Research article
J. Adv. Nutri. Sci. Technol. 5(1-2): 12 - 17, 2025 **DOI:10.15228/ANST.2025.v05.i01-2.p02**

Immune Response in Domestic Fowl Against Oil-Based Vaccine Produced from Local Isolates of *Mycoplasma gallisepticum*

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Abstract
The global economic importance of poultry for the production of meat, eggs, and related by-products, either through commercial poultry farms or by small-family owned producers, is undeniably crucial. *Mycoplasma gallisepticum* (MG) is the causative agent of highly transmissible and persistent Chronic Respiratory Disease (CRD). The clinical and economic burden of CRD causes substantial losses to the poultry industry and is particularly devastating to smaller producers. Imported vaccines are costly, difficult to obtain, and less effective due to frequently emerging mutant strains. The present study aimed to synthesize an oil-based inactivated vaccine using local isolates of *Mycoplasma gallisepticum* (MG) as a strategic control measure. This vaccine has the potential to serve as an effective alternative to currently imported vaccines. MG strains were isolated from infected broilers and obtained from poultry farms located in the Tret, Tarli, and Sehala areas of Islamabad. Effective Inactivation Dose (EID 70) of the cultures was calculated. The antigen was inactivated by 0.1% formalin and then washed in sterile PBS. The vaccine was prepared in montanide oil in 40:60 ratios. A commercial ELISA kit detected an antibody titer. Three vaccines, A, B, and C, were prepared from three different isolates. Two weeks old broilers were divided into 3 groups, each containing 10 birds and vaccinated. Unvaccinated birds were maintained as negative and positive control groups. Post-inoculation antibody titer after 15 and 30 days was 4 and 7, respectively. Birds were challenged with the EID 70 dose of respective antigens and an antigen mixture. The vaccines prepared from local isolates were found to be effective against CRD. The current study proves the effectiveness of locally produced vaccines against CRD; however, a larger scale is required in this respect.
Key Words: Poultry, *Mycoplasma gallisepticum*, Chronic Respiratory Disease, Vaccine

Highlights
- *Mycoplasma gallisepticum* (MG) is the causative agent of highly transmissible and persistent Chronic Respiratory Disease (CRD) in poultry.
- The clinical and economic burden of CRD causes substantial losses to the poultry industry and is particularly devastating to smaller producers.
- Imported vaccines are costly, difficult to obtain and less effective due to frequently emerging mutant strains.
- Vaccine from local isolates of inactivated MG was prepared in montanide oil.
- The vaccines prepared from local isolates were found to be effective against CRD.

1.0. Introduction
Mycoplasma gallisepticum (MG) is the causative agent of chronic respiratory disease (CRD) and infectious sinusitis in domestic fowls (Yadav et al., 2022). CRD is an economically significant infection that results in extensive losses for the poultry industry as the disease can cause reduced feed conversion, a decrease in egg production and hatchability, dwarfing of chicken embryos, significant downgrading of carcasses at slaughter, and even mortality, which is devastating for commercial poultry flocks (Malik et al., 2004; Shoaib, 2019). The symptoms of CRD in chickens include nasal discharge, coughing, sneezing, lameness of the joints, air saculitis, and occasionally conjunctivitis (Umar et al., 2017). Hygiene is an essential factor in disease etiology. Disease transmission is rapid throughout the flock and occurs by inhalation of air-borne droplets, contaminated eggs, or through infected equipment (Mugunthan et al., 2023).

Infection of MG in chicken embryonic membranes induces lymphotactin secretion that results in the migration and accumulation of lymphocytes to the infection sites (Lam and DaMassa, 2003). Infiltration of mononuclear cells and hyperplasia of mucous glands are exhibited in infected tissues of MG-inoculated chickens, resulting in the thickening of the mucosa (Majumder and Silbart, 2016). The avian T-cell response against mitogens fully develops at the age of 1 week (Liu et al., 2024). The humoral response to MG infection and antigenic stimulation by Bovine Serum Albumin (BSA) has been reported in 2-week old birds (Gaunson et al., 2000; Beaudet et al., 2019). Despite several studies documenting the development of avian immune response at 2 weeks of age, a number of vaccines have been shown to be effective in Ovo administrations (Alqhtani et al., 2023). Local antibody-mediated responses, as well as cytotoxic T cells and natural killer

cells, have been reported to have primary importance in MG infection, triggering oxidative stress, apoptosis, and histopathological changes (Hu et al., 2021).

