

In Process Quality Control Factors Affecting Efficacy of Avian Influenza (H₅N₁) Vaccine

Saeed Khan, Khushi Muhammad*, Atif Hanif, Masood Rabbani, Irshad Hussain and Muhammad Sarwar Khan

Department of Microbiology (SK, KM, AH, IH), University Diagnostic Laboratory (MR) and Department of Clinical Medicine and Surgery (MSK), University of Veterinary and Animal Sciences, Abdul Qadir Jilani Road, Lahore, Pakistan.

Abstract.- Avian Influenza (AI) is causing heavy economic losses in domestic poultry all over the world. It is caused by a highly pathogenic virus H₅N₁. The present study was aimed at assessing the quality control parameters during the process of vaccine production. The virus was recovered from lungs, trachea and fecal contents of the infected birds in chicken embryos. Binary ethylenimine (5mM) inactivated the virus within 16 hours of incubation at 25°C. The AI vaccine without adjuvant induced poor anti-AIV-HA antibody titer (3.5 GMT) in the vaccinated broilers. The vaccine containing aluminum hydroxide gel (AHG) induced the antibody response (36.8 GMT) that reached the peak level on 18 days post priming and declined thereafter. Montanide ISA 70 based AI vaccine induced increasing trend of the antibody titer (90.5 GMT) on 42 days of age. Boosting of the birds primed with AHG-AIV vaccine improved the antibody response (111.4 GMT on 42 days of age), while boosting of birds primed with montanide based AIV showed undetectable effect. The vaccines containing higher biological titer induced higher antibody titer in the vaccinated broilers. Serial passages of the AIV through embryos improved its HA and biological titer but tremendously decreased its antigenicity. It is concluded that use of the montanide based AIV vaccine prepared from its passage number <4, could be an effective way of the immuno-prophylaxis.

Key words: Avian influenza, vaccine, quality control, Montanide, biological titer.

INTRODUCTION

Avian influenza (AI) has significant potential to disrupt commercial poultry production resulting in extensive losses to the poultry farmers (Shane, 1995). Cause of the fatal outbreaks of avian influenza during 1995 and 1998-1999 was H₇N₃ and H₉N₂ types of the virus, respectively (Muhammad *et al.*, 1997; Naeem *et al.*, 1999; Muhammad *et al.*, 2001). However, outbreaks of bird flu were also recorded in northern areas of Pakistan. It is caused by H₅N₁ type of the virus. Chickens of all age groups ranging from 3 to 65 weeks are susceptible to the virus infection. Incubation period of AI is quite variable and it ranges from few hours to three days in the susceptible flocks (Calnek, 1997).

The AI virus consists of eight segments of negative sense single stranded RNA, which codes for ten proteins including haemagglutinin (H) and neuraminidase (N) (Bean *et al.*, 1985). There are

sixteen types of H and nine types of N antigens. There could be more than one hundred and forty four antigenic variants of the virus. The RNA has high frequency of mutation that induces antigenic changes in H and N molecules (Hinshaw *et al.*, 1981; Cowen, 1985). Moreover the pathogenicity and immunogenicity of AI virus is directly associated with the type of H and N antigen (Jordan, 1990). 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 in antibody formation in the host (Shane, 1995).

Avian influenza in poultry flocks could be controlled effectively, by eradication and compensation policy, clamping proper bio-security measures, limited or mass scale vaccination. Bird flu outbreaks are continuously reported from all across the country even in the vaccinated flocks. 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

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so farmers have to vaccinate their birds twice in broilers. Various factors such as quality of the vaccines, concurrent diseases, poultry management, nutrition etc., are incriminated to be the cause of poor antibody response. There are many factors during process of biologics production that affect quality of the vaccines. The present project is aimed at optimizing the in process quality control factors to improve the efficacy of the bird flu vaccine.

