# Evaluation of inactivated AIV vaccines in conjunction with antiviral drugs in chickens challenged with Egyptian H5N1 HPAIV

Ali, A. Salama; El-Bakry, M. Ismail; Fatma, M. Abdallah and Gemelat, K. Farag

Virology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt
mm.fatma @yahoo.com

**Abstract:** The goal of the presented research was to synthesis focuses on the effectiveness of vaccines and antiviral drugs in the prevention and treatment of avian influenza virus (AIV) in chickens. Antibody responses, and virus shedding were evaluated after challenge with Egyptian H5N1 HPAIV (A/chicken/faquos/amn/2/2011 (H5N1)). The results revealed that, the antibody titers in sera of the broiler chickens vaccinated with AI H5N1 vaccine alone or in combination with potent neuraminidase inhibitor antiviral drugs (NAI) were higher than antibody titers in sera of the broiler chickens vaccinated with AI H5N2 vaccine alone or in combination with NAI antiviral drugs against AIV with significant difference ($p< 0.05$). Furthermore, NAI antiviral drugs provided significant protection and reduction the duration and titer of virus shedding especially in vaccinated chickens. These investigations showed that NAI antiviral drugs used in conjunction with vaccination strategies in chicken farms reduced the risk of avian influenza virus.

[Ali, A. Salama; El-Bakry, M. Ismail; Fatma, M. Abdallah and Gemelat, K. Farag. **Evaluation of inactivated AIV vaccines in conjunction with antiviral drugs in chickens challenged with Egyptian H5N1 HPAIV.** *Life Sci J* 2013;10(1):1962-1968] (ISSN:1097-8135). http://www.lifesciencesite.com. 281

**Key Words**: HPAIV, H5N1, NAI, H5N2, broiler chickens, antiviral drugs, vaccines.

## 1. Introduction

Avian influenza (AI) is a contagious viral disease, worldwide in distribution caused by a single stranded, negative-sense RNA virus in the family Orthomyxoviridae, genus influenza virus A with the genome divided into eight gene segments**.** The surface is covered by two types of glycoprotein projections; rod shaped timers of haemagglutinin (HA) and mushroom shaped tetramers of neuraminidase (NA) **[1].** Influenza A virus is further categorized by serological reaction of the two surface glycoproteins into 16 different hemagglutinin (H1–16) and 9 different neuraminidase (N1–9) subtypes **[2].** Protection is primarily the result of humoral immune response against the hemagglutinin (HA), and secondarily against the neuraminidase **[3].** Avian influenza (AI) viruses vary in virulence either being of low or high pathogenicity **[4].** It affects the chickens of all ages with variable morbidity and mortality. With the HPAI viruses, morbidity and mortality rates are very high (50–89%) and can reach 100%in some flocks **[5].**

Vaccines have been used in AI control programs achieve one of three broad goals: (1) prevention, (2) management, or (3) eradication. The best protection is produced from the humoral response against the hemagglutinin (HA) protein **[6].** Vaccination has been shown to increase resistance to field challenge and reduce virus shedding levels in vaccinated birds and subsequently reduce transmission **[7].** However, vaccines have not been a universal solution in the control of AIV in the field **[3]** and as described previously, M2 ion channel inhibitors[ amantadine and rimantadine] & neuraminidase inhibitors [ oseltamivir (Tamiflu®) and zanamivir (Relenza®)] have comparable effectiveness in the prevention and treatment of influenza **[8].** Thus, in the present study, we investigated the protective efficacy of available inactivated oil emulsion whole-virus H5 ( H5N1 & H5N2 ) influenza vaccines against Egyptian H5N1 HPAIV in conjunction with neuraminidase inhibitors (oseltamivir® & zanamivir®) as anti-influenza A virus drug therapies in chickens.

## 2. Material and methods

**Chickens:**

One hundred and eighty one day old commercial Hubbard chicks were purchased from (Dakahlia Poultry Company). The chicks were reared in isolation cabinets with continuous light exposure and were individually identified by means of a numbered wing tag. Chickens were fed with water and feed *ad libitum* daily with commercial compound suitable for their age.

**Vaccines and vaccine administration**:

a. The inactivated oil emulsion reassortant avian influenza vaccine (H5N1 subtype, Re-1 strain), China. The vaccine strain is (H5N1 subtype, Egy/PR8-1 strain).

b. Volvac® AI KV avian influenza killed virus. The inactivated oil emulsion LPAI H5N2 vaccine. The vaccine strain is H5N2, A/ chicken / Mexico / 322/94/CPA.

The vaccine dose of the two vaccines under study was 0.5 ml /bird, it were inoculated in the lower (dorsal) part of the neck by the subcutaneous route.

