

## Influence of immunogen count to antibody response of inactivated avian influenza virus oil based vaccine in broilers

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### Abstract

Primary prerequisite of effective influenza virus immunization is homology of vaccinal strain to the wild type and appropriate immunogen count in each dose of vaccine to induce protective immune response. The purpose of current project is to study the effect of immunogen potency in inactivated monovalent and bivalent avian influenza vaccines. Eight AIV (Monovalent & Bivalent) oil based vaccines with different immunogen level were prepared and evaluated for potency in AIV susceptible broilers through Haemagglutination Inhibition (HI) test on 18 and 36-day post vaccination. It was observed that 0.3ml dose of inactivated avian influenza oil based AIV-H9 vaccine having infectivity titer EID<sub>50</sub> 1×10<sup>9.2</sup>/ml and (HA= 512) induced high mean anti AIV-H9 antibody titers (72±22.62) as compared to the vaccine containing EID<sub>50</sub> 1×10<sup>8.2</sup>/ml and (HA= 256). Similarly, same dose of bivalent avian influenza inactivated vaccine containing infectivity titer EID<sub>50</sub> 1×10<sup>9.2</sup>/ml and (HA= 512) induced better immune response against each antigen H7 and H9 respectively, that of 0.3 ml dose of the vaccine comprising infectivity titer EID<sub>50</sub> 1×10<sup>8.2</sup>/ml and (HA= 256) where H7 and H9 were documented. It was concluded that infectivity titers in 0.3 ml dose of inactivated adjuvanted vaccine shall be ≤ 10<sup>8.2</sup>/ml or 256 HAU to achieve protective anti body titers to get protection against avian influenza virus and its complications over a long period of time thus contributing to disease control in epidemics.

**Keywords:** Haemagglutination (HA), Infectivity titer (EID<sub>50</sub>), Avian influenza virus (AIV).

### Introduction

High-pathogenicity avian influenza (HPAI) virus has become endemic in poultry and voiced as potential source of virus for birds and mammals, including humans. Vaccination has become a part of the poultry control strategy, but vaccine failures have occurred in the field. This study identified possible causes of vaccine failure particularly at producer end, which included the use of an unlicensed virus seed strain and induction of low levels of protective antibody associated with insufficient quantity of vaccine antigen. However, the most important cause of vaccine failure was the appearance of drift variant field viruses that partially or completely overcame commercial vaccine-induced immunity. Furthermore, experimental vaccines using inactivated wild-type virus or reverse genetics-generated vaccines containing the hemagglutinin and neuraminidase genes of wild-type drift variant field viruses were protective. These studies indicate the need for surveillance to identify drift variant viruses in the field and update licensed vaccines when such variants appear.<sup>1</sup>

Influenza commonly known as flu is an infectious disease caused by RNA viruses of family Orthomyxoviridae that affects birds and mammals. The virus consists of eight segments negative sense single stranded RNA, which codes for ten proteins including haemagglutinin (H) and neuraminidase (N).<sup>2</sup> Moreover, the pathogenicity and immunogenicity of AI virus is directly associated with the type of H and N antigen.<sup>3</sup> The H projection is responsible for attachment of the virus to the host cells. The N activity disrupts neuraminic acid in the receptors of the host cells, allowing release of newly propagated virus. Both H and N proteins are important for antibody formation in the host. Avian influenza (AI) has significant potential to disrupt

commercial poultry production resulting in extensive losses to the poultry farmers.<sup>4</sup> Low pathogenic avian influenza (LPAI) in chicken causes ruffled feathers, temporary drop in egg production or loss of weight with respiratory signs.<sup>5</sup> Nili and Asasi recorded 20-60% mortality in broilers due to H9N2 virus infection. However, high mortality may occur in these birds due to invasion by secondary pathogens. LPAI virus infection causes deleterious effects in digestive, respiratory, reproductive and urinary organs of affected birds. The most frequent signs are sneezing, coughing, rales, and excessive eye discharge. The circulation of LPAI emerged into HPAI which results high mortality in flocks.<sup>6</sup> Bowes isolated LPAI H7N3 virus from broiler and breeder flocks in British Columbia.<sup>7</sup> The disease was associated with decreased egg production, respiratory disturbance and high mortality.<sup>8</sup>

Vaccine failure occurs due to rapid mutation of avian influenza virus as a result of genetic re-assortment, the quality control factors affecting the potency of vaccine during the production process and the genetic potential of various species such as domestic and wild birds. However, short life of broilers is always been considered as potential risk to the pathogens where active immunization particularly killed adjuvanted vaccines do not find ample time to express themselves effectively. Early exposure in such vaccinated broilers showed high morbidity followed by mortality. The current attempt was made to investigate the role of avian influenza virus immunogen count based on biological titer in inactivated vaccine to production of antibodies in short period of time.

