

Full Length Research Paper

Dosage regimen associated immuno modulatory effect of levamisole on humoral response of broilers against inactivated avian influenza virus H7N3 adjuvanted vaccines

Muhammad Ismail¹, Muhammad Danish Mehmood^{1*}, Huma Anwar¹, Sana Noreen¹, Amir Qazi², Muhammad Usman Ghani¹ and Mahreen Gul³

¹Ottoman Pharma (Immuno Division) Lahore, Pakistan, Pakistan.

²Institute of Molecular Biology and Biotechnology, University of Lahore, Pakistan, Pakistan.

³Post Graduate Medical Institute, Lahore, Pakistan.

Received 05 September, 2018; Accepted 18 October, 2018

Inactivated oil based avian influenza vaccines are being used to confront viral outbreaks but seem to be ineffective due to the shorter life span of broiler birds. In such situations immunostimulating agents may play a vital role to overcome the problem. The current study was designed to investigate the co-stimulatory effect of levamisole on humoral response of chicken against inactivated oil based influenza vaccines in broiler birds. The efficacy of levamisole (LMS) hydrochloride, a standard immune modulator was evaluated on one hundred broilers in association with inactivated adjuvanted avian influenza vaccines. Avian influenza susceptible broilers were divided into nine groups, each having ten birds. The birds in groups G1, G2, G3, G5, G6 and G7 were offered 30, 20 and 15mg kg⁻¹ bwt levamisole for four consecutive days after being vaccinated with oil and gel based inactivated influenza vaccines respectively; birds in group G9 were kept as control. Whereas, birds in Group G4 were kept as oil base vaccinated control and Group G8 were kept as gel base vaccinated control without levamisole medication. Blood samples were obtained from wing vein on 14th, 28th and 36th days post vaccination. Anti-influenza antibody response was measured using haemagglutination inhibition (HI) technique. Data analyzed by one way ANOVA and DMR test showed that levamisole in both regimens had appreciable effect on antibody titers ($p < 0.05$). In conclusion, LMS can stimulate immune system which causes better response to vaccination. Further studies are needed to evaluate other effective factors for each of the best results of LMS.

Key words: Levamisole, AIV H7N3, Immunomodulation.

INTRODUCTION

Poultry industry is the second largest industry after textile in Pakistan. It contributes significantly to GDP of the country. The availability of chicken table eggs and poultry meat has been increasing day by day which sufficiently almost every family and in urban areas every 5th family is

directly or indirectly associated with poultry related business (Sadiq, 2004).

The poultry industry has always been threatened by number of factors including environmental fulfills the requirement of animal protein. In rural areas hazards,

nutritional deficiencies, lack of advanced scientific management expertise, various pathogen infections and adjustment of imported breeds in local environment (Sandhu et al., 2009). Infectious viral diseases like Avian Influenza (AI), Hydropericardium Syndrome Virus (HPS), Infectious Bronchitis (IB), Newcastle Disease Virus (NDV) and Egg Drop Syndrome (EDS) play an important role in damaging socio economic status of livestock farmers in terms of high mortality in broilers, loss of production and poor hatchability in layer and breeder birds (Alexander, 2000). Avian influenza virus is highly infectious disease which can affect the birds at any age while the highly pathogenic avian influenza viruses can reach 50-89% morbidity and 100% mortality rate in some poultry flocks (Capua et al., 2000).

Avian influenza virus infection and bird flu outbreaks are continuously reported from all across the country even in the vaccinated flocks. Avian influenza in poultry flocks can be controlled effectively by eradication and compensation policy, clamping proper bio-security measures and mass scale vaccination. Inactivated AI virus vaccines and biosecurity measures have not exhibited results up to the mark in terms of control of bird flu in Pakistan (Naeem et al., 2007). Similarly, antibody response of commercial poultry to single bird flu vaccine is poor so farmers have to vaccinate their birds twice in broilers. Various factors such as quality of the vaccines, concurrent exposure to AI viruses, poultry management and nutrition etc., are incriminated to be the cause of poor antibody response.