**Potent neuraminidase inhibitor antiviral drugs:**

**a.** Oseltamivir capsule (Tamiflu®) is 75 mg/capsule manufactured by the Nile Company for pharmaceutical and chemicals industries, Cairo. It was administrated orally as 10 mg/bird for three successive days.

**b.** Zanamivir powder (relenza®) for inhalation, each blister contains zanamivir 5 mg manufactured by the Glaxowellcome. It was administrated by inhalation as 10 mg/bird for three successive days.

**Challenge avian influenza virus (AIV):**

Avian influenza virus (A/chicken/faquos/amn /2/2011 (H5N1)) with accession number JQ627585 was kindly supplied by Virology Department, Faculty of Veterinary Medicine, Zagazig University.

**Experimental design:**

One hundred and eighty day-old broiler chicks were randomly divided into 18groups, each group containing 10 chicks. Six groups (I-VI) of chicks were vaccinated with H5N1 and other six groups (VII-XII) were vaccinated H5N2 AI vaccines at 7days-old via subcutaneous injection with dose 0.5 ml / chick. Groups (I- XII) were treated differentially either with oseltamivir or zanamivir (10 mg/bird for three successive days) at 24 and /or 48hrs post challenge. The groups of (XIII-XVI) were administered oseltamivir and zanamivir (10 mg/bird for three successive days) without vaccination at 24 and /or 48hrs post challenge. The chickens of group XVII&XVIII were left as control groups. All chickens were challenged intranasally with 0.1 ml viral suspension containing $10^6EID_{50}$/ml of the challenge locally isolated AIV strain (A/chicken/faquos/amn/2/2011 (H5N1)) after three weeks post vaccination. Whereas chickens of (VI and XII) were vaccinated and remained unchallenged & untreated .Thereafter, the experimental chickens were observed daily over a period of two weeks & the clinical signs and the mortality rate were recorded. In order to monitor virus shedding after challenge, Oropharyngeal and cloacal swabs were collected at 3rd, 5th, 7th, and 9th day post challenge for analysis of viral shedding. Swab samples were subjected to RRT-PCR analyses and at the same time they were processed for virus titration in 10-day-old SPF - ECEs. To determine the level of specific antibodies against AIV by Hemagglutination inhibition (HI) test and Commercially available H5 avian influenza (AIV) antibody ELISA test kit, blood samples were taken at 7 days ( pre vaccination), 14 & 21 day post vaccination, and 14 day post challenge.

**Sampling:**

Chickens were observed daily for clinical signs throughout the duration of the study. Following challenge, oropharyngeal and cloacal swabs were processed for attempted virus isolation in SPF-ECEs and were analyzed by RRT-PCR. After collection, oropharyngeal and cloacal samples were placed in 1 ml phosphate buffered saline (PBS). 100 µL from each sample were used for RNA extraction. RNA was analyzed by real-time RT-PCR. The remaining sample was mixed with an equal volume of PBS containing penicillin (2000 IU/ml), streptomycin (2 mg/ml), gentamicin (0.05 g/ml) and mycostatin (1000 IU/ml) for virus isolation attempts. Also blood samples were collected from wing vein and kept in a slope position at 4°C overnight. Sera were then separated by centrifugation at 3000 rpm for 10 minutes and stored at -20°C. Sera were inactivated at 56°C for 30 minutes before testing **[9].**

**Reference AIV antiserum:**

Anti-avian influenza hyper immune serum against H5N1 AIV was kindly provided by Virology Department, Veterinary Medicine, Zagazig University.

**Washed Chicken red blood cells (RBCs):**

Blood samples were collected from wing veins of 2 – 3 apparently healthy of 4-6 weeks old chickens. Blood was received in sterile tubes containing 4% sodium citrate solution, and was subjected to three successive washing cycles by centrifugation at 1200 rpm for 10 minutes using PBS. For haemagglutination inhibition test (HI) test, the RBCs were used as 1 % suspension in PBS **[9].**

**Hemagglutination inhibition (HI) test:**

Hemagglutination units (HAU) of the H5N1 AIV were determined before each test using twofold dilutions. Sera were serially diluted twofold in 50 µl PBS, and 4 HAU of H5N1 were used in 50 µl. The contents of each well were gently mixed with a micropipettor and the plates were incubated for 30 min at room temperature. Finally, 50 µl of a 1 % chicken erythrocyte suspension was added to each well. The highest serum dilution capable of preventing hemagglutination was scored as the HI titer. The test was applied to quantify AIV antibodies in chicken sera and the data were reported as $\log_2$ titer according to OIE Manual[10]**.**

**Enzyme-Linked Immunosorbent Assay (ELISA):**

Commercially available H5 avian influenza (AIV) antibody ELISA test kit (ProFLOK® PLUS, Synbiotics Corporation, San Diego, CA, USA) was used under the manufacturer's instructions. Optical density values were read at 450 nm using an ELISA reader (Behring EL311). The kits used for detection of antibodies to haemaggltinins (HA) of influenza A virus, H5 strain.

