

ANTIBODIES RESPONSE OF BROILERS TO LOCALLY PREPARED OIL BASED *MYCOPLASMA GALLISEPTICUM* VACCINE

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ABSTRACT

In the present 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 10^7 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. The oil base vaccine did not interfere with the maternal antibody titer of the birds.

Key words: *Mycoplasma gallisepticum*, CRD, Immunogen, Montanide ISA70, MG Bacterin

INTRODUCTION

Mycoplasmas are present in every species including avian but various types of Mycoplasmas are present in poultry, among which four pathogenic Mycoplasmas are *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Mycoplasma meleagridis* (MM) and *Mycoplasmas iowae* (MI: Bradbury *et al.*, 2001). MG is the most important pathogen of poultry causing chronic respiratory disease (CRD) (Timms 1967; Soeripto *et al.*, 1989; Ley 2003; Nascimento *et al.* 2005), which is characterized by rales, nasal discharge, coughing, sneezing, conjunctivitis and air sacculitis with low mortality in uncomplicated cases in chickens (Ley *et al.* 1997). Clinical signs ranges from coughing to respiratory disturbances in chicken and decrease in production is prominent (Bradbury, 2005). Postmortem lesions of the disease are having mucopurulent exudates in trachea, bronchi, air sacs and nasal cavities (Kleven *et al.* 1984). Economic impact of the disease is highly significant even in the absence of clinical signs (Levisohn and Kleven, 2000). In the condition where keeping MG flock is impossible, live attenuated vaccine containing ts11 and MG 6/85 are used in layers and breeders with varying results (Whithear *et al.*, 1990; Whithear, 1996; Whithear *et al.*, 1996; Kleven, 2008). Immuno prophylaxis failure is due to difference in antigen

variation. The imported vaccines are costly and are not readily available. There is extensive pertinent information on mycoplasma in commercial poultry over the entire world (Ley, 2003) but related information for Pakistan is scarce. In Pakistan currently there is no organized surveillance and control systems exist for avian mycoplasma. Avian mycoplasma was first time isolated and characterized on cultural basis in Pakistan during 1986. In Pakistan MG vaccination is not common practice but vaccines are used only in the northern areas. Out breaks are common even in the vaccinated flocks that necessitate the need for developing MG vaccine from local isolated organisms. Present study is therefore designed to prepare inactivated oil base vaccine and to evaluate its potency in broilers.

MATERIALS AND METHODS

1. Production of *Mycoplasma gallisepticum* inactivated vaccine: PCR confirmed positive local *Mycoplasma gallisepticum* (MG) isolate seed, in active form, was inoculated @ 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. After 48 hrs of incubation the cultured media tubes were taken out of incubator and observed for presence of rich growth of MG. Ten milliliter of growth suspension was transferred to

graduated Hopkin's centrifuge tube (Kimax^R, USA). The tube was centrifuged at 3000xg 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 in the Hopkin's tube using phosphate buffer saline (pH 7) as diluent. For inactivation of growth of MG bacteria, 0.125 per cent of 37 per cent formaldehyde (Scharlau, Spain) 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 Frey's media and broth, incubated 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 confirmation of growth inactivation process, the montanide oil adjuvant (SEPPIC, Singapore) 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).

Evaluation of MG vaccine: Sixty (one-day-old) broilers were procured from Hi-Tech Hatchery, Multan Road, Lahore, and reared under standard management conditions in the experimental facility of Department of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore. The feed (Hi-Tech Feed, Lahore) and fresh water was provided *ad libitum* during whole period of experiment. Routine vaccination program for broiler chicken was implemented. All the chickens were tested for serum antibodies against MG up to 14th days of age through indirect ELISA kit (Pro-Flock, Synbiotics, USA) prior to inoculation of both the oil based vaccines (indigenous-IN-VAC and imported-IM-VAC). 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 was inoculated with 0.3 ml of sterile Frey's broth and kept as negative control. Each bird of group B was injected with 0.3 ml of IN-VAC subcutaneously (s/c) and each bird of group C was injected with 0.3 ml of IM-VAC (VaxFact-USA) s/c at mid neck region. Chickens in all groups were monitored daily up to 60 days of experiment and observations were recorded for their general conditions, clinical signs of disease or any mortality (Yoder and Hopkin, 1985). Blood samples (1ml) was collected from jugular vein of each bird of each group on 15, 30, 45 and 60 days post vaccination. The serum from each of the blood samples was separated, transferred to properly label serum vials and stored at -20°C till further processing. Anti MG ELISA antibody titer of each serum samples was determined through ELISA kit (ProFLOK Synbiotics, USA; Avakian *et al.*, 1988). The data thus obtained was analyzed using Analysis of Variance followed by DMRT