Use of immune stimulants for the prevention of diseases in poultry is considered an effective and improving area. Currently, avian influenza inactivated emulsified vaccine has not been successful in controlling avian influenza outbreaks in broiler birds due to shorter life span whereas, layer and breeder flocks are protected. The reason could be that oil based vaccine requires much time to induce protective antibody titer. For the reason different immuno stimulators were developed so that protective antibodies could be induced in shorter period of time by triggering the process of immunogenesis. Immuno stimulants are the natural or synthetic substances that can enhance specific and non- specific immune response in vaccinated flocks (Anderson, 1992). Levamisole is anthelmintic in nature and can be also used as immunostimulator in association with vaccine and non-specific immunostimulator to counter the immunosuppressive effect of infection (Singla and Juyal, 1992). It can also be administered as dietary supplement to enhance the immune response of hepatitis B vaccine in humans. Levamisole stimulated both humoral and cellular immune response when injected with DNA vaccine in association with strong interferon production (Jin et al., 2004). Hence the present study was planned to investigate the co-stimulatory effect of Levamisole on

humoral response of chicken against inactivated oil based influenza vaccines in broiler birds.

MATERIALS AND METHODS

Source of chicks

One hundred- day old broiler chicks were purchased from A&S Poultry breeding company located at Raiwind Lahore, Pakistan. These chicks were shifted to clean and fumigated KMNO₄+2% formalin environmental control experimental house of Ottoman Pharma (Immuno Division) Lahore. The chicks were offered feed and water *ad libitum*.

Source of virus

200 ml of characterized inactivated AI-H7N3 (A/chicken-broiler/OP-Karachi/03H7N3-06 (Tahir et al., 2016) virus having biological titer of 512 HAU and 1×10^{9.2}/ml EID₅₀ was obtained from the Ottoman Pharma (Immuno Division), a veterinary biological production unit; it was carried in ice packed plastic container to the center for research in Molecular Medicine, University of Lahore.

Sterility and safety testing

5 ml of the sample is filtered through 0.2 um membrane and eluted with the help of sterile normal saline solution. A loopful culture of inactivated filtered antigen was streaked on Tryptic soya agar (TSA), Macconkey agar (MA) and Salmonella shagella agar (SSA) (Oxoid-USA) separately. 500 ul from the rest sample was dispensed into the test tube containing autoclaved Fluid thioglycolate medium (FTM) (Oxoid-Germany) and Tryptic soya broth (TSB) (Oxoid-Germany). The streaked plates and inoculated test tubes were incubated at 37°C for 1 week and results were recorded. For safety testing 0.1 ml of the inactivated viral fluid was injected into nine day old chicken embryonated eggs and incubated at 37°C for 72 h to check HA activity as described by King (1991).

Preparation of vaccine

Montanide and Aluminium hydroxide gel based vaccines were prepared in the class II Biohazard safety cabinet using following prepositions and the mixture is sheared at 2700 RPM for 10 min to form a uniform emulsion.

AIV oil based vaccine (AIVOB)

AIVH7 infected Fluid: Montanide ISA 70MVG
35 parts : 75 parts

AIV gel based vaccine (AIVGB)

AIVH7 infected Fluid: 10% aluminum hydroxide gel
50 parts : 50 parts

Quality control testing

AIVH7 inactivated oil based vaccine was subjected to the following

*Corresponding author. E-mail: drdanishmehmood@gmail.com.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

Table 1. Experimental design.

Group (n= 8)	Marking	Vaccine type	Levamisole treatment
G1	Right Blue	AIVH7 OBV	30 mg/kg b.wt
G2	Right Purple	AIVH7 OBV	20 mg/kg b.wt
G3	Right Red	AIVH7 OBV	15 mg/kg b.wt
G4	Left Purple	AIVH7 OBV	No Treatment
G5	Right Black	AIVH7 GBV	30 mg/kg b.wt
G6	Right Orange	AIVH7 GBV	20 mg/kg b.wt
G7	Right Green	AIVH7 GBV	15 mg/kg b.wt
G8	Left Green	AIVH7 GBV	No Treatment
G9	No Marking	No Vaccine	No Treatment

quality control tests:

Drop test

One drop of vaccine was dropped on the surface of water for the detection of type of emulsion.

Microscopy

A loopful of vaccine was spread on the sterile slide and observed under 40X lens of microscope (Nikon, JAPAN) and the distribution of particles in the suspension was observed.

Viscosity

100 ml of the emulsion was measured with the help of digital viscometer (Sigma Germany).

