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Evaluation of the Immunogenicity of Oil-Based *Mycoplasma gallisepticum* Killed Vaccine in Broiler Chickens Experimentally Prepared from Local Isolate of Bangladesh

[1]Monira Noor, [1]Md. Shamsul Islam Basit, [2]Sultan Ahmed, [2]Dewan Muhammad Mahbobur Rajib, [1]Shobhan Das and [1]Md. Masudur Rahman
[1]*Department of Pathology, Faculty of Veterinary, Animal and Biomedical Sciences,*
[2]*Department of Microbiology and Immunology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet 3100, Bangladesh*

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Corresponding Author:
Md. Masudur Rahman
Department of Pathology, Faculty of Veterinary Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet 3100, Bangladesh

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Abstract: The study was aimed to evaluate the immunogenicity of experimentally prepared *Mycoplasma gallisepticum* killed vaccine in broiler chickens under field conditions in Bangladesh. Isolation and identification of *Mycoplasma gallisepticum* was performed by cultural, biochemical and PCR assays. Oil-based formalin killed mycoplasma vaccine was prepared from one field isolate. To evaluate the immunogenicity of the vaccine, day-old broiler chicks (n = 60) were randomly divided into three equal groups namely Group-A (non-vaccinated control), Group-B (trial vaccine) and Group-C (imported vaccine). Immunogenicity of locally prepared trial vaccine and imported vaccine was evaluated by ELISA. In chickens of control group (Group-A), the mean anti-MG ELISA antibody titer gradually decreased whereas that of trial vaccine (Group-B) and imported vaccine (Group-C) increased gradually after 15 days post-vaccination. The oil-adjuvant MG trial vaccine conferred anti-MG-ELISA antibodies in broilers after 15 days of vaccination that reached to the maximum level (2213.75±3.126) at 45 days post priming which is comparable with the imported vaccine (2121.75±3.431). The maternal antibody in chicks did not interfere with the trial and imported vaccines as both were killed vaccines. We conclude that the prepared vaccine candidate might be promising to control mycoplasmosis in Bangladesh. However, the protective efficacy of the vaccine needs to be evaluated before recommending the vaccine finally.

INTRODUCTION

Mycoplasmas can infect poultry but few are pathogenic namely *Mycoplasma Gallisepticum* (MG), *Mycoplasma Synoviae* (MS), *Mycoplasma Mmeleagridis* (MM) and *Mycoplasmas iowae*[1]. MG infection in chickens is commonly known as Chronic Respiratory Disease (CRD)[2-3]. It is characterized by coughing,

sneezing, respiratory rales, nasal discharge, etc. and the mortality rate in chickens is low in uncomplicated cases[4]. MG infection may cause great economic losses in poultry industry if proper diagnostic and preventive measures cannot be taken in time.

In Bangladesh the prevalence of Mycoplasmosis may be increased up to 61.45% in the winter season imposing a serious threat to the poultry farmers[5]. Vaccination in layer and breeder farms with a live attenuated vaccine containing its 11 and MG 6/85 confer varying protection[6-9]. Vaccination failure in Mycoplasmosis commonly occurs in Bangladesh which may be due to antigenic variation between vaccine candidate and field isolates (unpublished observation). In addition, imported MG biologics are very expensive and less available in the country that has limited their use by the poor farmers. These situations have favored the existence of mycoplasmosis in poultry flocks and act as a major hindrance in the development of the poultry sector in Bangladesh[10]. Furthermore, control of mycoplasmosis by vaccination has become more difficult with the advent of multi-age commercial layer complexes in Bangladesh. The development of low cost mycoplasma vaccine using local isolates might be promising to control mycoplasmosis in Bangladesh. Therefore, the present research was conducted to assess the immunogenicity of *Mycoplasma gallisepticum* killed vaccine in broiler chickens experimentally produced from local isolates of Bangladesh.

MATERIALS AND METHODS

Ethical approval: The research was implemented at the Department of Pathology and the Department of Microbiology and Immunology, Sylhet Agricultural University, Sylhet 3100, Bangladesh. The handling of chickens in the experiment was performed following the guidelines of current Bangladesh legislation (Cruelty to Animals Act 1920, Act No. 1 of 1920 of the Government of the People's Republic of Bangladesh). The specific experiments were performed with the approval of the Ethics Committee of the Sylhet Agricultural University, Bangladesh (Approval No. NATP-2/PIU-BARC-24/Research/2016/403).