to compare the antibodies response of the birds to local and imported vaccines (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Mycoplasma gallisepticum infection in commercial poultry is controlled by proper surveillance, control and eradication program. The local isolate (*Mycoplasma gallisepticum*) grew well in Frey's broth within 24 hours at 37°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 Hopkin's 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. The washed bacterial suspension re-suspended in phosphate buffered saline was inactivated using formaldehyde. The suspension was therefore inactivated through mixing formaldehyde (37 per cent: Merck) @ 0.05 per cent (Koski *et al.*, 1976; Rimler *et al.* 1978; Hildebrand *et al.* 1983; Glisson *et al.* 1984; Glisson and Kleven, 1985; Sasipreeyajan *et al.* 1985; Karaca and Lam. 1987; Yagihashi *et al.* 1987). Use of antimicrobials and mass scale vaccination program (Yoder, 1991). Vaccination program is only effective with strict biosecurity measures Live and killed vaccines are commonly used all over the world (Whithearils, 1996). Formaldehyde molecules bind with amine group of amino acids in protein molecule of the organisms and thus inactivate its viability. Higher concentration of the inactivant may reduce antigenicity of the organisms. The killed antigen with out adjuvant presumably absorbs from injection site with in few hours and do not undergo for induction of antibodies. The bacterial suspension was therefore admixed with Montanide (ISA70) that enhanced its absorption. Oil based bacterin presumably causes irritation at inoculation site and induces granuloma formation / development of lymphoid tissue. The macrophages or antigen presenting cells (APC) in the granuloma ingest the microbial antigen from oily suspension and present the microbial protein antigen on their surface in association with self MHC II (immune associated antigen: Ia). The T helper cells of the vaccinated birds recognize their specific antigens on surface of the APC and undergo the process of blast formation, proliferation and differentiation into effector and memory T lymphocytes (Tizard, 2004; Abbas *et al.*, 2007; Suling *et al.*, 2012; Kreslavsky *et al.*, 2012). The effector T lymphocytes secrete cytokines such as interleukin (IL) 2, IL-3, IL-4, IL-5, interferon gamma (Kita *et al.*, 1992; Tizard, 2004; Abbas *et al.*, 2007). The production of these cytokines is antigen specific but their action is antigen nonspecific (Boehm, 1997; Tizard 2004; Abbas *et al.*, 2007; Kreslavsky *et al.*, 2012; Suling *et al.*, 2012). These cytokines activates antigen stimulated B-lymphocytes, macrophages, cytotoxic T cells, natural killer (NK) cells (Sen and Lengyel, 1992; Boehm *et al.*,

1997; Tizard, 2004; Abbas *et al.* 2007). B-lymphocytes of the vaccinated birds recognize their specific antigen from inoculation site and undergo the process of blast formation, proliferation and differentiation into plasma and memory cells under the influence of the cytokine. The plasma cells secrete specific immune-globulins including IgM and IgG and release into circulation. The oil based MG vaccine induces detectable level of anti-MG-ELISA antibodies. The broilers when vaccinated with IN-VAC vaccine on 14 days of age 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, while the broilers vaccinated with IM-VAC on 14 days of age 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. However, non-vaccinated birds (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. Anti-MG ELISA titer in day old chicks indicates that the breeder flock was either vaccinated against MG or carrier of MG infection. Moreover, killed vaccine did not interfere the maternally derived anti-MG ELISA antibody titers. Use of MG bacterin in chickens is a common practice to protect them against respiratory signs, airsacculitis, egg production losses and reducing egg transmission (Rimler *et al.*, 1978; Hildebrand *et al.*, 1983; Glisson *et al.*, 1984; Sasipreeyajan *et al.*, 1985; Karaca and Lam, 1987; Sasipreeyajan *et al.*, 1987; Yagihashi *et al.*, 1987). The MG vaccine induces specific immune responses in the vaccinated birds in the form of production of specific antibodies and production of nonspecific factors / cytokines particularly interferon gamma that activate antigen stimulated B cells, macrophages, cytotoxic T-cells, Natural Killer (NK) cells (Mohammed *et al.*, 1987; Boyaka and McGhee, 2001; Hilton *et al.*, 2002; Tizard, 2004; Abbas *et al.*, 2007). Immunoglobulin G (IgG) binds

