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ANTIBODY RESPONSE OF BROILERS TO OIL BASED COMBINED AVIAN INFLUENZA (H_9 N_2) AND *MYCOPLASMA GALLISEPTICUM* VACCINE

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ABSTRACT

Mycoplasmosis caused by *Mycoplasma gallisepticum* (MG) and *Avian influenza* virus (AIV: H_9N_2) infection are serious health hazards for poultry. The AIV is low pathogenic but is cause of high morbidity and mortality in immunocompromised birds. On 7th day of age, each bird of group A, B and C (n=10) was vaccinated (0.3ml: I/M) with oil based O-MG, O-AIV and O-MG+AIV vaccine, respectively. Serum samples collected on 28, 35 and 42 days of age were processed for determination of anti-MG-ELISA antibodies and anti-AIV-HI antibodies. Antibody response of the birds to either immunogen of monovalent vaccines (O-MG and O-IV) was not significantly different (P>0.05) from antibody titers against both the immunogens in combined vaccine (O-MG+AIV). It is concluded that oil based combined O-MG+AIV vaccine induces effective antibody response in the vaccinated birds.

Key words: *Mycoplasma gallisepticum*, *Avian influenza* (H_9N_2), immunogen, vaccine.

INTRODUCTION

Avian mycoplasmosis is an important infectious disease of commercial birds which hampers their production. Among 20 different species, *Mycoplasma gallisepticum* (MG) is one of the avian bacterial pathogen responsible for chronic respiratory disease (CRD). In Pakistan, the disease is quite common and it causes enormous economic losses in the form of mortality, carcass condemnation, drop in egg production, poor hatchability and feed conversion ratio (Mukhtar *et al*., 2012). The causative agent of the disease multiplies in respiratory tract (trachea, lungs and air sacs) and reproductive organs such as ovules and oviduct (Ahmed *et al*., 2009; Indikova *et al*., 2013). The bacteria also multiply in macrophages of internal organs particularly in immunocompromised birds (Gondal *et al*., 2013). The disease is further complicated with other poultry pathogens such as Avian influenza, New Castle disease, infectious bronchitis, fowl cholera, coryza, *E. coli* etc. (Liu *et al*., 2001). The treatment of the disease in chronic form is not effective and economical. The disease is only controlled through effective immunoprophylaxis with both killed and live MG vaccines (Olanrewaju *et al*., 2011).

Avian influenza virus (AIV) is endemic in Pakistan with a quite different situation in comparison to other viral infections (Ahmed *et al*., 2009). The control efforts directed against low pathogenic AIV (H_9N_2) in poultry is not much successful to implement eradication programme, together with the use of vaccines and depopulation of sick birds. In some countries including USA vaccines are being used as a part of control programme of AIV (H_9N_2), but use of these vaccines is limited (Haddipur *et al*., 2011). On the other hand, vaccines used for control of the disease have been gaining much support and AIV vaccines are used all over the world (Mosleh *et al*., 2009).

Antibody response is specific to the immunogen. Currently development and application of multiple vaccine (multiple immunogens) in inactivated form in a single vaccine dose is common practice in commercial poultry and livestock farming. In poultry, oil based NDV+IB, Coryza+Fowl cholera+*E. coli*, NDV+AIV, etc., are prepared and commonly used (Pavic *et al*., 2010; Vemula *et al*., 2013). MG and AIV infections are main cause of economic loss to poultry industry so are issue of main concern for poultry farmers. Present study was therefore designed to prepare and evaluate antibody response of broilers to oil based combined MG and AIV vaccine.

MATERIALS AND METHODS

Source of organisms: *Mycoplasma gallisepticum* and *Avian influenza* (H_9N_2) virus (AIV) seed culture were obtained from University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences (UVAS), Lahore. The MG seed in its active form was confirmed through polymerase chain reaction (PCR) (Liu *et al*., 2001; Ramadass *et al*., 2006).