## Materials and Methods

### Source of Birds

80- day old broiler chicks were purchased from well reputed poultry breeding company located at Raiwind Lahore, Pakistan. These chicks were shifted to clean and fumigated (KMNO<sub>4</sub>+2% formalin) environmental control experimental house of Ottoman Pharma (Immuno Division) Lahore. The chicks were offered feed and water *ad libitum*.

### Source of Virus

The characterized inactivated Avian influenza H<sub>7</sub>N<sub>3</sub> (A/Breeder-Chicken/OP/OVG/17) EID<sub>50</sub>-10<sup>8.1</sup> /ml, HA-264 HA unit/ml and H<sub>9</sub>N<sub>2</sub> (A/Breeder-Chicken/OP/OVI/12) EIID<sub>50</sub>-10<sup>8.6</sup>/ml, HA-264 HA unit/ml virus<sup>9</sup> were obtained from Ottoman Pharma (Immuno Division) licensed veterinary vaccine manufacturing company located at 10-km Raiwind road, Lahore, Pakistan.

### Sterility and Safety Testing

5ml of the sample was filtered through 0.2um membrane and eluted with the help of sterile normal saline solution (0.9% NaCl). A loop full culture of inactivated filtered antigen was streaked on Tryptic soya agar (TSA), Macconkey agar (MA) and Salmonella shigella agar (SSA) (Oxoid-USA) separately. While, 500 ul each from rest of the 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 0.1ml of the inactivated viral fluid was injected into nine day old chicken embryonated eggs and incubated at 37°C for 72 hours to check HA activity as described by.<sup>10</sup>

### Confirmation of Virus

AIV infected harvest was confirmed by the method of Haemagglutination Inhibition Test as described by Hirst, 1942 using confirmed monoclonal antisera (GD Diagnostic-Holland) and through PCR.<sup>9</sup>

### Preparation of Vaccine

Four different concentrations of antigen were used in the preparation of monovalent and bivalent AIV vaccine preparation. First and second category vaccines were prepared on the basis of embryo Infectivity dose 50 (EID<sub>50</sub>). Whereas, third and fourth category vaccines were prepared using different biological titer of inactivated antigen (HAU). All the vaccines were prepared in class II biohazard safety cabinet using following prepositions and the mixture was homogenized at 2700rpm for 10 minutes to form a uniform emulsion Table 1.

**Table 1: Avian influenza montanide based vaccine formulation**

Vaccine Type	AIV Montanide based vaccine							
	Embryo infectivity Titer 50 (EID <sub>50</sub> / ml)				Haemagglutination Units /50ul of one ml			
	Aqueous 10 <sup>9.2</sup> /ml	Oil	Aqueous 10 <sup>8.2</sup> /ml	Oil	Aqueous 1024	Oil	Aqueous 256	Oil
Monovalent (AIVH9)	40	60	40	60	40	60	40	60
Bivalent (AIV H7&H9)	20+20	60	20+20	60	20+20	60	20+20	60

### 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 two weeks and the results were recorded.<sup>11</sup>

### Vaccine Sterility Testing

A loop full vaccine was streaked on tryptic soya agar (TSA), Macconkey agar (MA), Salmonella Shigella agar (SSA) and Sabouraud dextrose agar (SDA) plates and also check the sterility of vaccine in Tryptic soya broth (TSB) and Fluid thioglycolate medium (FTM). The plate and test tubes were incubated at 37°C for 14 days.<sup>11</sup>

### Experimental Design

The birds were divided into different groups identified by their respective marking. Each bird of group G1, G2, G3 and G4 were vaccinated with monovalent AIOBV while, group G5, G6, G7 and G8 were vaccinated with bivalent AIOBV on 7<sup>th</sup> day of age using 0.3ml of AIV inactivated vaccine respectively through subcutaneous route (S/C). Moreover, G9 was kept as unvaccinated negative control while G10 was declared as positive control injected with commercially available AIV vaccine. Details of the groups and their respective vaccines injected are given in Table 2.