Stability

It was evaluated by centrifugation of 10 ml of sample at 5000 rpm for 30 min (80-3 Centrifuge, CHINA).

Vaccine safety testing

0.6 ml of the vaccine suspension was inoculated into three weeks old AIV susceptible chicks. Broilers were observed for any sign and symptoms of avian influenza virus for one week and the results were recorded (OIE, 2014).

Vaccine sterility testing

A loopful vaccine was streaked on tryptic soya agar (TSA), Macconkey agar (MA), Salmonella Shigella agar (SSA) and Sabouraud dextrose agar (SDA) plates and also the sterility of vaccine in Tryptic soya broth (TSB) and Fluid thioglycolate medium (FTM) was checked. The plate and test tubes were incubated at 37°C for 14 days (OIE, 2014).

Experimental design

The birds were divided into nine different groups identified by their respective color marking. Each bird of group G1, G2 and G3 was vaccinated with AIOBV while, group G5, G6 and G7 were vaccinated

with AIGBV on 7th day of age using 0.3 ml of AIV inactivated vaccine through subcutaneous route respectively. Each bird from group G1, G2, G3, G5, G6, G7 was treated orally at day 5th, 6th, 7th, 8th and 17th of age with 30, 20 and 15 mg/kg levamisole respectively for five consecutive days. Whereas, G4 and G8 were vaccinated with AIOBV and AIGBV without any treatment of levamisole. Moreover, G9 served as non-vaccinated and non-levamisole treated. Details of the groups and their respective vaccines are given in Table 1.

Evaluation of immunostimulatory effect of Ims in aiv inactivated inoculated broiler birds

Blood collection

3 ml of blood from each of the bird of every group was collected on 7, 21, 34 and 42 days of age in sterile syringes. The syringes containing blood were kept at slant position and room temperature for overnight to separate. The serum thus separated was stored at -60°C till further use.

Haemagglutination inhibition test

The serum samples thus collected were subjected to haemagglutination inhibition (HI) test following the procedure described by Hirst (1942) to find anti AIH7 antibody titers.

RESULTS

The candidate avian influenza oil based and gel based vaccine showed no growth in either of the media and inoculated birds remained healthy up to 14 days (Table 2). Avian influenza H7N3 emulsion was stable after centrifugation and showed uniform particle size and its distribution on slide under microscopy. The viscosity of the emulsion sample was 70 mPaS/sat 60 rpm (30°C). Whereas, by drop test, it is evident that emulsions belongs to water in oil emulsion category. Montanide and Aluminium hydroxide gel based vaccines in broiler birds in association with different doses of levamisole, using one way ANOVA, and subsequently Duncan multiple range test (DMR) showed mean antibody response (0.6±.4) to Avian Influenza H7 oil based vaccine treated with 15 mg/kg body weight levamisole was significantly lower than that of the vaccine treated with 20 mg/kg body weight levamisole (0.9±.0) or 30 mg/kg body weight (1±0.9)

Table 2. Vaccine safety and sterility.

Vaccine type	Safety test (72 h PI*)				Sterility test (2 weeks PI)			
	Bird Status	Eggs	TSA	TSB	FTM	MA	SSA	SDA
AIV OBV	Live and Healthy	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
AIV GBV	Live and Healthy	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth

*PI=post inoculation.

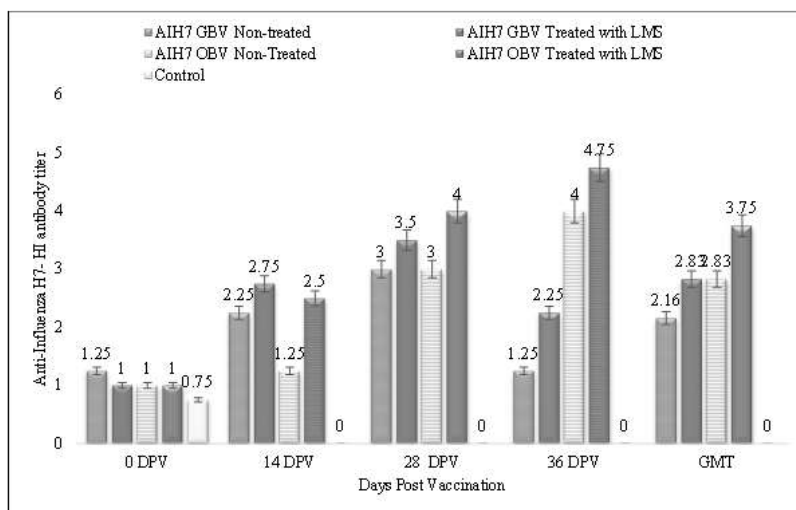


Figure 1. Effect of 15 mg/kg b.w LMS on inactivated avian influenza immunized broilers.