Cultivation of organisms: MG was grown in laboratory (Hennigan *et al*., 2012). The active culture (10ml) of the organism (48 hours at 37ºC incubated culture) was inoculated in a flask of 2.5 liter capacity containing 100ml sterile Frey's broth with 10 percent horse serum.

The inoculated broth was incubated at 37ºC at 5 percent CO_2 tension and observed daily for any growth. After every 48 hours, 300ml of the broth was added in the culture flask till to attain 1000ml of its final volume. The grown MG culture was centrifuged at 3000xg for 20 min. The final pellet resuspended in 10ml sterile phosphate buffer saline (PBS) was centrifuged in Hopkin's tube (Kimax^R, USA) at 3000xg for 20 min (Biro *et al*., 2005). Final sediment in the Hopkin's tube was resuspended in PBS to make one percent bacterial suspension.

AIV inoculated in 10 days-old chicken embryos through allantoic sac route were incubated at 37ºC for 48 hours (Rota *et al*., 1990). Allanto-amniotic fluid (AAF) was harvested from the embryos and processed for embryo infected dose 50 (EID_{50}) (Reed and Muench., 1938).

Inactivation of cultures: Bacterial and viral cultures suspensions were admixed with 0.125 percent of 37 percent formaldehyde (Scharlau, Spain) separately and each of the mixtures was incubated at 37ºC for 24 hours. Inactivation of MG mixture was confirmed by culturing the suspension on Frey's medium and broth, incubating at 37ºC at 10 percent CO_2 tension and observing for seven days for appearance of any specific growth of Mycoplasma colonies or color change, respectively (Koski *et al*., 1976).

The inactivation of AIV suspension was evaluated by growing the culture in 10 days-old chicken embryos. The inoculated embryos were incubated at 37ºC for 72 hours. No hem-agglutinating activity (HA) in AAF indicated inactivation of the virus. Sterility of the culture was determined on tryptose broth and blood agar. The inoculated broth and plates were incubated at 37ºC for 48 hours. No bacterial growth in the broth and the plates indicated sterility of the AAF.

Preparation of vaccines: For preparing 0.3ml dose of oil based MG vaccine (O-MG), 0.06ml of the chemically inactivated one percent MG suspension was mixed in 0.06ml PBS and 0.18ml of Montanide ISA-70 (SEPPIC, Singapore) oil.

For preparing oil based AIV vaccine (O-AIV), 0.06ml of the chemically inactivated AIV (H_9N_2) suspension in AAF was mixed with 0.06ml PBS and 0.18ml montanide ISA-70 oil.

For preparing 0.3ml dose oil based combined MG and AIV vaccine (O-MG+AIV), 0.06ml of one percent MG suspension in PBS was mixed with 0.06ml of AIV suspension in AAF and subsequently mixed with 0.18ml montanide ISA-70 oil.

Source of broilers: Forty broiler chicks (day-old) were procured from Hi-Tech Hatchery, Sunder, Multan road, Lahore and reared in experimental poultry sheds, Department of Microbiology, UVAS, Lahore. Fresh water and feed were given ad lib. At 7th day of age, the chicks were divided into A, B, C and D groups (n=10).

Vaccination schedule: O-MG, O-AIV and O-MG+AIV vaccine were injected (0.3ml: subcut: mid-dorsal side of neck) to each bird of group A, B and C, respectively and birds of group D served as non-vaccinated control.

Collection of blood: Blood sample (1ml) was collected from wing vein of each of the birds of each group on 28, 35 and 42 days of age. Each of the blood samples was kept at room temperature (30ºC±2) for 24 hours. The serum thus separated from each sample was transferred to properly labelled serum vials and stored at -40ºC for further processing.