**Specific pathogen free embryonated chicken eggs (SPF – ECEs):**

Eleven day old SPF-ECEs were purchased from poultry farm at Qom Osheem- Al Fayoum, Egypt.

**Avian influenza virus titration in (SPF – ECE):**

Virus containing oropharyngeal and cloacal swab samples were titrated and expressed as the 50% egg infectious dose ($EID_{50}$) using SPF–ECEs as previously described **[11].** Briefly, 200µl of each dilution (ten fold serial dilution) of swab samples suspended in PBS was inoculated into five10-day-old SPF–ECEs and incubated for 5 days or until death of the embryo. The allantoic fluids were collected and subjected for the hemagglutination activity . Titration was applied to quantify AIV in swabs and the data were reported as $\log_{10}EID_{50}$/ml according to Reed & Muench[12]

**Real-time reverse transcriptase polymerase chain reaction (RRT-PCR):**

For quantitation, swab samples were run together with known amounts of control viral RNA. To prepare control RNA, the reference virus used in this study was titrated using SPF-ECEs as described above and RNA was extracted from serially diluted virus ($10^4$–$10^8$ $EID_{50}$/ml). Standard curves were generated with this control viral RNA.

Sequences of the primer and hydrolysis probe sets specific for the H5 gene **(Table 1)** has been previously described by Spackman *et al.* [13].

**Table 1. primer and hydrolysis probe sequences used for TaqMan RRT-PCR*(Metabion Company).***

| | |
|---|---|
| H5+ 1456 | ACG TAT GAC TAT CCA CAA TAC TCA G |
| H5 - 1685 | AGA CCA GCT ACC ATG ATT GC |
| H5+ 1637 | FAM-TCA ACA GTG GCG AGT TCC CTA GCA-TAMRA |

The probe was labeled at the 5′ end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3′ end with the 6-carboxytetramethylrhodamine (TAMRA) quencher dye.

**Extraction of the RNA using QIAamp viral RNA mini kit (QIAGEN, Valencia, Calif, USA):**

Ribonucleic acid was extracted using the RNeasy mini kit (Qiagen Inc., Valencia, CA) from fluid containing swabs following the instructions of the manufacturer. Briefly, 500µL of swab fluid was mixed with 500 µL of the kit-supplied RLT Buffer and the entire sample was applied to the RNeasy spin column. The column was washed with buffers and then RNA was eluted in 50 µL of nuclease free water. 5µL per RRT-PCR reaction was used as a template.

**One step RRT-PCR using TaqMan probe:**

One-tube RRT-PCR was performed using the Qiagen one-step RRT-PCR kit in a 50µl reaction mixture containing 25 µL of the kit-supplied mix and 0.5 µL from 30 pmol of each primer, 0. 5 µL from H5 probe 50 pmol, 0. 5 µL from Access Quick RT-Enzyme and 18 µL nuclease free water and 5 µL of RNA that amplified using Stratagen PCR machine. The RT-PCR program consisted of 30 min at 50°C and 10 min at 95°C and a three-step cycling protocol was used as 95°C for 30 s, 50°C for 1min and 72°C for 30 Sec for 35 cycles. Fluorescence data were acquired at the end of each annealing step. The result of the avian influenza H5N1 one step real-time RT-PCR assay showed positive amplification signals with FAM dye for the original isolate and the first four dilutions from $10^{-4}$ to $10^{-8}$. Since samples with threshold cycle (Ct) values lower than 35 were counted as indicative of the presence of virus.

**Statistical Analysis:**

The egg infective dose fifty ($\log_{10}EID_{50}$ /ml) of virus shed from cloaca and oropharynx in each group was determined for consecutive days postchallenge and compared between groups by ANOVA.The $\text{logarithm}_2$ mean titre ($\log_2$) of H5 HI and ELISA antibody responses to H5N1 HPAIV were compared within and between groups postvaccination and postchallenge by ANOVA.

## 3. Result

- **Serological analyses:**

The goal of the presented research was to evaluate immunogenicity of the commercially available inactivated influenza vaccines either alone or in combination with NAI antiviral drugs (oseltamivir and/or zanamivir) of (Gp I - XII) and the efficacy of NAI antiviral drugs (oseltamivir and/or zanamivir) alone without vaccination of (Gp XIII- XVI) against the highly pathogenic avian influenza virus (A/chicken/faquos/amn /2/2011(H5N1)) in broiler chickens. All pre vaccination sera were negative for H5 antibodies in the HI and ELISA test.