Isolation and identification of *Mycoplasma galisepticum* by cultural and biochemical tests: A total of 95 poultry farms located in Sylhet division of Bangladesh with mycoplasmosis outbreaks were investigated in the study. After post-mortem examinations of 95 chickens (one chicken from each farm), different tissue samples (lungs, air sacs and tracheas) showing grossly visible lesions of mycoplasmosis were collected aseptically. In the laboratory, homogenous tissue suspensions (n = 95; tissues collected from a chicken were considered for preparing a homogenous suspension) were prepared and subjected to pass through a sterile membrane filter having pore size of 0.45 μm. Finally, the filtrates were inoculated on to the Hayflick's agar 25 plates and broth media containing supplement that can prevent bacterial and fungal growth. Upon inoculation the Hayflick's agar plates and broth cultures were incubated under microaerophilic conditions (5% CO_2) at 37°C temperature. The plates were examined daily using a stereomicroscope (25×Labomed, USA) for any visible colony growth. Characteristic rounded colonies of *Mycoplasma* with central nipple were incised from the agar plates and sub-cultured on to the specific Hayflick's agar plate and in broth to get pure colonies of *Mycoplasma*. Pure colonies of *Mycoplasma* were further subjected to specific biochemical tests following standard procedures before going for PCR assay.

Confirmation of *Mycoplasma gallisepticum* by amplifying specific MGC_2 gene using PCR: To confirm *Mycoplasma gallisepticum*, one micro liter broth culture of the pure MG isolates was used for DNA extraction by using Favor Prep™ Blood Genomic DNA Extraction Mini Kit (Favorgen® Biotech Corporation, Taiwan) according to the manufacturer's instructions followed by the detection of *Mycoplasma gallisepticum* specific MGC2 gene by PCR assay using specific primer pairs[11]. Thermo Scientific Dream Taq Green PCR Master Mix (2X) was used for the PCR assay. The reaction mix comprised of 25 μL of mastermix, F Primer MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT C-3' (5 pmole μL^{-1})-0.5 μL, R Primer MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3' (2 pmole μL^{-1}) -0.5 μL and RNase free water 19 μL. In each PCR tube, a final volume of 45 μL reaction mixture was dispensed carefully. Then appropriate DNA sample (5 μL) to be tested was added to each tube. Extracted DNA from *Mycoplasma gallisepticum* of commercial vaccine and DNA free water were used as a positive and negative control, respectively in each run. The tubes were then placed in a thermal cycler (Applied Biosystem 2720, Thermo fisher Scientific, USA) for the following cycles: initial denaturation at 94°C/5 min for one cycle, 40 cycles: 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, 1 cycle (final extension): 72°C for 5 min and stored at 4°C (OIE, 2012; ISO/IEC 17025:2005 NVI-QMS SOP). Amplified PCR products were visualized using 2% agarose gel electrophoresis. Gene Ruler 100 bp DNA Ladder (Thermo Scientific) was used as DNA marker.

Preparation of MG killed vaccine: A positive local isolate of MG confirmed by PCR assay that grown well in broth media was named as MG-SAU 1 and considered as seed to prepare MG killed vaccine. The active MG seed was added into at 10% v/v into Frey's broth and incubated