Monitoring of antibodies: Anti-MG-ELISA and anti-AIV-HI antibodies were determined using indirect enzyme-linked immunosorbent assay (ELISA) (Pro-Flock Synbiotic, USA) (Avakian *et al*., 1988) and haemagglutination inhibition test (HI) (Rowe *et al*., 1999), respectively.

Statistical analysis: The data regarding HI test was processed for calculating geometric mean titer (GMT) on 21, 28 and 35 days post-vaccination (DPV). Each value of GMT was processed for calculation of cumulative geometric mean titer (CGMT). Whereas, each value of the experiment regarding anti-MG-ELISA titers was divided by 100 and processed for calculating GMT on 21, 28 and 35 DPV. Mean values of antibody titers of each group were compared using one way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (Steel *et al*., 1997).

RESULTS

Broilers at 7th day of age were vaccinated with either of O-MG, O-AIV and O-MG+AIV vaccine and antibody response of the birds to the respective vaccines was determined on 28, 35 and 42 days of age (Table-1 and 2). O-MG vaccine induced 102.18±63.41, 323.76±116.84 & 397.96±375.77 units of mean anti-MG-ELISA-antibody titers in vaccinated broilers on 21, 28 and 35 days post-vaccination (DPV), respectively. Cumulative mean anti-MG-ELISA antibody titer of the birds was 274.63±88.85. O-MG+AIV vaccine induced 130.03±45.24, 329.7875±91.94, 463.9875±171.47 units of mean anti-MG-ELISA antibody titers in the vaccinated broilers on 21, 28 and 35 DPV, respectively. Cumulative mean anti-MG-ELISA antibody titer of the birds was 307.93±92.02. The non-vaccinated birds showed 1±0.5, 2.9±3.17, 8.33±6.54 units of mean anti-MG-ELISA antibody titers on 28, 35 and 42 days of age, respectively. Cumulative mean anti-MG-ELISA antibody titer of the broilers was 4.07±2.20.

O-AIV vaccine induced 39.4, 128 and 128 units of geometric mean anti-AIV-HI antibody titers in the vaccinated broilers on 21, 28 and 35 DPV, respectively. CGMT of the birds was $\log_2^{98.5\pm51.15}$. O-MG+AIV vaccine induced 52, 137.2 and 104 units of geometric mean anti-AIV-HI antibody titers in the vaccinated broilers on 21, 28 and 35 DPV, respectively. CGMT of the birds was $\log_2^{97.7\pm42.9}$. The non-vaccinated birds showed 6.1, 4.6 and 3 units of geometric mean anti-AIV-HI antibody titers on 28, 35 and 42 days of age, respectively. CGMT of the birds was $\log_2^{4.53\pm1.22}$.

Antibody response of birds to either of the immunogens in the combined vaccine was not significantly different from that of birds vaccinated with either of the monovalent vaccines ($p<0.05$).

Table-1. Comparative mean anti-MG-ELISA antibody response of broilers to inactivated oil based MG vaccines.

Type of vaccine	Anti-MG-ELISA antibody titer (DPV)			CGMT ± S.D
	21	28	35	
O-MG	102.18±63.41	323.76±116.84	397.96±375.77	274.63^{a}±88.85
O-MG+AIV	130.03±45.24	329.79±91.94	463.98±171.47	307.93^{a} ±92.02
Control	1±0.5	2.9±3.17	8.33±6.54	4.07^{b}±2.20

Note: Cumulative mean values of anti-MG-ELISA antibody titer in last column with different superscripts differ significantly ($P<0.05$). MG: mycoplasma gallisepticum, AIV: Avian influenza virus, O: oil based, S.D: standard deviation, DPV: days post-vaccination, CGMT: cumulative geometric mean titer.