The mean HI titers were 7.1 $\log_2$ of H5N1 inactivated vaccine immunized chickens at 21days post vaccination were higher than the mean HI titers in sera of chickens at 14 days post vaccination of 3.6 $\log_2$. While, the mean HI titers were 6.5 $\log_2$ of H5N2 inactivated vaccine immunized chickens at 21days post vaccination were higher than the mean HI titers in sera of chickens at 14 days post vaccination of 3.1 $\log_2$ **(Table 2).** A marked increase

of H5-specific antibody HI titers (7.8 & 7.4 $log_2$) at 14 days post challenge were observed in surviving chickens of the (GP I & VII) immunized with H5N1 vaccine as well as H5N2 vaccine, respectively **.** Mean HI titers in sera were 7.9& 7.6 $log_2$ of chickens immunized with H5N1& H5N2 vaccines in combination with NAI drugs in (Gp II-V& GPVIII-XI) , respectively **.** At the end of the experimental trial, the uninfected vaccinated groups (GPVI&XII) showed a slight increase in HI titers (7.2& 6.0 to 7.4& 6.3), respectively when vaccinated with H5N1 &H5N2 vaccines

The data were analyzed by HI test **(Table 2)**, and reconfirmed by commercially available H5 avian influenza (AIV) antibody ELISA test kit.

The mean ELISA titers (1964) of H5N1 inactivated vaccine immunized chickens at 21days post vaccination were higher than the mean ELISA titers in sera of chickens at 14 days post vaccination (1633). While, the mean ELISA titers (1775 ) of H5N2 inactivated vaccine immunized chickens at 21days post vaccination were higher than the mean ELISA titers in sera of chickens at 14 days post vaccination of (1476) **.** A marked increase of H5-specific antibody ELISA titers (2522 & 2462) at 14 days post challenge was observed in surviving chickens of the (GP I & VII) immunized with H5N1 vaccine as well as H5N2 vaccine, respectively **.** Mean ELISA titers in sera of chickens immunized with H5N1& H5N2 vaccines in combination with NAI drugs increased slightly in (GpII-V& GPVIII- XI) was 2597& 2520 , respectively **(Table 2).** At the end of the experimental trial, the uninfected vaccinated groups (GPVI&XII) showed a slight increase in ELISA titers (1743& 1442 to 1939&1631), respectively when vaccinated with H5N1 &H5N2 vaccines.

- **Analyses of viral shedding**

During the period of 14 days, the challenged chickens were observed, oropharyngeal and cloacal swabs were collected at 3rd, 5th, 7th and 9th days post challenge to reveal possible virus shedding.

The highest value of mean $log_{10}EID_{50}$/ml of recovered AI challenge virus from all oropharyngeal swabs was frequently recorded at 3rd day post challenge. In (GPI-V) was $10^{5.2}$, $10^{4.6}$, $10^{5.0}$, $10^{4.3}$, and $10^{5.4}$ $log_{10}$ $EID_{50}$/ml. whereas in (GPVII-XI), the mean $log_{10}$ $EID_{50}$/ml was $10^{5.7}$, $10^{4.9}$, $10^{5.3}$, $10^{4.7}$, and $10^{5.6}$.The mean $log_{10}$ $EID_{50}$/ml were $10^{5.7}$, $10^{5.9}$, $10^{5.3}$, and $10^{5.7}$ in groups XIII- XVI. During the entire period of observation, the all non vaccinated chickens (GPXVII) were died at the 2nd day post challenge and the AIV was reisolated in high titers $10^{6.2}$ and $10^{5.7}log_{10}$ $EID_{50}$, respectively via oropharyngeal and cloacal swabs . The AI challenge virus was recovered less frequently and the viral titers were observed rather low from all cloacal samples in the experimental study than oropharyngeal swabs as shown in **(Table 3).** Recovered AI challenge virus titers were considerably reduced at 5th day post challenge. The AI virus was also detected from the oropharyngeal and cloacal swabs of chickens at day 7 with low titer rather than at 5 th days. While no virus was detected from any chickens on day 9th post challenge **.** However, (GPVI, XII, and XVIII), all chickens survived and no symptoms of disease were observed. Also, Virus could no longer be isolated from the pooled oropharyngeal and cloacal swabs of these chickens.