MATERIALS AND METHODS

Source of sample and preparation of inoculum

During 2007, dead broilers with history of respiratory syndrome were reported to University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences, (UVAS) Lahore, from Sheikhpura district, Punjab. Samples of lungs, trachea, spleen and fecal contents of the broilers were collected and transferred to WTO Quality Operations Laboratory (WTO-QOL), UVAS, Lahore.

Each of the samples (lungs: 5 g, trachea: 5 g and spleen: 5 g) was ground with 10 g of pre-washed and pre-sterilized sand in pestle and mortar for 10 minutes. Twenty five (25) ml sterilized normal saline (0.85% aqueous solution of sodium chloride: pH 7.2) was added in the ground material. Similarly, each fecal sample (5 g) was suspended in 25 ml of the normal saline. In both of the above mentioned cases, whole content was suspended and then transferred to a properly labeled glass beaker and kept undisturbed on the bench for 10 minutes. Clear material (10 ml) from upper layer of each of the beakers was centrifuged at 4000xg for 15 minutes and each of the supernatants was mixed with antibiotics (Gentamycin: 200 ug/ml; Penicillin: 10,000 units/ml; Streptomycin: one mg/ml) and antifungal (Amphotericin-B: 0.02 mg/ml) agents. Each of the samples was further filtered through a separate syringe filter of 0.2 micron porosity (Anonymous, 2009a)

Embryo inoculation and biological titration

Chicken embryos (Ten days old) were purchased from a reputable HI-TECH Sunder Hatchery, Multan Road, Lahore, at different intervals. Each time, the embryos were transferred

to laboratory egg incubator. Each of the samples was inoculated in the embryos through allantoic cavity, incubated for 48 hours and allanto-amniotic fluid (AAF) was harvested according to the method as described by (Hitchner *et al.*, 1980). The AAF containing virus suspension was processed for hemagglutination titer (Allan *et al.*, 1978) and biological titer (Villegas, 1998). The AI virus was passed through chicken embryos six times and HA as well as biological titer of the virus suspension was calculated after every passage.

Preparation and evaluation of vaccines

Formaldehyde (37%) at rate of 0.12% and Binary Ethyleneimine (BEI) at rate of 0.5 mM concentration was used to inactivate the virus (Bahnemann, 1975). The inactivated AI virus suspension (10 ml) passed through syringe filter (0.2 micron porosity) and was inoculated (0.1 ml) in 10 days old five chicken embryos for 1st safety test. The inoculated embryos were incubated at 37°C for 48 hours and then chilled overnight. The AAF of each embryo was harvested and pooled together and was subjected to HA activity. The AAF (0.1 ml) was inoculated in 10 days old five embryos for 2nd safety test. All the embryos were incubated at 37°C for 72 hours. The embryos were candled every day to record any embryonic mortality. On 72 hours post-incubation, the embryos were removed from the incubator and chilled overnight. The AAF harvested and processed for HA activity (Allan *et al.*, 1978). Lack of HA activity in the fluid was the indication that the virus suspension in the AAF had been inactivated properly and AAF qualifies for vaccine production.

The virus suspension (one ml: AAF) was streaked on the nutrient agar and mycoplasma broth. The nutrient agar plates were incubated at 37°C for 5 days, while the mycoplasma broth were incubated for 10 days (Elsken, 1996). The lack of growth on the bacterial culture media was the indication of the sterility of the virus suspension.

Effects of different passage number, biological titer, types of adjuvant, boosting, etc on the efficacy of the AI virus vaccines were determined. Effect of chemical on the virus inactivation was also determined. Chemically inactivated AI virus suspension was processed for

preparation of their respective vaccines. Thiomersal sodium was added at recommended amount in each batch of the virus suspension (Anonymous, 2009b).