MG infection is controlled through several attenuated live vaccines and inactivated MG bacterins (Ishfaq et al., 2020). However, commercially available vaccines have been shown to demonstrate remarkable differences in terms of post-vaccination reactions, serological responses, and persistence in the upper respiratory tract. Various strains of MG differ in virulence, which influences the potency of the vaccine (Ferguson-Noel et al., 2012). An imported vaccine is costly, and its availability is limited, which is partly the reason why inactivated vaccines from local isolates are increasingly becoming popular; notably, the effectiveness of oil bacterin has been reported by several studies for controlling MG outbreaks (Moura et al., 2012; Limsatanun et al., 2018; Ferguson-Noel et al., 2024).

The objective of the current study was to develop a killed vaccine of the local isolates of MG for a reduction in economic losses caused by CRD.

2.0. Materials and Methods

2.1. Ethical statement

The animals were housed and euthanized in accordance with International ethics standards for animal rights. Sodium pentobarbital (100 mg/kg body weight) was administered via intravenous route to minimize suffering during post-trial flock culling.

2.2. Experimental animals

Two weeks old, healthy broiler chickens were maintained for experimental vaccination and control. The vaccine trial lasted for a total of 30 days.

2.3. Sample collection

MG was isolated from chickens obtained from the broiler breeder farms located at the Tret, Tarli, and Sehala areas of Islamabad. Sterile cotton swabs with wooden applicators were used to collect tracheal swab samples from CRD-infected chickens. The swabs were dipped in mycoplasma broth. The infectious organism was identified and inoculated for 5 days on pleuropneumonia-like organism (PPLO) broth as previously described elsewhere (Hanif and Najeeb, 2007). The cultures were then centrifuged and washed with phosphate-buffered saline (PBS). The EID 70 (Egg Infectious Dose) was calculated. Optical density (O.D) was measured at 590 nm, and CFU/ml (Colony Forming Units per milliliter) was calculated.

$$\text{CFU/ml} = \frac{\text{Number of colonies counted}}{\text{Volume of culture plated (ml)} \times \text{Dilution factor}}$$

2.4. Preparation of vaccine

The antigen was rendered inactive in 0.1% formaldehyde (Koski et al., 1976) and then washed four times with sterile PBS. The inactive isolates were mixed with montanide oil in the ratio of 40:60 and stirred magnetically overnight (Fig, 1). Three vaccines, A, B, and C, were prepared from each of the three different isolates. The quantity of inoculated vaccine was 0.5ml. The emulsion was packed in vials. Antibody titer was detected with a commercial ELISA Kit.

2.5. Administration of vaccine

Three groups of broilers VG1, VG2, and VG3, each containing ten birds, were subcutaneously injected and inoculated with 0.5ml of A, B and C vaccines in the wings. Five groups, UVG1, UVG2, UVG3, UVG4, and UVG5, each having three unvaccinated broilers, were kept as control.

2.6. Antigen challenge experiments

The vaccinated groups VG1, VG2, and VG3 were challenged with EID 70 dose of respective antigens A, B, and C as well as a mixture of all three antigens (A+B+C). The unvaccinated positive control groups UVG1, UVG2, and UVG3 were challenged by antigens A, B, and C; UVG4 was challenged by a mixture of all the three antigens (A+B+C) whereas UVG5 was kept as negative control and challenged only with 0.3ml of buffer (as infraorbital eye drops). The antibody titer was recorded twice, after 15 and 30 days (Fig. 2).