Table-2. Comparative mean anti-AIV-HI antibody response of broilers to oil based AIV vaccines

Type of vaccine	Anti-AIV-HI antibody titer (DPV)			CGMT±S.D
	21	28	35	
O-AIV	39.4	128	128	98.5^{a}±51.15
O-MG+AIV	52	137.2	104	97.7^{a}±42.9
Control	6.1	4.6	3	4.53^{b}±1.22

Note: Cumulative mean values of anti-AIV-HI antibody titer in last column with different superscripts differ significantly ($P<0.05$). AIV: Avian influenza virus, MG: mycoplasma gallisepticum, O: oil based, S.D: standard deviation, DPV: days post-vaccination, CGMT: cumulative geometric mean titer.

DISCUSSION

Chronic respiratory disease is caused by *Mycoplasma gallisepticum* and is characterized by chronic respiratory distress, wasting and reduced egg production (Bradbury and Jordan 1971). Its variable lipoprotein (haemagglutinin) eludes and causes immunosuppression (Szczepanek *et al*., 2010). The disease is economically important even in the absence of clinical signs (Levisohn and Kleven, 2000). It is controlled through strict biosecurity measures and mass-scale vaccination. Inactivated bacterial vaccine without adjuvant absorbs from the inoculation site and is excreted out from the body without inducing any specific antibody response (Sarwar *et al*., 2013). The inactivated microbial antigens in the vaccines are made water-insoluble by adding chemicals such as mineral oil, alum and gel. Alum precipitates the immunogens and minimizes their absorption to induce immunity (Hyslop and Morrow, 1969; Park *et al*., 2014). Aluminium hydroxide gel is the least toxic and adsorbs the vaccinal immunogen to make it insoluble. In this way the gel increases retention time of the immunogen at inoculation site. Being least toxic, the gel is commonly used in veterinary and medical vaccine formulations (Franchi and Nunez, 2008). Antibody response to gel adsorbed vaccines is rapid, reaching peak level and declines thereafter. The duration of gel based vaccines is not more than 4-6 months (Vecchi *et al*., 2012; Kerkvliet *et al*., 2013). Mineral oil encapsulates the vaccinal macromolecules such as proteins, lipids, carbohydrates, nucleic acids etc. The oil retains the antigen at injection site for longer period than gel, alum or salts. Oil is commonly used in dairy and avian vaccines (Box and Furminger 1975; Fox *et al*., 2011). Oil adjuvants being mild irritant, recruit antigen presenting cells (APCs) which take up, process and present the antigen on their surface in association with major histocompatibility complex-II (MHC-II) antigens. T-helper cells recognize only those antigens which are presented on the surface of APCs in association with MHC-II antigens and undergo the process of blast formation, proliferation and differentiation into effector and memory T-helper cells. The effector cells secrete cytokines which modulate immunogenesis pathway of antigen-stimulated B-cells. The B-cells in the presence of cytokines induce immunity for longer time period. In the

present study oil based monovalent O-MG and O-AIV vaccines induced 274.63±88.85 and 98.5[a]±51.15 units of cumulative anti-MG-ELISA and anti-AIV-HI antibody titers in vaccinated birds, respectively.

Monovalent bacterial or viral vaccines have multiple antigens/epitopes. Each of the antigens is driven on its specific pathway without interfering immunogenesis of the others. Monovalent vaccine in literary term means the vaccine against one pathogen while bivalent or multivalent vaccine means the vaccine against two or more than two pathogens. Multivalent vaccines are commonly used in livestock (Celma *et al*., 2013), poultry (Pavic *et al*., 2010; Vemula *et al*., 2013) and small animals. Oil based bivalent O-MG+AIV vaccine induced 307.93±92.02 and 97.7±42.9 units of cumulative anti-MG-ELISA and anti-AIV-HI antibody titers in vaccinated birds, respectively.

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REFERENCES

Ahmed, A., T. Khan, B. Kanwal, Y. Raza, M. Akram, S. Rehmani and S. Kazmi (2009). Molecular identification of agents causing respiratory infections in chickens from southern region of Pakistan from October 2007 to February 2008. Int. J. Agric. Biol. 11: 325-328.

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.