Oropharyngeal and cloacal swab samples in which AIV was titrated in SPF-ECEs were subjected to TaqMan RRT-PCR. Since the mean threshold cycle (Ct) values were observed ranged between 24.0 - 35.0, all groups shed virus at a comparable level.The RRT-PCR was performed to quantify the titer and variations in AIV RNA levels over time in swabs of challenged chickens. High loads of viral RNA were frequently detected at 3rd day post challenge **(Table 3).** The mean $C_t$ in (GPI-V) were 29.3, 31.6, 30.7, 31, and 30 whereas the mean $C_t$ in (GPVII-XI) were 28.7, 29.8, 29, 29.5, and 29.01 In addition to, the mean $C_t$ were 28.3, 27, 28.07, and 26.3 in groups XIII- XVI. The viral load continued to decrease at 5th, 7th day post challenge. However, AIV RNA levels were dropped at 9 th day post challenge **.** However, (GPVI, XII, and XVIII), AIV could not be detectable. Also, Viral RNA could not be detectable in all the pooled cloacal swabs as shown in **(Table 3).**

**Table 2. Immune response of broiler chickens post vaccination with inactivated AIV vaccines and administration with NAI drugs using HI and ELISA.**

| Groups | 14 day post vaccination | | 21day post vaccination | | 14 day post challenge | |
|---|---|---|---|---|---|---|
| | HI | ELISA | HI | ELISA | HI | ELISA |
| I | 3.5± 0.28 | 1633± 37.52 | 7.0± 0.12 | 1964± 25.98 | 7.8± 0.17 | 2522± 11.54 |
| II | 3.5± 0.11 | 1566± 25.98 | 7.0± 0.12 | 1990± 11.54 | 7.9± 0.17 | 2577± 23.09 |
| III | 3.6± 0.12 | 1462± 23.09 | 7.1± 0.12 | 1943± 25.98 | 7.7± 0.17 | 2686± 23.09 |
| IV | 3.9± 0.11 | 1544± 11.54 | 7.4± 0.16 | 1934± 25.98 | 7.9± 0.17 | 2554± 17.32 |
| V | 3.4± 015 | 1484± 17.32 | 7.0± 0.12 | 1989± 11.54 | 7.8± 0.16 | 2574± 17.32 |
| VI | 3.0± 0.10 | 1366± 17.32 | 7.2± 0.16 | 1743± 25.98 | 7.4± 0.16 | 1939± 23.09 |
| VII | 3.4± 0.13 | 1476± 11.54 | 6.5± 015 | 1775± 11.54 | 7.4± 0.16 | 2462± 11.54 |
| VIII | 3.2± 0.11 | 1351± 23.09 | 6..7± 0.10 | 1739± 25.98 | 7.6± 0.16 | 2578± 23.09 |

| | | | | | | |
|---|---|---|---|---|---|---|
| IX | 2.9± 0.16 | 1306± 11.54 | 6.5± 0.10 | 1668± 11.54 | 7.5± 0.16 | 2490± 17.32 |
| X | 3.0± 0.17 | 1390± 17.32 | 6.3± 015 | 1624± 25.98 | 7.6± 0.12 | 2512± 23.09 |
| XI | 3.0± 0.21 | 1310± 22.80 | 6.5± 0.10 | 1569± 17.32 | 7.4± 0.12 | 2500± 11.54 |
| XII | 3.0± 0.12 | 1105± 22.80 | 6.0± 015 | 1442± 17.32 | 6.3± 0.12 | 1631± 23.09 |
| XIII | - | 207± 1.15 | - | 272± 1.57 | 4.2± 0.05 | 708± 1.57 |
| XIV | - | 102± 1.15 | - | 256± 1.57 | 4.6± 0.12 | 939± 1.15 |
| XV | - | 302± 1.57 | - | 308± 1.15 | 3.7± 0.22 | 801± 1.57 |
| XVI | - | 207± 1.57 | - | 256± 1.57 | 4.5± 0.16 | 708± 1.15 |
| XVII | - | 117± 1.15 | - | 200± 1.15 | 4.0± 0.17 | 649± 1.57 |
| XVIII | - | 132± 1.57 | - | 184± 1.57 | - | 184± 1.15 |

