

Comparative Immunological Study between the Efficacy of Bivalent Inactivated H5N1-ND and Baculovirus Expressed H5N1-ND Inactivated Vaccine (BEST) in Broiler Chickens

Raheel, I.A.R.¹, Abed, A.H.², Hassan, K.E.³, Orabi, A.⁴

^{1,2} Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suef University, Egypt

³ Department of Poultry Diseases, Faculty of Veterinary Medicine, Beni-Suef University, Egypt

⁴ Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt

Abstract: Outbreaks of highly pathogenic avian influenza H5N1 (HPAIH5N1) virus have been observed in the commercial poultry industry. The use of inactivated vaccines to control it is widely used. This work was planned to compare the efficacy of conventional bivalent inactivated H5N1-ND and Baculovirus Expressed H5N1-ND (BEST) vaccine in protection of broiler chickens under field conditions by measurement of humoral and cellular immune responses and screening of challenge and shedding of field H5 carrying AI virus using real time-PCR (RT-PCR) as well as measurement of some parameters concerning mortality and performance. Three chick flocks (one day old) were used in the experimental infection. Two flocks were vaccinated with BEST vaccine while the third was vaccinated with bivalent inactivated H5N1-ND vaccine at 10 days of age. Thirty nine tracheal and cloacal swabs were collected at 35 days of age for RT-PCR as well as 30 blood samples were collected at 10, 25 and 31 days of age for measurement of humoral (HI assay) and cellular immune response (INF- γ and IL-6 assays). HI titers against of both NDV and AIV-H5 vaccine in the first and second flocks significantly increased reaching the maximum protective level at 21 day post vaccination. In the 3rd flock, HI titers against NDV reached to protective level at 21 day post vaccination while AIV-H5 titers did not. INF- γ and IL-6 concentrations in BEST vaccinated groups were higher than bivalent inactivated H5N1-ND vaccinated group. Results of RT-PCR of examined samples revealed that 60, 40 and 70% showed H5 virus blood circulation in the flocks, respectively. It was concluded that BEST vaccine was suggested as an important factor in activation of innate immunity which has been reflected on lowering mortalities, lowering clinical signs of disease and increasing body weight gain.

Keywords: Broilers, H5N1-ND vaccine, INF- γ , IL-6, RT-PCR

1. Introduction

Emergency management of animal diseases is required when there are outbreaks of contagious diseases in a country or region that is free of infection. The prompt identification of an infectious disease is a prerequisite for the appropriate management of such emergency situations. This is, however, useful only if the infrastructure can respond quickly and adequately to the emergency situation. The management of a suspected in der case of highly pathogenic Avian influenza (AI) or Newcastle disease (ND) is crucial to subsequent actions aimed at limiting the spread of infected and, ultimately, in prompt eradication of the virus (Capua and Alexander, 2009). Egypt has had a big increase in the reported poultry outbreaks of H5N1 recently, according to the FAO (2015). Outbreaks of highly pathogenic avian influenza H5N1 (HPAIH5N1) virus have been observed in the commercial poultry industry in Asia, Europe, Middle East and Africa. The use of inactivated vaccines to control HPAIH5N1 is widely used. Despite, in attempts to control the disease in Egypt, by destroying millions of birds, HPAIH5N1 virus has become endemic in several regions in domestic and wild birds (Taha et al., 2006 and Smith et al., 2006).

This work was planned to compare the efficacy of conventional Bivalent inactivated H5N1-ND and Baculovirus Expressed H5N1-ND (BEST) vaccine in

protection of broiler chickens under field conditions by measurement of humoral and cellular immune responses as well as screening of challenge and shedding of field H5 carrying AI virus using real time-PCR (RT-PCR), also, measurements of some parameters concerning feed consumption, weight gain and mortalities in flocks under study.

2. Materials and Methods

Chicks: Three chick flocks (one day old Cubb broiler chicks) obtained from El-Ahram poultry Company. Chicks were fed on commercial ration and water ad libitum. Ten randomly selected one day old chicks from each flock were used for collection of serum samples for detection of maternal humoral immunity using HI test. All flocks under study as well as control group were vaccinated against infectious bronchitis and infectious bursal disease with the same programme. Lasota strain (live attenuated ND) vaccine was given to all flocks and control group at 16 days of age in drinking water.

Field Samples. Three flocks were used for collection of serum samples as well as tracheal and cloacal swabs during the period from January to March 2016 as follow:

First flock (El-Fayoum Governorate): Ten thousands chicks were vaccinated with BEST vaccine at 10 days of age

(0.5ml/bird subcutaneously). Thirty nine tracheal and cloacal swabs (10 from each flock and 3 from each group as a control) were collected at 35 days of age for rRT-PCR for detection of viral shedding as well as 30 blood samples were collected at 10, 25 and 31 days of age (i.e. at 0, 15 and 21 days post vaccination; p.v) for measurement of humoral and cellular immune response.