One hundred and fifty two day-old broilers were purchased from HI-TECH Sunder Hatchery, Multan Road, Lahore, and were reared in the Experimental Animal House of WTO-QOL, UVAS, Lahore. Feed and water were given *ad libitum*. On 10th day of age, the birds were divided into 18 groups each contained 8 birds. Each of the vaccine @ 0.3 ml was injected, subcutaneously (S/C) at mid dorsum of the neck to each bird of the respective group, while 8 birds were kept as un-vaccinated control. The blood samples from each bird were collected on 21, 28, 35 and 42 days of age. The serum from each sample was processed for monitoring anti-H₅N₁ virus antibody titer using hem-agglutination inhibition (HI) test as described by Allan *et al.* (1978).

The data thus collected were subjected for statistical analysis by calculation of geometric mean titre (GMT; Brugh, 1978).

RESULTS AND DISCUSSION

Avian influenza (AI) virus particularly highly pathogenic type (H₅N₁) is causing substantial losses in domestic poultry throughout the world for last few decades. Avian influenza in commercial layers or broilers is characterized by severe respiratory signs, facial swelling, blackening comb/wattles, shank and lower side of feet. It causes 100% morbidity and 30-70 % mortality (Yaqub *et al.*, 1998; Naeem *et al.*, 1999). The causative agent (H₅N₁) of bird flu in commercial breeding flock was detected through cultivation of morbid samples (lungs, spleen, tracheal swabs and fecal content) in 10 days old chicken embryos. The AI virus grew well in the embryos. The virus inoculated via allantoic sac route disseminates to different parts of embryo via hematogenous route. It replicates in chorio-allantoic membrane, embryo and on the lining of allantoic and amniotic cavity. Its replication in these parts was detected through hem-agglutination (HA) activity and lesions on the embryo. Moreover, it induced death of chicken embryos within 36-48 hours post inoculation like other AI viruses as recorded by Naeem *et al.* (1999)

and Muhammad *et al.* (2001). Perdue *et al.* (1990) also recorded that non pathogenic strains of AIV induced death in 9 days old embryos more rapidly than that in 12 or 13 days of age. The bird flu virus when inoculated at 9 days of chicken embryos induced death with in 48 hours (11th day) and poor yield of AAF (6 ml/embryo). In contrast the virus inoculated in 10 days old embryos, induced embryonic death at 12 days of age and resulted an average 8 ml of AAF/embryo. Similar observations were recorded by Yaqub *et al.* (1996) during growth of AIV-H₇N₃ in the embryos of different ages. The AAF when mixed with 5% washed chicken erythrocytes, caused agglutination. Due to hem-agglutinin, AIV are capable to agglutinate RBCs of different mammalian and avian species. This agglutination results from adsorption of virus particle to the mucoprotein receptors on the surface of RBCs (Buxton and Fraiser, 1977). The bird flu virus was confirmed as H₅N₁ using hem-agglutination inhibition test and specific antiserum as well as by RT-PCR using H₅ and N₁ specific primers (Broomand *et al.*, 2005). Serial passages of the bird flu virus in the embryo improved its hem-agglutination potential as well as its infectivity titer (Fig. 1). It could be due to the development of new mutants having more potential of hem-agglutination and more sensitivity of replication in embryonic

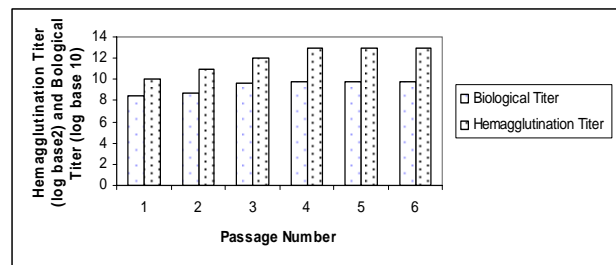


Fig. 1. Effect of virus passage through chick embryo on its hem-agglutination and biological titer. The bird flu virus was passed through chicken embryo six times and its hem-agglutination titer and biological titer (Egg Infective Dose 50) after every passage was determined.