**Table 3. The efficacy of inactivated AIV vaccines and NAI drugs against challenging H5N1 HPAIV.**

| Titration of excreted H5N1HPAIVafter challenge in SPF-ECEs& RRT-PCR | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | 3 days post challenge | | | 5 days post challenge | | | 7 days post challenge | | | 9 days post challenge | | |
| | $log_{10}EID_{50}$ | | Ct[c] | $log_{10}EID_{50}$ | | Ct | $log_{10}EID_{50}$ | | Ct | $log_{10}EID_{50}$ | | Ct |
| | Op[a] | Cl[b] | | Op | Cl | | Op | Cl | | Op | Cl | |
| I | 5.2± 0.17 | 4.6± 0.15 | 29.3± 1.57 | 4.0± 0.21 | 2.5± 0.05 | 31.73± 0.31 | 2.0± 0.13 | 1.0± 0.18 | 33.34± 0.98 | - | - | 34.62± 2.87 |
| II | 4.6± 0.15 | 4.0± 0.18 | 31.6± 2.12 | 3.5± 0.11 | 2.8± 0.21 | 32.82± 0.18 | 1.8± 0.21 | 1.0± 0.07 | 34.15± 1.02 | - | - | 35.0± 1.89 |
| III | 5.0± 0.21 | 4.3± 0.21 | 30.7± 0.98 | 3.5± 0.11 | 2.9± 0.05 | 32.0± 0.31 | 1.8± 0.31 | 1.0± 0.13 | 33.18± 0.57 | - | - | 34.05± 2.87 |
| IV | 4.3± 0.15 | 3.9± 0.15 | 31.0± 1.64 | 3.2± 0.11 | 2.5± 0.07 | 32.1± 0.18 | 1.5± 0.13 | 1.0± 0.07 | 33.34± 1.47 | - | - | 34.62± 1.89 |
| V | 5.4± 0.21 | 4.0± 0.18 | 30.0± 2.12 | 4.0± 0.21 | 3.0± 0.21 | 32.01± 0.18 | - | - | 33.97± 1.02 | - | - | 34.06± 2.87 |
| VI | - | - | - | - | - | - | - | - | - | - | - | - |
| VII | 5.7± 0.31 | 4..4± 0.21 | 28.7± 2.12 | 4.3± 0.31 | 3.3± 0.11 | 30.21± 0.31 | 2.5± 0.05 | 1.0± 0.31 | 32.0± 0.98 | - | - | 33.7± 0.57 |
| VIII | 4.9± 0.07 | 4.4± 0.21 | 29.8± 1.57 | 4.0± 0.07 | 3.0± 0.11 | 32.85± 0.18 | 2.0± 0.21 | 1.0± 0.13 | 33.92± 1.02 | - | - | 34.62± 1.49 |
| IX | 5.3± 0.31 | 4.6± 0.18 | 29.0± 1.57 | 3.5± 0.18 | 2.9± 0.21 | 31.5± 0.18 | - | - | 32.44± 1.47 | - | - | 34.0± 0.57 |
| X | 4.7± 0.18 | 3.9± 0.18 | 29.5± 1.57 | 4.0± 0.31 | 3.0± 0.11 | 32.12± 0.18 | 2.6± 0.05 | 1.5± 0.13 | 33.89± 1.47 | - | - | 34.17± 0.57 |
| XI | 5.6± 0.07 | 4.4± 0.21 | 29.01± 1.57 | 4.0± 0.18 | 3.0± 0.21 | 31.44± 0.31 | 2.5± 0.31 | 1.5± 0.13 | 32.7± 0.98 | - | - | 33.54± 1.49 |
| XII | - | - | - | - | - | - | - | - | - | - | - | - |
| XIII | 5.7± 0.31 | 4.2± 0.21 | 29.3± 1.64 | 4.1± 0.07 | 3.2± 0.11 | 30.32± 0.31 | 3.0± 0.21 | 1.5± 0.13 | 31.35± 0.57 | - | - | 33.0± 0.84 |
| XIV | 5.9± 0.31 | 4.5± 0.21 | 27.0± 2.12 | 4.0± 0.13 | 2.9± 0.21 | 29.0± 0.18 | 2.5± 0.05 | 1.8± 0.18 | 30.0± 1.02 | - | - | 32.0± 0.57 |
| XV | 5.3± 0.18 | 4.0± 0.21 | 28.07± 1.64 | 3.9± 0.31 | 2.9± 0.05 | 30.45± 1.64 | 2.0± 0.07 | 1.0± 0.31 | 32.0± 0.98 | - | - | 33.0± 0.97 |
| XVI | 5.7± 0.31 | 4.3± 0.21 | 26.3± 2.12 | 4.0± 0.13 | 3.1± 0.11 | 28.0± 0.07 | 2.0± 0.31 | 1.0± 0.18 | 30.0± 0.57 | - | - | 32.0± 0.57 |
| XVII | 6.2± 0.18 | 5.7± 0.18 | 24.0± 1.64 | 4.5± 0.21 | - | - | - | - | - | - | - | - |
| XVIII | - | - | - | - | - | - | - | - | - | - | - | - |

[a] Op= oropharyngeal swab [b] Cl= cloacal swab [c] Ct= threshold cycle

## 4. Discussion

The objective of this study was to evaluate in a comparative setting the protective efficacy of available inactivated H5N1 and H5N2 AIV vaccines either alone or in combination with NAI antiviral drugs (oseltamivir® and/or zanamivir®) in chickens against H5N1 HPAIV.