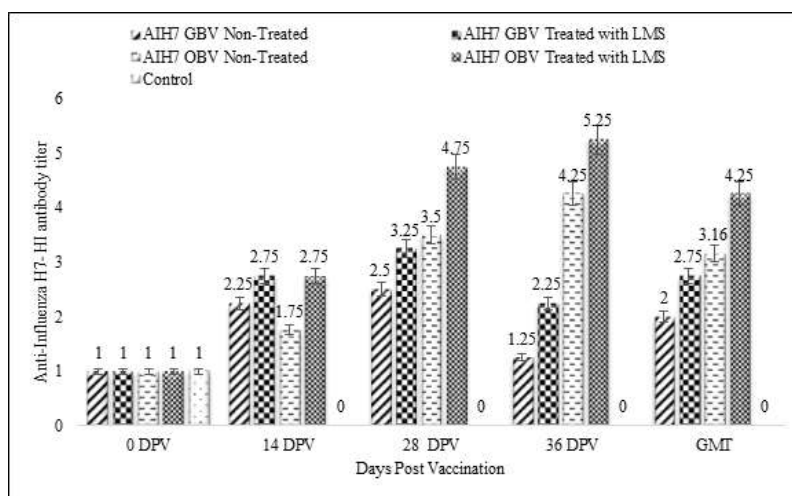


Figure 2. Effect of 20 mg/kg b.w LMS on inactivated avian influenza immunized broilers.

($P < 0.05$) (Figures 1 and 2). Mean antibody response of birds to Avian Influenza H7 Gel base vaccine treated with

levamisole at 30 mg/kg body weight was significantly higher than the birds treated with levamisole AT 15 mg/kg

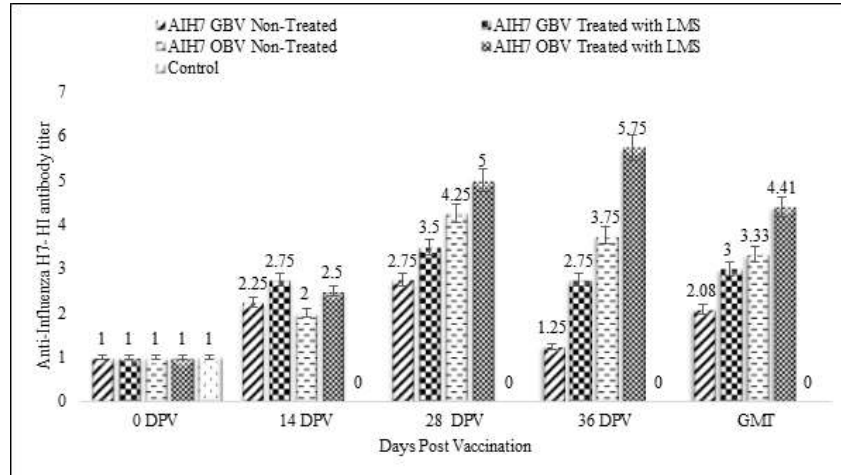


Figure 3. Effect of 30 mg/kg b.w LMS on inactivated avian influenza immunized broilers.

body weight and significantly lower than that of the vaccinated birds treated with 20 mg/kg body weight ($P < 0.05$) (Figures 1 and 3). The mean antibody response of birds to oil based vaccine was significantly higher than that of the vaccine containing aluminium hydroxide gel ($P < 0.05$).

DISCUSSION

Poultry industry in Pakistan and all over the world is the major source of cheap animal protein (Alexander, 2000); however currently the progress of the industry is hindered by many factors including nutritional and infectious disease such as infectious bronchitis, infectious bursal disease, Newcastle disease, hydropericardium syndrome and avian influenza, etc.