**Table 2: Experimental Design**

S. No	Group (n=08)	Immunogen Count	Vaccine Type	Marking
1	G1	EID <sub>50</sub> 1×10 <sup>9.2</sup>	AIV H9 OB Monovalent	Right Blue
2	G2	EID <sub>50</sub> 1×10 <sup>8.2</sup>	AIV H9 OB Monovalent	Right Black

3	G3	HA 1024	AIV H9 OB Monovalent	Right Green
4	G4	HA 256	AIV H9 OB Monovalent	Right Purple
5	G5	EID <sub>50</sub> 1×10 <sup>9.2</sup>	AIV H9+H7 OB Bivalent	Left Blue
6	G6	EID <sub>50</sub> 1×10 <sup>8.2</sup>	AIV H9+H7 OB Bivalent	Left Black
7	G7	HA 1024	AIV H9+H7 OB Bivalent	Left Green
8	G8	HA 256	AIV H9+H7 OB Bivalent	Left Purple
9	G9	Negative control	No Vaccine	White
10	G10	Positive control	Commercial vaccine	Black

### Evaluation of Seroconversion

#### Blood Collection

3ml blood from each bird of every group was collected on 0, 18 and 36 days of age in sterile syringes. The syringes containing blood were kept at slant position at room temperature for overnight. 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 to determine anti H7 and H9 antibody titers.<sup>12</sup>

### Results

The candidate avian influenza monovalent and bivalent oil based vaccine showed no growth in either of the media and inoculated birds remained healthy up to 14 days (Table-3). 0.3ml dose of each avian influenza monovalent and bivalent oil based vaccine containing 9HA units/ml, injected to six days old broilers induced detectable anti AIV-HI antibody titers on 18<sup>th</sup> and 36<sup>th</sup> DPV. The 0.3 ml dose of each monovalent vaccine containing 256HAU/50ul, 1024 HAU/50ul AIVH9 virus showed 8.0±0.0 and 40±14.8 mean standard deviation (M=SD) respectively on 18 day post vaccination to that of 34±13.35, 72±22.62 containing 256HAU/50ul, 1024HAU/50ul AIVH9 virus induced on 36 DPV. Similarly, monovalent vaccine having infectivity titer EID<sub>50</sub> 1×10<sup>8.2</sup>/ml, EID<sub>50</sub> 1×10<sup>9.2</sup>/ml of AIVH9 virus in 0.3 ml dose showed 10.50±8.79 and 34±13.35 respectively on 18

DPV as compared to 30±5.65 and 72±22.62 M=SD induced on 36 DPV.

Whereas, in case of AIV bivalent vaccines where 0.3ml dose of inactivated vaccine contain 256 HAU of each AIV-H7 and AIV-H9 virus induced 7±1.85, 9±2.82 anti-influenza HI antibody titers simultaneously on 18 day post inoculation as compared to 26±8.28 and 34±13.35 on 36 day post injection. Similarly, mean anti HI antibody titer was recorded against bivalent vaccine containing biological titer of 1024 HAU of each AIV-H7 and AIV-H9 in 0.3ml dose showed 20±7.40 and 24±8.55 respectively on 18 day PV as compared to 44±16.56 and 52±16.56 on 36 day PV. Moreover, AIV bivalent vaccines where 0.3ml dose of inactivated vaccine contain EID<sub>50</sub> 1×10<sup>8.2</sup>/ml of each AIV-H7 and AIV-H9 virus induced 7±4.14, 10.50±4.75 anti-influenza HI antibody titers simultaneously on 18 DPV as compared to 24±8.55 and 30±15.85 on 36 day post injection. Furthermore, mean anti AIV-HI antibody titers was recorded against bivalent vaccine containing biological titer of EID<sub>50</sub> 1×10<sup>9.2</sup>/ml of each AIV-H7 and AIV-H9 in 0.3ml dose showed 20±7.40 and 22±8.28 respectively on 18 day as compare to 44±16.56 and 52±16.56 on 36 DPV.