Hemagglutination inhibition test and enzyme-linked immunosorbent assay were used to evaluate immunogenicity of the available inactivated avian influenza vaccines either alone or in combination with NAI antiviral drugs .The mean HI titer of inactivated vaccines in immunized chickens at 21day post vaccination were higher than the mean HI titer in sera of chickens at 14 day post vaccination. These data were analyzed by HI test, and reconfirmed by available H5 avian influenza (AIV) antibody ELISA test kit as shown in **(Table2).** Previous studies had indicated that the vaccinated chickens could be completely protected from highly pathogenic AIV challenge when the antibody titers to the challenge virus equaled or were greater than $4log_2$ at three weeks after vaccination **[14].** A mean HI titer in the sera of broiler chickens vaccinated with AI H5N1 vaccine alone or in combination with NAI antiviral drugs was significantly higher ($P < .05$) than

antibody titers in sera of the broiler chickens vaccinated with AI H5N2 vaccine alone or in combination with NAI antiviral drugs. As previously mentioned by Tian *et al.*[14]**,** Most consistent reduction in respiratory shedding was afforded when the vaccine was more similar to the challenge virus. Significant increase of antibody titers were observed in vaccinated and treated chickens two weeks after challenge. This is in accordance with the other Studies **[16]** and Could possibly represent a lack of replication of the challenge virus in the surviving birds.

The efficacy evaluations have been based on a challenge study performed few weeks post vaccination. Oropharyngeal and cloacal swabs for virus titration were taken at the peak of replication, i.e. 3 day post challenge. As **Swayne and Halvorson, 2003** were taken Oropharyngeal and cloacal swabs at the peak of virus shedding, day 3 post-inoculation, for virus isolation attempts in 10 days embryonating chicken eggs .

After challenge , protective efficacy was evaluated based on clinical observations and the magnitude of viral shedding. The challenge virus was highly pathogenic for the control group as causing 100 % mortalities within 48 hours .Challenge of other groups showed difference in immune response and protective efficacy of vaccines and drugs. Also our results were in agreement with Villegas & Swayne [17] who reported that all unvaccinated challenged birds died within 2 days, whereas 90% and 100% of chickens vaccinated with H5N1and H5N2 respectively were protected against morbidity and mortality.

The data obtained from this study show a significant increase of the survival rate in chickens, a significant reduction in sick /dead chickens, and a significant reduction in the number of chickens shedding the challenge virus , which results in an overall reduction of shedding leaves in vaccinated treated chickens **(data not shown)** is consistent with previous studies where chemotherapy may be useful in the treatment of a highly pathogenic influenza virus outbreak in humans or other animals when used in combination with vaccine**[19,20].**

The quantitation of virus shed from infected chickens was done by titration in ECE-SPFs and expressed as 50% embryo infective dose **[18].** RRT-PCR also has been successfully applied in the quantitation of AIV samples and is a reliable alternative to virus isolation in ECEs **[13].**

The AI challenge virus was recovered less frequently and the viral titers were observed rather low for all cloacal samples in the experimental study than oropharyngeal swabs. **Tian and his colleague,2005** recorded the viral titers shed from the trachea were higher than from the cloaca and is believed to be related to a shift in replication efficiency for the upper respiratory tract after infection with challenge virus **[14, 21].**

The AI challenge virus shedding peaked at day 3 post challenge, which might be related to reisolation of inoculum, and remained significantly lower ($P < .05$) in consecutive days postchallenge. while no virus was detected in chickens on day 9$^{th}$ post challenge by titration in ECE-SPFs as reported by Webster *et al.* [19].Also, High loads of viral RNA were frequently detected at 3$^{rd}$ day post challenge and the viral load continued to decrease at 5$^{th}$, 7$^{th}$ day post challenge. However, AIV RNA levels were dropped at 9 $^{th}$ day post challenge.This is in accordance with other studies**[22,19]** that demonstrated, high titers of virus were detected in birds at 3 day post challenge, However, the titer of virus decreased significantly and the number of virus positive birds also decreased at 7 day post challenge. By contrast, Viral RNA could not be detectable in all the pooled cloacal swabs of any challenged chickens in this experiment.The failure of detection of viral RNA in cloacal swabs in comparison to SPF-ECEs titration might relate to inhibitory substances present in fecal specimens that reduce or block PCR amplification and most commercial RNA extraction kits have limited capacity to remove inhibitors from these clinical samples **[18].**

## Conclusion

The serological response and protection percentage in vaccinated chickens were improved following administration of potent neuraminidase inhibitor antiviral drugs.Thus, chemotherapy may be useful in the treatment of a highly pathogenic influenza virus when used in combination with vaccine.