cells. The bird flu virus is highly pathogenic and has a high risk of mutation. This could be plausible reason that its live attenuated vaccines cannot be

prepared. Its subunit or recombinant or chemically inactivated vaccines are available (Beard *et al.*, 1992). The inactivants such as formaldehyde and binary ethylenimine inactivated the bird flu virus effectively. Formaldehyde at rate of 0.12 percent concentration inactivated the virus at 25°C within 24 hours post incubation. It was further observed that the 0.12% formaldehyde inactivated the virus 8 hours post-interaction time at 37°C. Formaldehyde mitigated the HA activity of the virus as recorded by King (1991). BEI at concentration ≥ 5 mM inactivated the virus effectively 24 hours post incubation at 37°C. It was further recorded that the same concentration of BEI did not inactivate the virus at 4°C even 32 hours post-interaction time but inactivated the virus at ambient temperature (25°C) and at 37°C, effectively. However, inactivation at 25°C was preferred because higher temperature (37°C) may degrade the immunogen. BEI based inactivation has minimum effect on HA activity of the virus. Sodium thiosulphate at 2% can completely neutralize the residual BEI. Formalin influences the antigenicity of the virus (Mark and Tauraso, 1970), and is carcinogenic in nature hence BEI is preferred to inactivate the vaccinal virus (Buonavoglia *et al.*, 1988). In the present study the safety test in chicken embryos showed that the BEI is an effective virucidal.

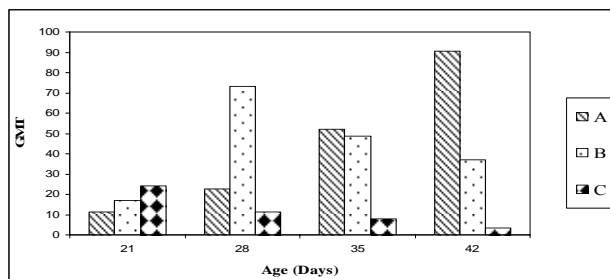


Fig. 2. Effect of adjuvant on the efficacy of bird flu (H_5N_1) virus vaccine. The birds were primed at 10 days of age with oil based (A), gel based (B) and non adjuvant (C) bird flu vaccine. Anti-bird flu virus HI antibody titer was determined at 21, 28, 35 and 42nd days of age. GMT: Geometric Mean Titer.

Adjuvant play major role in induction of antibody response of birds to the vaccines (Fig. 2). In birds primed with non-adjuvant vaccine showed

high level of anti-AIV-HI antibodies on 11 days post priming and decline upto undetectable level on 32 days post priming. Non adjuvant inactivated AI virus vaccines are presumably absorbed from inoculation site with in short period of time of administration without providing a suitable stimulus to immuno-competent cells. These antigens might have been processed by antigen presenting cells (APC) within few days resulting low thresh hold and short life of antibody titer. Similar observations were recorded by Yaqub *et al.* (1996) and Ruat *et al.* (2008). Such birds when challenged with virulent virus, showed high morbidity and mortality. It is possible to slow the rate of antigen elimination by mixing it with an insoluble adjuvant. Adjuvant containing vaccines form a depot at inoculation site and enhance the antibody production (Ninomiya *et al.*, 2007). As there is no lymphoid system in birds (Jeurissen *et al.*, 1988) and adjuvant containing vaccines cause irritation, recruit immunocompetent cells (lymphocytes and APC) at the injection site (Unanue, 1984). These cells phagocytose, process and present antigen on their surface in association with self immune associated (Ia) antigen. The thymus dependent lymphocytes (T-cells) can only recognize the antigen when presented on the surface of APC with Ia antigen (Vanio *et al.*, 1988). These antigen stimulate T-cells, transform into lymphoblasts and populate in different primary and secondary lymphoid organs such as spleen, MALT (mucosal associated lymphoid tissue) etc. (Vanio *et al.*, 1988). These cells secrete lymphokines, which potentiate the activity of bursal dependent lymphocytes (B-cells) and induce cell mediated immunity (Vanio and Ratcliffe, 1984). The cell mediated immunity is important for control of virus infections (Kaufman, 1988). This could be a logical reason of high level of HI antibodies in sera of birds vaccinated with adjuvant containing vaccines. Aluminium hydroxide is produced in the form of a colloidal suspension to which the antigenic material adsorbed and they produce a small local granuloma on inoculation site (Tizzard, 1996). Aluminium hydroxide gel based bird flu vaccines when injected into different groups of birds at 10 days of age, released the gel adsorbed antigen, produced antibodies that reached to peak at 28th day of age and last immuno-competent cells were transformed