Monovalent positive control influenza vaccine induced 56±14.81 M=SD HI antibody titer on 36 day post vaccine. Whereas, bivalent influenza vaccine showed 30±5.65, 36±11.31 serum anti AIV-H7 and H9 respectively on 36 day post vaccination. Moreover, birds included in negative control group did not show any anti AIV-HI antibody titer 0.75±1.03 on 48 day of their age.

**Table 3: Vaccine Safety and Sterility**

Safety Test (72 hrs. PI*)			Sterility Test (2 weeks PI)					
Vaccine Type	Bird Status	Eggs	TSA	TSB	FTM	MA	SSA	SDA
AIH9 OBV	Live and Healthy	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
AIH9+H7 OBV	Live and Healthy	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth

\*PI=post inoculation

## Discussion

In present study, different dose dependent avian influenza inactivated vaccines were evaluated in AIV susceptible broiler birds using haemagglutination inhibition test. Six inactivated vaccines containing different infectivity titers were evaluated for their serological potency in broiler birds and were found satisfactory ( $\text{GMT} \geq 10$ ) as compliant to international standard for immune potency with a  $\text{GMT} \geq 32$ . In our studies, vaccine containing  $\text{EID}_{50} 1 \times 10^{9.2}$ , HA-256 surpass international standards. Previous studies revealed that  $\geq 8$ <sup>13</sup> or  $\geq 10$  (Kumar) is specific for anti AIHI geometric mean titers in blood serum of birds to antigenically similar vaccinal strain can resist field of genetically similar influenza virus as compared to survival. In another study GMTs of  $\geq 40$  anti AIHI geometric mean titers prevents virus shedding in majority of vaccinates.<sup>14</sup> According to OIE terrestrial manual anti AIHI  $\text{GMT} \geq 32$  compromises protection from mortality as compared to  $\text{GMT} \geq 128$  offers protection from virus shedding.<sup>15</sup>

It was observed that vaccine with higher biological immunogen count induced high antibody titers in broilers. Highest anti AIV-HI antibody titer  $72 \pm 22.62$  was achieved with immunogen count  $\text{EID}_{50} 1 \times 10^{9.2}/\text{ml}$  in oil based vaccine at 32 days post vaccination. It was observed that avian influenza oil based vaccine having infectivity titer of  $\text{EID}_{50} 1 \times 10^{9.2}/\text{ml}$  induced significantly higher anti AIV HI antibody titer ( $72 \pm 22.62$ ) as compared to inactivated AIV oil based ( $30 \pm 5.65$ ) vaccine having immunogen count  $\text{EID}_{50} 1 \times 10^{8.2}/\text{ml}$  (Fig. 1). Moreover, inactivated oil based AIV-H7&H9 bivalent vaccine with higher immunogen level  $\text{EID}_{50} 1 \times 10^{9.2}/\text{ml}$  induced higher anti AIV HI antibody titer ( $52 \pm 16.56$ ) as compared to inactivated oil based AIV bivalent vaccine containing H9N2 and H7N3 with lower immunogen level  $\text{EID}_{50} 1 \times 10^{8.2}/\text{ml}$  (Fig. 4). We also found that anti AIV-H7 antibody titer  $44 \pm 16.56$  was found significantly lower than that of AIV-H9  $52 \pm 16.56$  in oil based bivalent vaccine post 36<sup>th</sup> DPV. The possible reason of lower level of anti AIV-H7 antibody titer could be low infectivity titer of the virus. The results of current study regarding role of infectivity titer to the antibody production is almost similar to the findings of Khan who reported that avian influenza formalized vaccine containing ( $\text{EID}_{50} 1 \times 10^9$ ) showed significantly higher anti AIHI antibody titers in the broilers after 42 days of injection as compared to the vaccine having immunogen level of  $\text{EID}_{50} 1 \times 10^8$ ,  $\text{EID}_{50} 1 \times 10^7$  and  $\text{EID}_{50} 1 \times 10^6$ .<sup>16</sup> Antigen infectivity titer ( $\text{EID}_{50} 1 \times 10^9$ - $10^8$ - $10^7$ ) in aqueous part of candidate AIV vaccines showed descending pattern of anti AIHI antibody titer in the vaccinated broilers. It is observed that immunogen count has positive co-relation with production of antibodies. High level of AIV antigen count in vaccine association with mineral oil as adjuvant may efficiently potentiate antigen presentation which in result elicit high antibodies in blood serum. Our findings also corroborates findings of a previous which revealed that avian influenza count in vaccine is directly proportional to the antibody titer and vice versa (Pour *et al.*, 2006). The magnitude of serological responses to the antigen is