## References

1. Swayne, D.E., Halvorson D.A. 2003.Influenza. In: Saif, Y.M., Barnes, H.J., Fadly, A.M., Glisson, J.R., McDougald ,L.R. Diseases of poultry. Ames, IA, Iowa State University Press, p. 135–160.
2. Easterday, B.C., Hinshaw, V.S., Halvorson, D.A., 1997. Infuenza. In: Calnek, B.W., Barnes, H.J., Beard, C.W.,McDougald, L.R., Saif, Y.M. (Eds.), Diseases of Poultry 10th Edition, Iowa State University Press, Ames,IA, pp. 583-605.
3. Swayne,D.E. 2009. Avian influenza vaccines and therapies for poultry. Comparative Immunology, Microbiology and Infectious Diseases 32 , 351–363.
4. Perdue, M.L., Suarez, D.L., Swayne, D.E., 2000. Avian Infuenza in the 1990s. Poultry and Avian Biol. Rev., 10(in press).

5. Capua, I. F., Mutinelli, M.A., Bozza, C., Terregino, G. Cattoli, C., 2000. Highly pathogenic avian influenza (H7N1) in ostriches (Struthiocamelus). Avian Pathol., 29, 643–646.
6. Swayne, D.E., Akey, B. 2005. Avian influenza control strategies in the United States of America. In: Schrijver R.S., Koch, G., editors. Avian influenza. Prevention and control. Dordrecht: Springer; p. 113–30.
7. Swayne, D.E., Beck, J.R., Garcia, M., Stone, H.D. 1999. Influence of virus strain and antigen mass on efficacy of H5avian influenza inactivated vaccines. Avian Pathol., 28,245–55.
8. WHO.2004. Guidelines on the Use of Vaccines and Antiviral during Influenza Pandemics. Geneva, World Health Organization, (http://www.who.int/csr/resources/publications/influenza/1129_01_A.pdf, accessed 17, November 2005).
9. OIE Manual, 2005. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter, 2.7.12 .
10. OIE Manual, 2008. Avian influenza .In, Manual of Diagnostic tests and Vaccines for terrestial animals, OIE (Ed) .6th Edn. OIE, Paris, France.
11. OIE Manual, 2009. Manual of diagnostic tests and vaccines for terrestrial animals purtz, section 2-1. Chapter 2-1.
12. .Reed, L.J. , Muench, H. 1938. A simple method of estimating fifty percent end point. Am .J. Hyg. 27, 493 – 497.
13. Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L., Suarez, D.L. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology, 40(9), 3256-3260.
14. Tian ,G.,Zhang, S.,Li ,Y., Buc Zh, Liu, P., Zhoub ,J., Li Ch, Shi, J., Yua, K., Chen ,H . 2005. Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. Virol, 341, 153 – 162.
15. Swayne,D. E., Michael, L., Perdue, Joan R. Beck, Maricarmen Garcia ,David L. Suarez.2000.Vaccines protect chickens against H5 highly pathogenic avian infuenza in the face of genetic changes infield viruses over multiple years.Veterinary Microbiology. 74 ,165-172.
16. Ellis, T.M., Leung, C.Y., Chow, M.K., Bissett, L.A., Wong, W., Guan, Y., Peiris, M. 2004. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol. 33, 405–412.
17. Villegas, P., Swayne, D.E. 1998. Titration of biological suspensions. A Laboratory Manual for the Isolation and Identification of Avian Pathogens.fourth ed. American Association of Avian Pathologists,Kennett Square, PA, 248–254.
18. Das, A., Spackman, E., Mary, J., Pantin-Jackwood, David ,L. ,Suarez,1. 2009. Removal of real-time reverse transcription polymerase chain reaction(RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of Avian influenza virus by RT-PCR..J .Vet .Diagn .Invest 21,771–778 .
19. Webster,R. G., Webster, Y. Kawaoka , Bean, W. J. 1986 . Vaccination as a strategy to reduce the emergence of amantadine- and rimantadine-resistant strains of A/Chick/Pennsylvania/83 (H5N2) influenza virus. *Antimicrob. Chemother.* 18 (Supplement B), 157-164.
20. Lee, D.H., Lee, Y.N., Park, J.K., Yuk, S.S., Lee, J.W., Kim, J.I., Han, J.S., Lee, J.B., Park, S.Y., Choi, I.S., Song, C.S. 2011. Antiviral efficacy of oseltamivir against avian influenza virus in avian species. Avian Dis. 55(4):677-9.
21. Webster,R.G.,Guan,Y.,Peiris,M.,Chen,2006.H5N1 influenza contiuous to circulate and change. Microbe,1(12):559-565.
22. Lee, C.W., Suarez, D.L.2004. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J .Virol. Methods 119,151–8.

1/20/2013