into plasma cells that continue production of the antibodies 18 days post priming. Later on due to excessive decay of antibodies, the titer declined. These antibodies appeared faster and latter on disappeared at the same rate (Yaqub, 1996). Boosting of birds, primed with gel based bird flu virus vaccines improved the production of antibody titer. Same results were recorded by Philipa *et al.* (2005) when they were working on alum precipitated vaccines and the birds which were primed, showed low antibody level as compared to those which were vaccinated two times (boosted) at 2 weeks interval. The aluminum salts especially aluminum hydroxide is extensively used in microbial vaccines for veterinary and the only adjuvant approved for human use (East *et al.*, 1992).

Birds primed with oil based H₅N₁ virus vaccine at 10 days of age induced high and consistent but increasing trend of anti-AIV-HI antibody titer till 42 days of bird's age. Similar information was recorded by Stone (1987) who observed that oil based vaccines (AIV and NDV) induce the highest and the most consistent antibody response in the vaccinated birds. The oil adjuvant are readily adapted to many poultry disease antigens and are widely used in water-in-oil emulsion vaccines. Oil adjuvant is costly but gives prolong immunity. The adjuvant may consist of light mineral oil containing one or more emulsifiers (Stone, 1987). The oil phase of the vaccine causes a noticeable granuloma on account of irritation at the site of inoculation (Ahmad *et al.*, 1974). This irritation recruits APCs and immuno-competent cells. The oils release antigen slowly over a long period of time and hence prolong duration of the immunity. Boosting of the birds primed with oil based bird flu virus vaccine showed un-detectable boosting effect (Fig. 3). This could be due to increasing trend of antibody in the birds primed with oil based vaccine. This could be due to auto-boosting effect of oil based vaccines. Avian influenza is controlled with adjuvant inactivated AIV vaccines (Naeem and Hussain, 1995; Yaqub *et al.*, 1996; Muhammad *et al.*, 1997). Outbreaks due to AIV (H₇N₃) are controlled with inactivated monovalent adjuvant containing vaccines (Naeem *et al.*, 1999; Yaqub *et al.*, 1996). Adjuvants are essential if long term immunity is to be established (Ruat *et al.*,

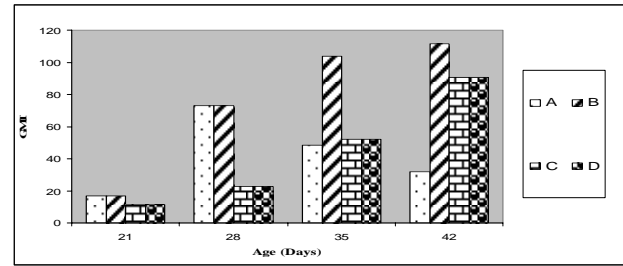


Fig. 3: Effect of boosting on the efficacy of bird flu (H₅N₁) Vaccine. The broilers (10 days of age) were divided into 4 groups. Birds of group 1st and 2nd were primed (P) with gel based and 3rd and 4th with oil based bird flu vaccine. Birds of group 2nd were boosted (B) with gel based and birds of group 4th with oil based bird flu vaccine at 28th days of age. The anti-bird flu virus HI antibody titer was determined at 21, 28, 35 and 42nd days of age. A: Priming with gel based vaccine, B: Priming and boosting with gel based vaccine, C: Priming with oil based vaccine, D: Priming and boosting with oil based vaccine.