Biro, J., J. Povazsan, L. Korosi, R. Glavits, L. Hufnagel and L. Stipkovits (2005). Safety and efficacy of Mycoplasma gallisepticum TS-11 vaccine for the protection of layer pullets against challenge with virulent Mycoplasma gallisepticum R-strain. Avian Pathol. 34 (4): 341-347.

Box, P.G. and I.G. Furminger (1975). Newcastle disease antibody levels in chickens after vaccination with oil emulsion adjuvant killed vaccine. Vet. Rec. 96: 108-111.

Bradbury, J.M., and F.T.W. Jordan, (1971). The influence of pH of the culture medium on the sensitivity of Mycoplasma gallisepticum antigens for use in certain serological tests. J. Hyg. 69(4): 593-606.

Celma, C. C., M. Boyce, P. A. Vanrijn, M. Eschbaumer, K. Wernike, B. Hoffmann, M. Beer, A. Haegeman, K. De-Clercq, and P. Roy (2013). Rapid generation of replication-deficient monovalent and multivalent vaccines for bluetongue virus: protection against virulent virus challenge in cattle and sheep. J. Virol. 87: 9856-9864.

Fox, C.B., S.L. Baldwin, M.S. Duthie, S.G. Reed, and T.S. Vedvick (2011). Immunomodulatory and physical effects of oil composition in vaccine adjuvant emulsions. Vacc. 29: 9563-9572.

Franchi, L. and G. Nunez (2008). The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1β secretion but dispensable for adjuvant activity. Eur. J. Immunol. 38: 2085-2088.

Gondal, M. A., M. Rabbani, K. Muhammad, T. Yaqub, M. A. Babar, A. A. Sheikh, A. Ahmad, M. Shabbir, and M. I. Khan (2013). Antibodies response of broilers to locally prepared oil based Mycoplasma gallisepticum vaccine. The J. Anim. Plant Sci. 23(4): 1094-1098.

Haddipur, M., G. Habibi, and A. Vosoughi (2011). Prevalence of Antibodies to H_9N_2 Avian Influenza Virus in Backyard Chickens around Maharlou Lake in Iran. Pakistan Vet. J. 31(3): 192-194.

Hennigan, S.L., J.D. Driskell, N.F. Noel, R.A. Dluhy, Y. Zhao. R.A. Tripp, and D.C. Krausea (2012). Detection and differentiation of avian mycoplasmas by surface-enhanced raman spectroscopy based on a silver nanorod array. Appl. Envir. Microbiol.78: 1930–1935

Hyslop, N.S. and A.W. Morrow (1969). The influence of aluminium hydroxide content, dose volume and the inclusion of saponin on the efficacy of inactivated Foot-and-Mouth disease vaccines. Res. Vet. Sci. 10: 109-120.

Indikova, I., P. Much, L. Stipkovits, K. Siebert-Gulle, M.P. Szostak, R. Rosengarten, and C. Cittia (2013). Role of the GapA and CrmA Cytadhesins of *Mycoplasma gallisepticum* in Promoting Virulence and Host Colonization. Infect. Immunol. 81: 1618-1624.

Kerkvliet, E., N. Sinnige, C. Franso, R. Van-Ree, and R. Vandenhout (2013). Antibody-based assays for potency determination of mite allergoids and for stability studies of alum-adsorbed mite vaccines. Arb. Paul Ehrlich Inst. Bundesinstitut Impfstoffe Biomed. Arzneim Langen Hess. 97: 106-111.

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., and S. H. Kleven (2000). Avian mycoplasmosis (*Mycoplasma gallisepticum*). Revue scientifique et technique (International Office of Epizootics). 19(2): 425-442.

Liu, T., M. Garcia, S. Levisohn, D. Yogev, and S. H. Kleven (2001). Molecular variability of the adhesin-encoding gene pvpA among *Mycoplasma gallisepticum* Strains and Its Application in Diagnosis. J. Clinic. Microbiol. 39: 1882-1888.