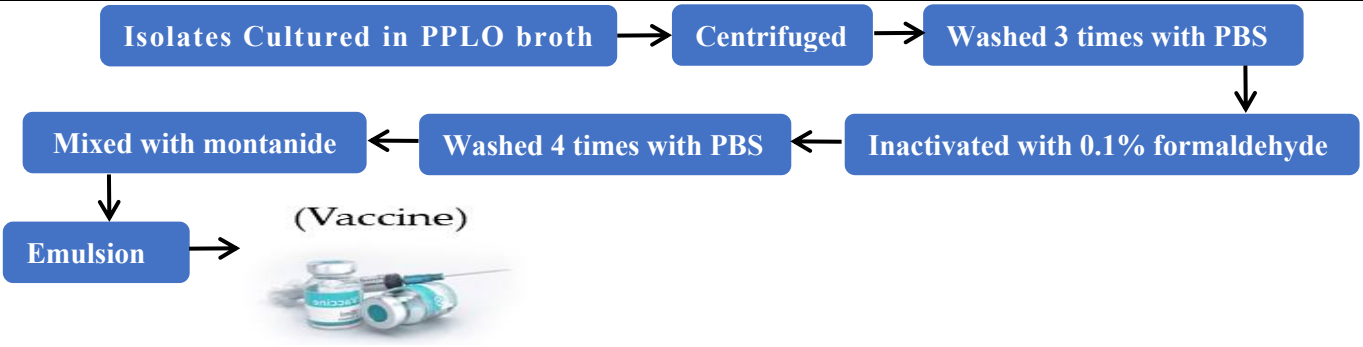

Figure 1: Preparation of vaccine

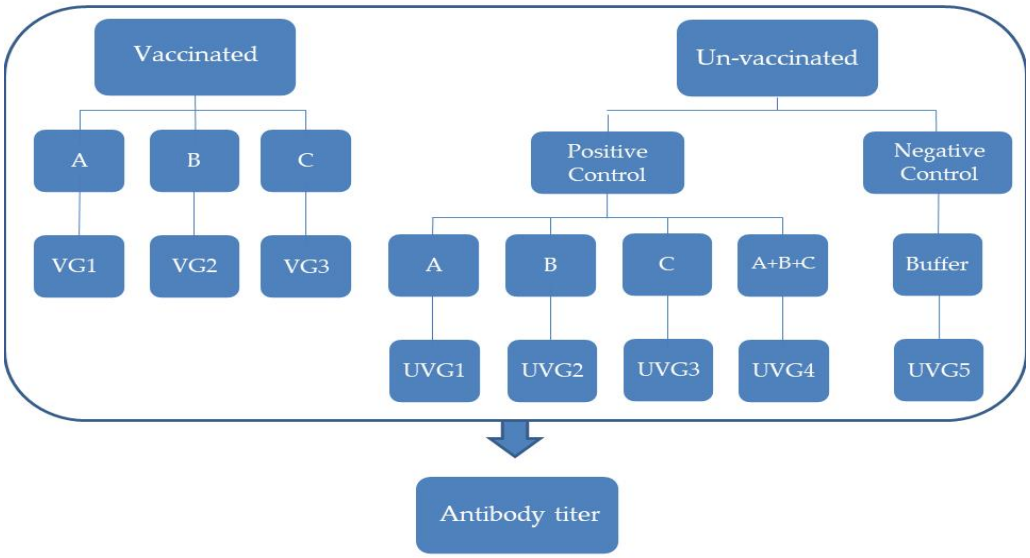

Figure 2: Antigen challenge experiments

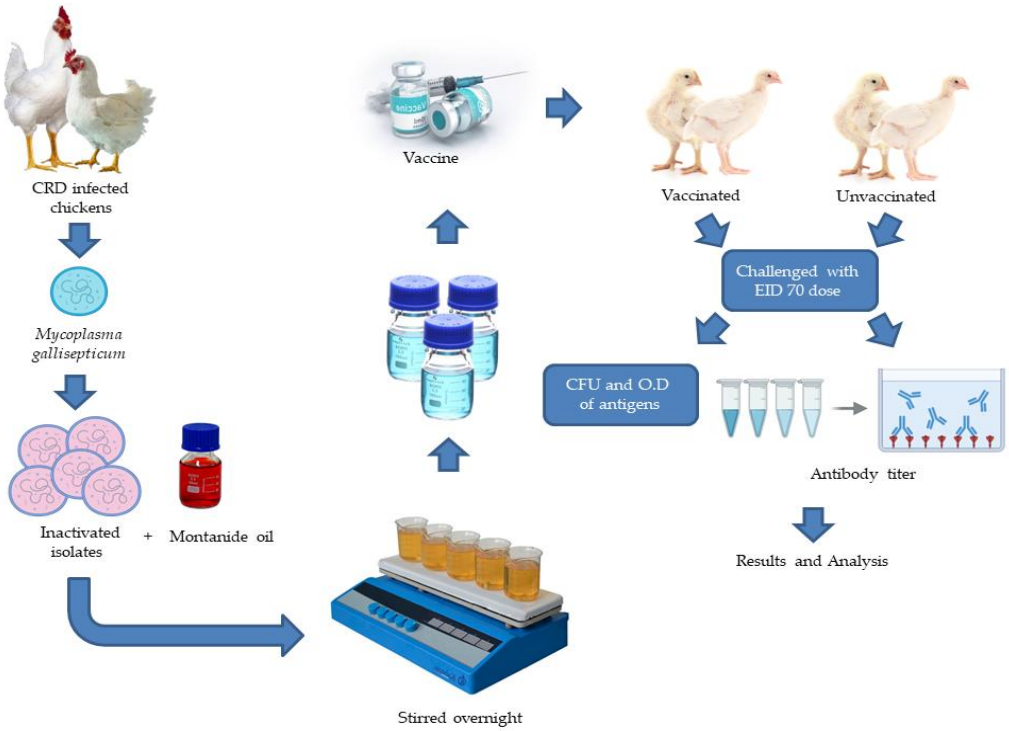

Figure 3: Plan of work

3.0. Results and Discussion

The CRD vaccine produced from local isolates of MG proved to be effective. The CFU and O.D of antigens A, B, and C were 3.5x107, 4.5x107, 1.5x108, and 1.03, 1.12, and 1.3, respectively (Table 1). The vaccinated groups VG1, VG2, and VG3 showed no signs or symptoms of CRD when challenged with antigens A, B, C, and antigen mixture A+B+C. Meanwhile, the unvaccinated groups UVG1, UVG2, UVG3, and UVG4 developed CRD-related disease symptoms (Table 2). Post-inoculation antibody titer after 15 and 30 days was 4 and 7, respectively (Table 3).

Table 1: CFU and O.D of antigens A, B and C

Observations	Vaccinated groups		
	VG1	VG2	VG3
CFU	$3.5x10^7$	$4.5x10^7$	$1.5x10^8$
O.D	1.03	1.12	1.3

Table 2: Antigen challenge experiments

Antigen Challenge EID 70	Vaccinated group			Unvaccinated control group				
				Positive				Negative
	VG1 Antigen A	VG2 Antigen B	VG3 Antigen C	UVG1 Antigen A	UVG2 Antigen B	UVG3 Antigen C	UVG4 Antigen A+B+C	UVG5 Buffer
	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve

Table 3: Post-vaccination antibody titer after 15 and 30 days in three groups of vaccinated and unvaccinated control group broilers

	Vaccinated groups			Control groups
	VG1	VG2	VG3	No vaccination
Antibody titer after 15 days	4	4	4	0
Antigen titer after 30 days	7	7	7	0

MG strains exhibit variable levels of virulence, with some capable of causing severe respiratory disease in poultry. Highly virulent strains can rapidly spread within breeder and commercial flocks, leading to significant economic losses due to reduced egg production, poor weight gain, and increased mortality. The ability of MG to persist and transmit easily makes it a substantial concern in poultry farming, necessitating stringent biosecurity measures, vaccination programs, and early detection strategies to prevent outbreaks and minimize their impact (Soeripto et al., 1989; Tulman et al., 