Avian influenza is one of the most important infectious poultry disease causing having economic losses to the poultry industry. The first avian influenza epidemic reported in Pakistan causes mortality of over more than one million birds causing serious economic losses to the poultry industry.

Avian influenza characterized virus (EID_{50} : $1 \times 10^{9.2}$) obtained from Ottoman Pharma (Immuno Division) was processed for Avian influenza oil based (AIOB) and avian influenza gel based (AIGB) vaccines following instruction of the supplier. Levamisole (LMS) is an isomer of the phenylimidothiazole salts of tetramisole with immunomodulatory effect. It is synthetic product traditionally used as anthelmintic agent in animals. Moreover, LMS has proven as immuno-stimulant properties (Cuesta et al., 2002).

Our results revealed that, oral administration of LMS at 30 mg/kg to avian influenza vaccinated broilers from 5th to 9th day of age (2 days post vaccination) daily for four successive days resulted in potentiation of chicken

immune response to inactivated oil base avian influenza disease vaccination. This was evidenced by significant increase in HI-geometric means in LMS treated groups (G-1 and G-5) versus non-treated groups (G-4 and G-8). In current study it was observed that 30 mg/kg dose of LMS when offered to AI vaccinated broiler birds at the age 9th day potentiated maximum anti influenza HI antibody titer (4.41) at 36 days post vaccination (Figure 3); observed effect of 20 mg/kg dose of LMS augmented protective anti influenza HI antibody titer (4.25) but the extent was less than 30 mg/kg dose. Moreover, 15 mg/dose of LMS showed less augmentation in the production of specific anti influenza HI antibody (3.75) (Figure 1).

Our results are in accordance with Kulkarni et al. (1973) who concluded that LMS treated groups showed a significant increase in HI titers than non-treated groups. Effect of LMS as an immunomodulator in birds vaccinated with Newcastle disease vaccine.

It is reported that levamisole is isomer of phenylimidothiazole salt showing anthelmintic activity in nematodes (Janssen, 1976). It showed immunomodulatory when used with vaccines (Renoux, 1971). It can enhance the cell mediated immunity by enhancing the maturation of macrophages and maturation of cells involved in immune system (Kelly, 1978). Immuno stimulating effect of levamisole is under observation to see its mechanism of action but it is well known that it can boost the immune response either humoral or cell mediated in vaccinated flocks to viral antigens. Vaccinated flocks treated with levamisole showed high level serum antibodies. It is also revealed that levamisole injected with the Hitchner B1 vaccine subcutaneously in post vaccinated birds showed increased level of serum antibodies (Bastami et al., 1991). Levamisole enhanced protection in mice when treated with Brucella vaccine. Levamisole was immune stimulant in many species by

modulation of phagocytosis and leukocyte cytotoxic activity (Cuesta et al., 2002). The use of LMS as adjuvant in vaccine is debated (Morrison et al., 2000). Studies showed that LMS in killed viral vaccines or in DNA vaccine enhanced the cell-mediated immunity (Kang et al., 2005 and Jin et al., 2004).

Immuno stimulants are the natural or synthetic substances that can enhance specific and non-specific immune response in vaccinated flocks (Anderson, 1992). Levamisole can also be administered as dietary supplement to enhance the immune response of hepatitis B vaccine in humans. Levamisole stimulated both humoral and cellular immune response when injected with DNA vaccine in association with strong interferon production (Jin et al., 2004).

It is concluded that levamisole offered to the immunosuppressed birds makes them able to mount immune response. Immuno modulatory effect of levamisole was only seen in those chickens which already had undergone immunosuppression. Levamisole increased the proliferation of lymphocytes in chickens and in mice (Yin et al., 2006) which indicated that it enhanced the cellular immunity in the form of interferon- γ . Moreover levamisole enhanced both cell-mediated immunity and humoral immunity in inactivated vaccinated birds. It shows that LMS increases the cellular immune response by activating the T lymphocytes and T cells to produce protective antibodies.

Conclusion

In conclusion, use of 30 mg levamisole/kg b.wt showed enormous response to AIOB, AIGB vaccinated birds. The humoral response to inactivated influenza vaccine in broilers could be enhanced by addition of levamisole treatment in brooding and rearing schedule of broilers for induction of rapid anti influenza anti body titer and better performance of feed conversion ratio (FCR).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors of the paper are highly grateful to the officials of Ottoman Pharma for providing laboratory facilities to execute the research and Prof. Dr. Mahmood Hussain Qazi, (late) Director Center for research in molecular medicine for building the research strategies. The author would also express my gratitude to the anonymous reviewers who help me to make this manuscript available for readers.