dependent on the amount of antigen in each dose of the vaccine.<sup>17</sup>

Inactivated monovalent H9N2 vaccine with biological titer of  $1024\text{HAU}/50\mu\text{l}$  induced significant anti AIV HI titers ( $72 \pm 22.62$ ) as compared to inactivated monovalent H9N2 vaccine ( $34 \pm 13.35$ ) with comparatively low immunogen count ( $256\text{HAU}/\mu\text{l}$ ). AIV bivalent vaccine containing AIV-H7N3 and AIV-H9N2 each with high immunogen count ( $1024\text{HAU}/\mu\text{l}$ ) in single dose induced significantly high anti AIV HI titers ( $44 \pm 16.56$ ,  $52 \pm 16.56$ ) as compared to inactivated bivalent vaccine containing AIV-H7N3 and AIV-H9N2 with comparatively low biological titer ( $256\text{HAU}/\mu\text{l}$ ,  $M \pm \text{SD} = 26 \pm 8.28$ ,  $34 \pm 13.35$ ). Similar observation were reported by Anees.<sup>18</sup> Monovalent vaccine with high infectivity titer  $\text{EID}_{50} 1 \times 10^{9.2}/\text{ml}$  was able to generate better antibody response ( $72 \pm 22.62$ ) when compared to the monovalent vaccine with lower infectivity titer  $\text{EID}_{50} 1 \times 10^{8.2}/\text{ml}$ , ( $30 \pm 5.65$ ). AIV HI antibody response of vaccinated birds were correlated with the antigen levels of candidate vaccines. The maximum anti AIV-H7 and H9 HI antibody titer was observed in bivalent oil based vaccine having  $\text{EID}_{50} 1 \times 10^{9.2}/\text{ml}$  of infectivity titer on 36 day post vaccination. The minimum anti AIV HI antibody titer that could protect chicken from HPAI virus infection related symptoms and death is considered to be 64 HIU. In contrast, chickens with anti AIV HI titers of 5HIU or higher, survive without symptoms of AI and there is no viral shedding after the challenge. Khan reported that AIV vaccine containing infectivity titer ( $\text{EID}_{50} 1 \times 10^{9.2}/\text{ml}$ ,  $\text{EID}_{50} 1 \times 10^{8.2}/\text{ml}$ ) of AIV antigen correspondingly induce protective anti AIV-HI titers in broilers.<sup>16</sup>

Oil based inactivated vaccines have been used extensively in commercial poultry particularly in broiler farming for immuno-prophylaxis against avian influenza viruses. Despite of multiple vaccine injections birds do not induce protective antibodies in limited age period. In such situation different factors play a critical role in induction of minimal immune response and failed to protect birds from disease during influenza outbreaks. Fast re-assortment in influenza viruses, chemical nature of adjuvants, quality of feed, health status and breed of birds may play role in uncertain immune response to the vaccines.<sup>19-25</sup> On the contrary oil based vaccines immunogenic potential is evidently associated with time period which requires ample time to mount protective immune response. However, life span of broilers is comparatively short where presence of high level maternal antibodies are always another contradictory aspect in immunology. Inactivated antigen normally injected in subcutaneous part of the bird skin accumulates cause irritation and form granuloma which attract antigen presenting cells (APC) and ultimately enhance antibody production.<sup>26</sup> The immuno competent cells move towards stimulus, phagocytose antigen and present them on their surface in association with immune associated antigen (Ia).<sup>27</sup> These cells secrete cytokines (IL-1, IL-2, IL-3, IL-4 and IL-5) which subsequently activates B and T-lymphocytes, induce humoral and cell mediated immunity respectively.<sup>28</sup>