at 37°C with 10% CO_2 tension and daily observation was made for any growth. The cultured media was taken out of incubator after 48 h of incubation and 10 mL of growth suspension was transferred to graduated Hopkin's centrifuge tube. To estimate packed cell volume per ml of media, the growth suspension was then centrifuged at 3000×g for 20 min. The mass of MG antigen was adjusted to 1% in the Hopkin's tube using PBS (pH 7) as a diluent. In order to inactivate MG, 37% formaldehyde was used at 0.125% (v/v) and the tubes were subjected to incubation at 37°C with 10% CO^2 tension for 12 h of interaction time. To ensure whether MG was completely inactivated by formaldehyde, the broth cultures after inactivation were separately cultured on both Frey's media and broth, incubated at 37°C with 10% CO_2 tension and observed for consecutive 7 days for the appearance of any specific *Mycoplasma* colony or color change, respectively. The montanide oil adjuvant was admixed with the completely inactivated MG at 4:1 ratio to properly emulsify the mycoplasmal biomass. The inactivated MG was further tested according to the previously described method to ensure its sterility and safety[12]. Briefly, heat sterilization of the experimental oil adjuvant vaccine was performed at 160°C for 1 h. Sterility tests were performed using sterility media (Triptic soy broth, Tryptose agar and Sabroud agar) with incubation at 37°C for 7 days. No growth of any organism within the 7 days incubation period indicated the sterility of the vaccine. For safety test, 1 mL (double of the field dose) of the trial vaccine was inoculated s/c at mid-neck region of 6 chickens of 3 weeks of age and the chickens were kept under observation for 14 days for any visible clinical signs.

Evaluation of immunogenicity of MG killed vaccine in broiler chickens: To evaluate the immunogenicity of MG killed vaccine, 1 day-old broiler chicks (n = 60) were randomly divided into three groups (namely A-C), each having 20 chickens and reared in standard management conditions. The feed and fresh drinking water were supplied *ad ilibitum* during the experimental period. Routine vaccination was also performed in experimental broiler chickens to control other diseases. All the chickens of Group A, B and C were tested for serum antibodies against MG at 0 day of age and 14 days of age (i.e., 0 day post-vaccination) through indirect ELISA (ProFlok® MG ELISA Kit, Zoetis, USA) before vaccination with the oil-based MG vaccines (trial and imported vaccines). At the age of 14 days, each of the chickens of group A was injected subcutaneously (s/c) with 0.5 mL of sterile Frey's broth and kept as a Negative Control (NC). Each of the chickens of groups B and C were injected with 0.5 mL of Trail Vaccine (TrV group) and Imported Vaccine (ImV group), respectively through subcutaneous (s/c) route at mid-neck region. Chickens of all groups were closely observed daily up to 60 days of experiment and observations were recorded for their feed intake, growth conditions and clinical signs of any disease or any mortality. Blood samples (1 mL each) were collected from the jugular vein of each chicken from each group on 30, 45 and 60 days of age, i.e., 15-, 30- and 45-days post-vaccination. Sera from each of the blood samples were separated and transferred to properly labeled vials, heat inactivated and stored at -20°C till further testing. Serum anti-MG ELISA antibody titer of each serum sample was measured through ELISA kit (ProFlok® MG ELISA Kit, USA) according to the manufacturer's instruction. Data were analyzed using MS Excel and SPSS to determine the significant difference in the antibody titer among the chickens of control, TrV and I mV groups.

RESULTS AND DISCUSSION

Isolation, identification and molecular detection of Mycoplasma gallisepticum: In order to isolate mycoplasma, tissue samples (lungs, air sacs and trachea) from a total of 95 mycoplasmosis suspected chickens from 95 poultry farms in Sylhet division were collected. Out of 95 samples, only 17 (17.89%) were positive for mycoplasma based on cultural examination and biochemical tests. Primary isolates with prominent *Mycoplasma* fried egg like microcolonies were found under a stereomicroscope (25×imagnification) after three to four days of incubation (Fig. 1a, b). Pure *Mycoplasma* microcolonies were obtained after five to six subsequent sub-culturing onto agar plates. In the glucose fermentation test, all the isolates fermented glucose as determined by a change of color from red to yellow (Fig. 1c). *Mycoplasma gallisepticum* was further confirmed by detecting specific MGC2 gene using PCR assay. In PCR assay among the 17 solates, 12(70.58%) were found strong positive and 5(29.41%) were weakly positive for MG that was evidenced by the migration of PCR amplified DNA products (bands) at 185bp position along with the positive control. Among the 17 positive isolates one strong positive isolate (Fig. 1d) was designated as MG-SAU 1 and used as a candidate isolate for vaccine preparation.