with bacteria and enhance its phagocytosis through macrophages (Muir *et al.*, 2000). The phagocytosed mycoplasma may not be killed or may show resistant to the microbicidal activity of respiratory burst metabolites in phagosome or lysozymes in lysosome of the chicken macrophages (Green *et al.*, 1991; Klasing, 1998; Lam and Damassa, 2000; Qureshi *et al.*, 2000; Qureshi, 2003). However, macrophages activated through interferon gamma may kill the endocytosed bacteria through microbicidal activity of its respiratory burst metabolites or lysozymes. One of the central cytokines in immunity is IFN-gamma (IFN-gamma). Cellular immunity is increased with help of IFN-gamma by activation of Th1 cells, CD8+ and NK cells. It plays an important role in enhancing the development of cellular immunity in birds including in the development of Th1 cells, CD8+ T cell responses, and natural killer cell cytotoxicity (Boehm *et al.*, 1997). MG vaccine activates cellular immune responses in tracheal mucosa including natural killer and cytotoxic T cell responses that are important for the immunity (Gaunson *et al.*, 2006). In the immune-compromised birds, interferon gamma is not produced effectively in response to the vaccine so macrophages are not properly activated that ultimately fail to kill phagocytosis mycoplasma (Muneta *et al.*, 2008). CD8+ cells activated in response to interferon gamma may inactivate the intracellular mycoplasma in macrophages or other cells (Gobel, 1996). Mycoplasma may be killed through activated NK cells through a process known as antibody dependent cell mediated cytotoxicity ADCC. IgG bind with NK cells through its Fc part and binds with mycoplasma through Fab parts. NK cells brought in contact with mycoplasma in free form may be killed through NK cells released perforins (Boyaka and McGhee, 2001; Hilton *et al.*, 2002). The major advantage of oil-emulsion bacterin is that protection against economic losses can be obtained without the introduction of a live-vaccine strain.

Table 1 Comparative anti-MG- ELISA antibody response of broilers to local and imported MG bacterin

Groups	Day 0	Day 15	Day 30	Day 45	Day 60
IN-vacc	29.38± 2.82 ^a	153.26±8.00 ^a	332.67± 9.05 ^a	475.78± 27.69 ^a	500.03± 25.38 ^a
IM-Vacc	28.57±2.25 ^a	106.77± 7.11 ^b	286.13± 10.04 ^b	353.45± 10.13 ^b	489.89± 18.76 ^a
Control	28.78±2.53 ^a	21.50± 2.23 ^c	14.55±1.17 ^c	7.18± 0.64 ^c	4.67± 0.37 ^c

Thirty day-old broilers (n=30) were reared for fourteen days and divided into three groups (A, B & C), each having ten birds. These birds were vaccinated on 14th day of age. Each bird of group A was injected (0.3ml: S/C, Mid dorsal side of neck) with IN-VAC. Each bird of group B was injected (0.3ml: S/C, Mid dorsal side of neck) with IM-VAC. Each birds of the group C were kept as unvaccinated control. Blood sample from each bird of each group was collected on 0, 15, 30, 45 and 60

days of age. Serum from each sample were separated and processed for monitoring of anti-MG-ELISA antibody titer. Results are shown with Mean± std. error. In columns means with different superscripts are significant at P< 0.05.

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