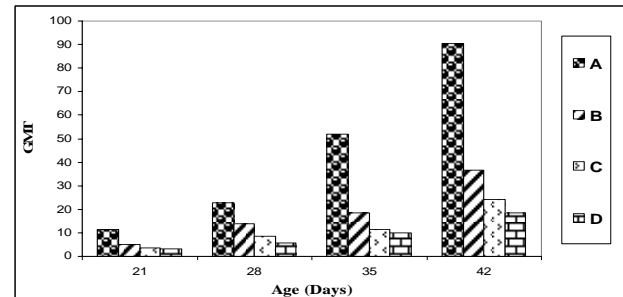


Fig. 4: Effect of biological titer on efficacy of bird flu virus (H₅N₁) vaccine. The birds were primed at 10 days of age with oil based bird flu vaccine of different biological titer. The vaccines were labeled as A, B, C and D containing 10⁻⁹, 10⁻⁸, 10⁻⁷ and 10⁻⁶ units of EID₅₀ (biological titer) respectively and anti-bird flu virus HI antibody titer was determined at 21, 28, 35 and 42nd days of age. GMT Geometric Mean Titer

2008). The immune response, being antigen driven, responds to the presence of antigen and terminates the response once antigen is eliminated (Pushko *et al.*, 2007). The aluminum hydroxide and oils are traditional adjuvants but are still in use and playing a role as the base of vaccine formulation because aluminum hydroxide is least toxic for

tissue, less irritant and adsorbs the immunogen. Other adjuvants such as mineral salt, oils, hydrophilic and hydrophobic block polymers, hydrocarbons, surface active agents, liposomal membranes, lipopolysaccharides etc, are also useful (Dalsgaard, 1987).

AI virus vaccines containing decreasing infectivity titer (10^{-9} , 10^{-8} , 10^{-7} and 10^{-6}) correspondingly induced decreasing anti-AIV-HI antibody titer in the vaccinated broilers (Fig 4). It is worth mentioning that serial passages of the virus through chicken embryo tremendously decreased its antigenicity (Fig. 5). AI virus vaccine prepared from passage number 5 or thereafter did not induce detectable level of anti-AIV-HI antibodies. It could be due to rapid development of its mutants in AI suspension on subsequent passages. The preservatives (thiomersal sodium) in the vaccine had undetectable effect on its efficacy.

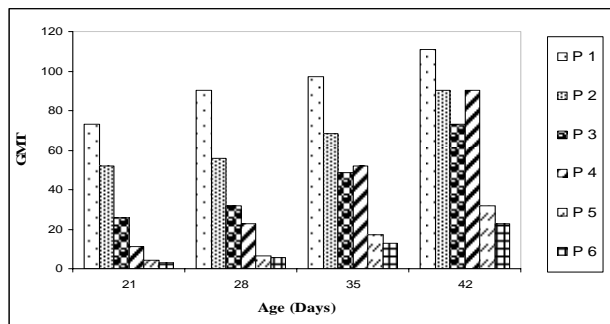


Fig. 5. Effect of virus passage through chicken embryo on efficacy bird flu (H_5N_1) vaccine. The birds were primed at 10 days of age with bird flu oil based vaccine containing virus of different passage. Anti-bird flu virus hem-agglutination inhibition (HI) antibody titer was determined at 21, 28, 35 and 42nd days of age. P: Passage number, GMT Geometric Mean Titer.

It is concluded from the results that higher the biological titer of the AI virus in the vaccine, higher the anti-AIV-HI antibody titer in the vaccinated birds. AI virus vaccine prepared from more than 4th passage showed undetectable level of antibodies. Adjuvant inactivated AI virus vaccines induced higher anti-AIV-HI antibody titer that could protect birds from bird flu over a long period of time thus providing a mean of disease control in high risk

areas. It means passage number and biological titer of AI virus, type of adjuvant in the vaccine and boosting of the birds are critical in process quality control factors affecting the efficacy of the AIV vaccines.

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