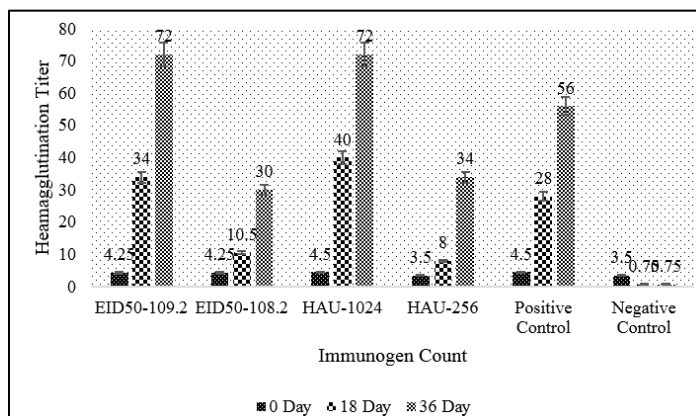


Fig. 1: Effect of immunogen count on anti AIV-HI antibody titers of inactivated monovalent H9N2 oil based vaccine containing different infectivity titers

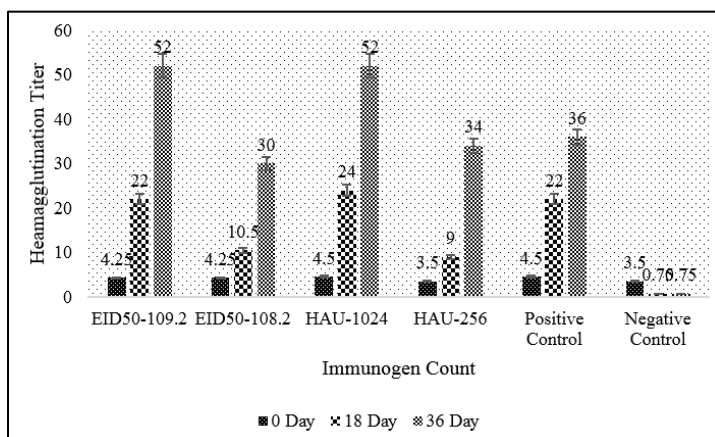


Fig. 2: Effect of immunogen count on anti AIVH9-HI antibody titers of inactivated bivalent AIV oil based vaccine containing H9N2 and H7N3

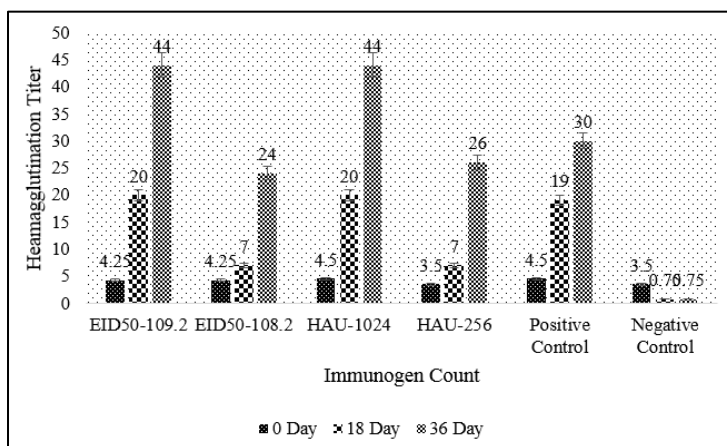


Fig. 3: Effect of immunogen count on anti AIVH7-HI antibody titers of inactivated bivalent AIV oil based vaccine containing H9N2 and H7N3

**Conclusion**

Regardless of the incriminated factors involved in low immunogenic response to inactivated influenza vaccination, high humoral reaction can be achieved in short span of broilers life by induction of high immunogenic count in

association with mineral oil adjuvants. High Infectivity (EID<sub>50</sub>) and biological titer (HA) were declared as effective method for quantification of influenza virus in the production of inactivated influenza vaccine. This practice may help us to improve quality of inactivated influenza vaccine primarily been developed for commercial broiler farming.

## Acknowledgement

The authors wishes to his gratitude to the CEO Ottoman Pharma, Lahore, Pakistan for authorization to my team for free access to R&D along with financial support in this regard. I am also thankful to our colleagues Muhammad Ismael, Usman Ghani and Dr. Sajjad Hussain who offered technical support and assisted the research.

**Conflict of Interest:** None.

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**How to cite this article:** Zia S, Mehmood MD, Anwar H, Gul S, Ejaz R. Influence of immunogen count to antibody response of inactivated avian influenza virus oil based vaccine in broilers. *Indian J Microbiol Res* 2019;6(1):44-49.