Mosleh, N., H. Dadras, and A. Mohammadi (2009). Molecular quantitation of H_9N_2 Avian influenza virus in various organs of broiler chickens using TaqMan real time PCR. J. Mol. Genet. Med. 3(1): 152-157

Mukhtar, M., M. Awais, Anwar M, Z. Hussain, N Bhatti, and S. Ali. 2012. Seroprevalence of *Mycoplasma gallisepticum* Among Commercial Layers in Faisalabad, Pakistan. J. Basic Appl. Sci. 8: 183-186.

Olanrewaju, H.A., S.D. Collier, and S.L Branton (2011). Effects of single and combined *Mycoplasma gallisepticum* vaccinations on blood electrolytes and acid-base balance in commercial egg-laying hens. Poult. Sci. 90: 358-363.

Park, M. E, S.Y. Lee, R. H. Kim, M. K. Ko, K. N. Lee, S. M. Kim, B.K. Kim, J.S Lee, B. Kim, and J.H, Park (2014). Enhanced immune responses of foot-and-mouth disease vaccine using new oil/gel adjuvant mixtures in pigs and goats. Vacc. 32: 5221-5227.

Pavic, A., P.J. Groves, and J.M. Cox (2010). Utilization of a novel autologous killed tri-vaccine (serogroups B [Typhimurium], C [Mbandaka] and E [Orion]) for *Salmonella* control in commercial poultry breeders. Avian Pathol. 39: 31-39.

Ramadass, P., R. Ananthi, T.M.A. Senthilkumar, G. Venkatesh and V. Ramaswamy (2006). Isolation and characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* from poultry. Ind. J. Ani. Sci. 76 (10): 796–798.

Reed, L. J., and H. Muench (1938). A simple method estimating fifty percent end points. Am. J. Immunol. 49: 263-271.

Rota, P. A, B. K. De., M. W. Shaw, R. A. Black, W. C Gamble, and A. P. Kendal (1990). Comparison of inactivated, live and recombinant DNA vaccines against influenza virus in a mouse model. Virus Resp.16: 83-93.

Rowe, R. A., Abernathy, J. Hu-Primmer, W.W. Thompson, X. Lu, W. Lim, K. Fukuda, N.J. Cox, and J.M. Katz (1999). Detection of antibody to avian influenza A(H_5N_1) virus in human serum by using a combination of serologic assays. J. Clin. Microbiol. 37: 937-943.

Sarwar, N., K. Muhammad, M. Rabbani, M. Younus, M. Sarwar, M. A. Ali, K. Hanif, and M. Kamran (2013). Optimization of Physico-chemical factors augmenting *in-vitro* biomass production of *Pasturela multocida*. The J. Anim. Plant Sci. 23(4): 1085-1088.

Steel, R. G. D., J. H. Torrie, and D. A. Dickey (1997). Principles and Procedures of Statistics. A Biometrical Approach. 3rd Ed. McGraw-Hill, New York.

Szczepanek, S. M., S. F. Jr, V. L. Schumacher, X. Liao, M. Padula, S. P. Djordjevic, and S. J. Geary (2010). Identification of lipoprotein MslA as a neoteric virulence factor of *Mycoplasma gallisepticum*. Infect. Immun. 78: 3475–3483.

Vecchi, S., S. Bufali, D.A. Skibinski, D.T O'Hagan, and M. Singh (2012). Aluminum adjuvant dose guidelines in vaccine formulation for preclinical evaluations. J. Pharm. Sci. 101: 17-20.

Vemula, S., Y. Ahi, A. Swaim, J. Katz, R. Donis, S. Sambhara, and R. Mittal (2013). Broadly protective Adenovirus-based multivalent vaccines against highly pathogenic Avian influenza viruses for pandemic preparedness. Plos One. 8: e62496.