Evaluation of safety and sterility of formalin killed oil-based mycoplasma vaccine: The local MG-SAU1 isolate (*Mycoplasma gallisepticum*) that was selected for vaccine preparation grew well in Frey's broth within 24 h at 37°C with 10% CO_2 showing10^7 Colony Forming Units (CFU) per ml of the medium. The mycoplasma packed cell was formed after centrifugation and was inactivated using formaldehyde. The results of sterility test indicated that the prepared inactivated MG vaccine was free from any contaminant. Concerning the safety of the prepared

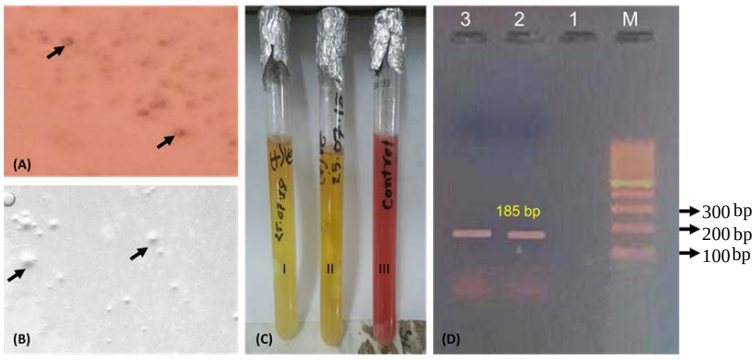

Fig. 1(a-d): Cultural and biochemical characters and PCR-based identification of *Mycoplasma Gallisepticum* (MG). (A) fried egg colony on Hayflick agar, (B) Mycoplasma colony under Steriomicroscope (25X), (C) Glucose fermentation test, Positive samples shows color changes from red to yellow. (D) Detection of MGC2 gene of MG. M-100 bp DNA ladder, lane-1 water control, lane-2 positive control, lane-3 mycoplasma field isolate (MG-SAU1)

Table 1: Comparative antibody response in broilers with Trail and imported MG vaccines

	ELISA antibody titer (Groups A-C)			
Age (days)	Control	Local vaccine	Imported vaccine	p-values
0 (day old chick)	245.35±2.924	244.95± 3.316	244.8±3.205	
14 (0 day of vaccination)	230.95±3.720	229.85±3.498	230.35±2.601	<0.001
30 (15 days post vaccination)	209.3±2.637	1103.1±6.632	1099.75±5.138	
45(30 days post vaccination)	201.9±2.90	1621.5±6.447	1545.35±4.416	
60 (45 days post vaccination)	193.8±2.94	2213.75±3.126	2121.75±3.431	

vaccine, it was found that chickens inoculated with even double doses of prepared vaccine didn't show any abnormalities or adverse reactions in vaccinated chickens.

Evaluation of serum anti-MG-ELISA antibodies in broiler chickens vaccinated with trial and imported vaccines: We measured the anti-MG-ELISA antibodies in the chickens of all three groups (Group A-C) on 0 day and 14 days old (i.e., 0 day post-vaccination) and results are shown in Table 1. On 0 day old, the mean anti-MG-ELISA antibody titer of Group A, B and C were 245.35±2.924, 244.95±3.316 and 244.8±3.205, respectively and no significant difference was existed in antibody titer among three groups. Similarly, chickens of all three groups (Group A, B and C) had nearly similar antibody titers on day 0 post-vaccination (i.e., at 14 days old) without any significant difference. However, after MG vaccination (trial vaccine in Group B and imported vaccine in Group C) mean serum anti-MG ELISA antibody titer in chickens of Group-B and Group-C was found to be increased gradually as evaluated at 15-, 30- and 45-days post-vaccination whereas in Group A (non-vaccinated control) mean serum antibody titer decreased gradually (Table 1). Significant difference ($p<0.001$) in mean serum antibody titer was existed between non-vaccinated (Group-A) and vaccinated groups (Group B and C) at all three time points. In Group-B (TrV group) the mean serum anti-MG antibody titers on -15, -30 and 45-days post-vaccinations were 1103.1±6.632, 1621.5±6.447 and 2213.75±3.126, respectively whereas mean antibody titers in Group-C (ImV group) were recorded as 1099.75±5.138, 1545.35±4.416 and 2121.75±3.431, respectively at the same time points (Table 1). When we compared serum MG antibody titers between two vaccinated groups (Group-B and Group-C) significant difference ($p<0.001$) in mean serum antibody titer existed at 30 and 45-days post-vaccination but not at 15-days post-vaccination.