REFERENCES

- Alexander DJ (2000). A review of avian influenza in different bird species. *Veterinary Microbiology* 74:3-13.
- Anderson DP (1992). Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. *Annual Review of Fish Diseases* 2:281-307.
- Anderson DP (1992). Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. *Annual Review of Fish Diseases* 2:281-307.
- Bastami MA, Aziza M, Amer, El-Kady MF (1991). Stimulation of chicken immune system to Newcastle disease vaccine by levamisole treatment. Beni-Suef, *Veterinary Medicine: Research and Reports* 1:161-169.
- Capua I, Mutinelli F, Bozza MA, Terregino C, Cattoli G (2000). Highly pathogenic avian influenza (H7N1) in ostriches (*Struthio camelus*). *Avian Pathology* 29:643-6.
- Cuesta A, Esteban M, Meseguer J (2002). Levamisole is a potent enhancer of gilthead seabream natural cytotoxic activity. *Veterinary Immunology and Immunopathology* 89(3-4):169-174.
- Hirst GK (1942). The quantitative determination of Influenza virus and antibodies by means of red cell agglutination. *Journal of Experimental Medicine* 75:49-64.
- Janssen PAJ (1976). The Levamisole story. *Progress in Drug Research* 20:347-383.
- Jin H, Li Y, Ma Z, Zhang F, Xie Q, Gu D, Wang B (2004). Effect of chemical adjuvants on DNA vaccination. *Vaccine* 22:2925-2935.
- Kang Y, Jin H, Zheng G, Xie Q, Yin J, Yu Y, Xiao C, Zhang X, Chen A, Wang B (2005). The adjuvant effect of levamisole on killed viral vaccines. *Vaccine* 23(48-49):5543-5550.
- Kelly MT (1978). Modulation of microphage function by levamisole. *Journal of the Reticuloendothelial Society* 24:139.
- King DJ (1991). Evaluation of different methods of inactivation of Newcastle disease virus and avian influenza virus in egg fluids and serum. *Avian Diseases* 35:505-514.
- Morrison R, Nowak B, Carson J (2000). The effect of levamisole as an adjuvant on the humoral immune response of Atlantic salmon (*Salmo salar* L.). *Bulletin of the European Association of Fish Pathologists* 20(3):101-105.
- Naeem K, Siddique M, Ayaz M, Jalalee MA (2007). Avian influenza in Pakistan: outbreaks of low- and high-pathogenicity avian influenza in Pakistan during 2003-2006. *Avian Diseases* 51:189-193.
- OIE (2014) Avian Influenza OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Ch. 2.3.4. OIE, Paris, France.
- Renoux G, Renoux M (1971). Effect immunostimulant d'un imidothiazole dans l'immunization des souris contre l'infection per *Brucella abortus*". *Cr. Acad. Sci. (D) Paris* 272:349-350.
- SADIQ M (2004). Pakistan poultry sector still. *World Poultry*. 20(8):10-11.
- Sandhu BS, Brar RS, Brar APS, Sood NK and Singla LD (2009) Prevalence and pathology of parasitic gastrointestinal infections of poultry in punjab. *Indian Veterinary Journal* 86:1276-1277.
- Singla LD, Juyal PD (1992) Immunomodulatory effects of levamisole against *Trypanosoma evansi* infection in cow-calves: Serum gamma-globulins. *Journal of Veterinary Parasitology* 6(2):9-14.
- Tahir MS, Mehmood D, Sultan U, Saeed MH, Khan AR, Ansari F, Salman MM, Majeed KA (2016). A modified strategy of multiplex RT-PCR for simultaneous detection of H5, H7 and H9 subtype of avian influenza virus based on common forward oligo. *Journal of Applied Poultry Research* 25:322-332.
- Yin J, Jin H, Kang Y, Xiao C, Zhao L, Li X, Ding Z, Yang F, Zhu Q, Wang B (2006). Efficacy of modified levamisole adjuvant on inactivated virus vaccine. *Viral Immunology* 19(3):525-535.