2012), which has both become a global health and economic concern. Prevention and control of MG are the most important modes of poultry farm management. Broad-spectrum antibiotic treatment is neither economically feasible nor commercially viable; furthermore, the infected birds do not fully respond to antibiotics (Emam et al., 2020) and may eventually lead to drug-resistant strains (Gautier-Bouchardon et al., 2002; Pakpinyo et al., 2007; Bottinelli et al., 2022). Vaccines are scarce, expensive, and mostly ineffective due to genetic differences in strains. Live attenuated or killed vaccines from local isolates have proven to be much more successful than commercial vaccines (Jacob et al., 2014; Bekele et al., 2018; Wu et al., 2024). Oil bacterin has been particularly effective in controlling MG (Limsatanun et al., 2016). The current study proves that vaccines A, B, and C produced from local isolates of MG protected CRD for immunized broilers. The immunized birds also showed cross protection when challenged by the antigen mixture A+B+C. The potential for production of an easily available, effective and cheap vaccine may be explored through a larger scale study in this respect.

4.0. Conclusion

An oil-based vaccine produced from local isolates showed promise against MG infections, potentially offering efficient immunity. While these isolates are promising vaccine candidates, large-scale field trials are essential to thoroughly evaluate the oil-based CRD vaccine's effectiveness and confirm its suitability for widespread use.

Acknowledgment

Authors would like to acknowledge the poultry farm owners for their cooperation throughout the current study in collection and transportation of samples. Authors declare that the housing of experimental animals, lab work and all related protocols were conducted at LCWU and GCU, Lahore, however no funding or financial support was provided by either institutions.

Conflicit of Interest

Authors declare no conflict of interest in this article.

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Received: December 30th, 2024. **Accepted: March 11th, 2025**