Avian mycoplasma is an important pathogen of poultry that causes serious economic impact in Bangladesh. MG infection usually progresses slowly and remains unidentified as long as stress conditions lead to an outbreak of the disease. In the present study, 95 tissue samples were collected from naturally infected poultry to isolate mycoplasma. Among the 95 samples, 17(17.89%) exhibited observable fried egg appearance on Hayflicks agar. Pathogenic avian mycoplasma show similar cultural characteristics in mycoplasma broth and agar medium. These 17 samples were also positive in PCR assay. Mycoplasmas are fastidious organisms. However, it is assumed that either the overuse of antibiotics in the poultry farms sampled or the difficulty in culturing mycoplasma could have kept the number of positive cases lower. For the preparation of killed vaccine, one PCR positive isolate designated as MG SAU1 that showed

strong positive band was inactivated by formaldehyde. 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. Based on sterility and safety test results, the prepared vaccine fulfilled all the criteria described previously. For sterility test of vaccine, similar procedure was used by other researchers[13-20]. Formaldehyde molecules bind with amino acids of protein molecules of the organisms and thus inactivate its viability. An inactivating chemical agent with higher concentration may reduce the antigenicity of the organisms. The killed antigen without adjuvant apparently absorbs from the injection site within a few hours and does not undergo the induction of antibodies. That's why montanide oil was mixed with the antigenic suspension as an adjuvant to enhance its absorption. The prepared trail vaccine (Group B) was evaluated and compared with the imported vaccine (Group C) and non-vaccinated chickens (Group A) were kept as control. All 3 groups had similar antibody titers at day 0 post-vaccination. After vaccination mean antibody titer of Group B and C increased significantly whereas in group A mean antibody titer was decreased gradually. Our results on serum antibody titer against MG killed vaccines were supported by previous findings[21-23]. Detection of anti-MG ELISA titer in day-old chicks stipulated that the breeder flock might be vaccinated earlier against MG or there might have a carrier of MG infection in the breeder flock.

Moreover, the killed vaccine did not neutralize the maternally derived anti-MG ELISA antibody titers in vaccinated chickens. MG killed vaccine is commonly used in chickens to protect them against respiratory signs, airsacculitis, egg production losses and reducing egg transmission[15-17, 19, 20, 24]. In the case of the oil-adjuvant vaccine, the major advantage is that protection against a specific disease might be achieved without the introduction of a live-vaccine strain in a flock.

CONCLUSION

It could be concluded that among the 95 samples collected from mycoplasma affected farms 17(17.89%) were found positive for mycoplasma by cultural characteristics. All of the 17 samples were also positive in PCR assay with *Mycoplasma gallisepticum* specific primers. Formalin killed mycoplasma vaccine was prepared from MG-SAU 1 isolate. After vaccination (at 45 days post-vaccination) with locally produced trial vaccine antibody titer in broiler chickens was found to reach at protection level 15-days post-vaccination and antibody titer continued to increase up to 45-days post-vaccination which was comparable with the imported commercial vaccine. Based on the study results, it could be concluded that the locally prepared low cost trial vaccine candidate might be useful to control *Mycoplasma gallisepticum* in Bangladesh. However, further study needs to be performed to evaluate cell-mediated immunity and protective efficacy in vaccinated chickens before vaccine recommendation.

