grew well in Hayflaick's broth within 24 hours at 37° C with 10% CO₂ and showed 10^{8} colony forming units (CFU) per ml of the medium that gave 1.5% packed cell volume (PCV) in the Hopkins tube. *Mycoplasma gallisepticum* bacterin was prepared from a concentrated suspension of whole cells that is emulsified into an oil adjuvant. For killed vaccines the most important characteristics for seeds 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 [9].

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. Virulent strains were desirable for challenge test.

In previous studies, formaldehyde inactivated Montanide ISA70 based *Mycoplasma gallisepticum* vaccine from the PCR confirmed positive local isolate was prepared and evaluated in broilers in other countries. The amount of immune gene per 0.3 ml 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; Vax Fact-USA) subcutaneously at mid neck region, respectively [22].

In this study, formaldehyde inactivated Montanide ISA70 based *Mycoplasma gallisepticum* vaccine from local isolated strain and identity was confirmed with known primer using PCR was prepared and evaluated in chickens using air sack lesions score method. All birds were necropsied at 10 days post-challenge and air sac lesions were scored. Therefore, by this study it is concluded that Oil based MG bacterin (killed *Mycoplasma gallisepticum* trial vaccine) protects infection from *Mycoplasma gallisepticum* in Ethiopia.

6. Conclusion and Recommendations

The previous prevalent studies revealed that *Mycoplasma gallisepticum* infection is widespread in both commercial and backyard chickens. The overall result recorded in the present study of oil based inactivated *Mycoplasma gallisepticum* trial vaccine formulated at National Veterinary Institute is useful for the near future of Ethiopia. This is the first report on developing Oil adjuvated (Montanide ISA 70) inactivated *Mycoplasma gallisepticum* vaccine in Ethiopia and the vaccine production department of National Veterinary Institute is potentially useful in the control of *Mycoplasma gallisepticum* and hence further work in validating the vaccine production should be considered.

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