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REFERENCES

01. Bradbury, J.M., C.A. Yavari and C.M. Dare, 2001. Mycoplasmas and respiratory disease in pheasants and partridges. Avian Pathol., 30: 391-396.
02. Ley, D.H., 2003. Mycoplasma Gallisepticum Infection. In: Diseases of Poultry, Saif, Y.M., H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. Mc-Dougald and D.E. Swayne (Eds.). 11th Edn., Iowa State University Press, Ames, IA., USA., pp: 722-743.
03. Nascimento, E.R., V.L.A. Pereira, M.G.F. Nascimento and M.L. Barreto, 2005. Avian mycoplasmosis update. Revista Brasileira Ciencia Avicola, 7: 1-9.
04. Ley, D.H., J.M. McLaren, A.M. Miles, H.J. Barnes, S.H. Miller and G. Franz, 1997. Transmissbility of live *Mycoplasma gallisepticum* vaccine strains TS-11 and 6/85 from vaccineated layer pullets to sentinel poultry. Avian Dis., 41: 187-194.
05. Sikder, A.J., M.A. Islam, M.M. Rahman and M.B. Rahman, 2005. Seroprevalence of Salmonella and *Mycoplasma gallisepticum* infection in the six model breeder poultry farms at Patuakhali district in Bangladesh. Asian Network Sci. Inf., 4: 905-910.
06. 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: 654-660.
07. Whithear, K.G., Soeripto, K.E. Harringan and E. Ghiocas, 1990. Safety of temperature sensitive mutant *Mycoplasma gallisepticum* vaccine. Aust. Vet. J., 67: 159-165.
08. Whithear, K.G., 1996. Control of avian mycoplasmosis by vaccination. Rev. Sci. Tech., 15: 1527-1553.
09. Kleven, S.H., 2008. Control of avian mycoplasma infections in commercial poultry. Avian Dis., 52: 367-374.
10. Bhuiyan, Z.A., M.Z. Ali, M.M. Moula, M.A. Bary, N. Arefin and M. Giasuddin, 2019. Seroprevalence of major avian respiratory diseases in broiler and sonali chicken in selected areas of Bangladesh. J. Adv. Vet. Anim. Res., 6: 561-566.

11. Bekele, L. and T. Assefa, 2018. Inactivated vaccine trial of *Mycoplasma gallisepticum* in Ethiopia. Open J. Vet. Med., 8: 75-85.
12. 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 *M. gallisepticum* R-strain. Avian Pathol., 34: 341-347.
13. David, S.A.W., D.V. Volokhov, Z. Ye and V. Chizhikov, 2010. Evaluation of *Mycoplasma* inactivation during production of biologics: Egg-based viral vaccines as a model. Applied Environ. Microbiol., 76: 2718-2728.
14. Koski, T.A., G.G. Christianson and F.L. Cole, 1976. Inactivation of mycoplasmas by use of phenol, formalin and beta-propiolactone. J. Biol. Standardization, 4: 151-154.
15. Rimler, R.B., R.B. Davis, R.K. Page and S.H. Kleven, 1978. Infectious coryza: Preventing complicated coryza with Haemophilus gallinarum and *Mycoplasma gallisepticum* bacterins. Avian Dis., 22: 140-150.
16. Hildebrand, D.G., D.E. Page and J.R. Berg, 1983. *Mycoplasma gallisepticum* (MG)-laboratory and field studies evaluating the safety and efficacy of an inactivated MG bacterin. Avian Dis., 27: 792-802.
17. 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 *M. synoviae*. Avian Dis., 28: 397-405.
18. Glisson, J.R. and S.H. Kleven, 1985. *Mycoplasma gallisepticum* vaccination: Further studies on egg transmission and egg production. Avian Dis., 29: 408-415.
19. 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.
20. 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.
21. Gondal, M.A., M. Rabbani, K. Muhammad, T. Yaqub, M.E. Babar *et al*., 2013. Antibodies response of broilers to locally prepared oil based *Mycoplasma gallisepticum* vaccine. J. Anim. Plant Sci., 23: 1094-1098.
22. Jacob, R., S.L. Branton, J.D. Evans, S.A. Leigh and E.D. Peebles, 2014. Effects of live and killed vaccines against *Mycoplasma gallisepticum* on the performance characteristics of commercial layer chickens. Poult. Sci., 93: 1403-1409.
23. Yoder Jr., H.W. and S.R. Hopkins, 1985. Efficacy of experimental inactivated *Mycoplasma gallisepticum* oil-emulsion bacterin in egg-layer chickens. Avian Dis., 29: 322-334.
24. Sasipreeyajan, J., D.A. Halvorson and J.A. Newman, 1987. Effect of *Mycoplasma gallisepticum* bacterin on egg-transmission and egg production. Avian Dis., 31: 776-781.