Second flock(Beni-Suef Governorate): Ten thousands chicks were used. Vaccination programme and sample collection was the same as the first flock (the only differences were the locality and ration used).

Third flock: Comprising 10,000 chicks vaccinated with bivalent inactivated H5N1- ND vaccine at 10 days of age (0.5ml/bird subcutaneously). Sample collection, locality and ration used were the same as the second flock.

Control group: Five hundreds birds from each flock were labeled and used as control group. These chicks were vaccinated with the same programme except using inactivated vaccine. Tracheal and cloacal swabs were collected in phosphate buffer saline (pH 7.2) containing gentamycin (50µg/ml) and mycostatin (1,000units/ml) in a 1:5 (w/v) dilution (OIE, 2014).

3. Immunological Parameters

Antigens: Inactivated H5N1 antigen (A/chicken/Egypt/18-H/2009) was used for detection of AIV-H5 antibodies and Lasota strains (8HA units) for detection of NDV antibodies. Haemagglutination inhibition (HI) assay was applied at 25 and 31 days of age (15 and 21 days p.v) in all flocks according to (OIE, 2014) with minor modification, it used for detection AIV-H5 and NDV antibodies titers after vaccination. 25µl of antigens (8 HA units) of each isolate were added to each well of a plastic U-bottomed microtiter plates, and then 25µl of antisera were added in the first well. Two fold serial dilutions of 25µl volumes of the antisera across the plates was done and left for 30 min. at room temperature. 25µl of 1% chicken RBCs were added to each well and the plates were incubated after gentle mixing for 30 min. at room temp. The HI antibody titer was determined as the reciprocal of the highest serum dilution that had complete inhibition of haemagglutination.

Interferon-gamma (INF-γ) assay (Karakolev et al., 2015). INF-γ concentrations were determined at 31 days of age (21 days p.v) by immunoenzymatic assay. Chicken INF-γ ELISA kits (Novatein Bio, Massachusetts, USA) were used. In the wells of the ELISA plate, 7 standards were added with concentrations of 0, 6.25, 12.5, 25, 50, 100 and 200pg/ml. Absorptions were measured at wavelength of 450nm. Interferon concentrations were calculated from the standard curve by means of a software product.

Interleukin-6 (IL-6). It was determined at 31 days of age (21 days p.v) using Sandwich-ELISA where the Micro-ELISA plate provided has pre-coated with antibody specific to chicken IL-6. The optical density (OD) was measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of chicken IL-6. The concentration of IL-6 in the sample was calculated by comparing the OD of the sample to the standard curve according to Helle et al. (1991); Rath et al. (1995) and Kalyuzhny (2005).

Statistical analysis: The results were presented as means ± SE. All given parameters were compared between the control group and the vaccinated groups using the one way ANOVA with fixed effects of the factors using statistica 6.0 (Start Soft INC.). Differences were considered significant at p<0.05.

Real-Time RT-PCR (rRT-PCR) for detection of AIV-H5 under field conditions. The viral RNA was extracted by Bioflux viral RNA Mini Spin column kit (Bioflux, China) in accordance with manufacturer's instructions. Single step rRT-PCR assays using Verso 1-Step qRT-PCR Kit Plus ROX 100 Vial (Thermo Scientific) were conducted using specific oligonucleotide primers and probes for each target virus. The final reaction volume was 25µl including; 5µl RNA template, 12.5µl 2X 1-step PCR ready mix, 1.25µl RT-enhancer, 0.25µl Verso enzyme mix, 1µl of each of the forward and reverse primers (Table 1), and 0.25µl probe for AI-H5N1 (Slomka et al., 2007) together with 3.75µl nuclease free water. Thermocycling rRT-PCR conditions were 50°C for 15 min, 95°C for 15 min, followed by 40 cycles at 95°C for 15 sec and 30 sec at 54°C (for AI-H5N1) with reading of fluorescence in this step.

Table 1: Primers used for RT-PCR for detection of AIV-H5

Virus	Target gene	Oligonucleotide	Primer Sequence (5'-3')	Probe sequence (5'-3')	Reference
AIV-H5	H5	LH1	ACA TAT GAC TAC CCA CAR TAT TCA G	CCCTAGCACTGGCAATCATG	(Slomka et al., 2007)
		RH1	AGA CCA GCT AYC ATG ATT GC		

Real-Time RT-PCR (rRT-PCR) for detection of velogenic NDV under field conditions. The viral RNA was extracted by Bioflux viral RNA Mini Spin column kit (Bioflux, China) in accordance with manufacturer's instructions. Single step rRT-PCR assays using NDV real-time detection kit (iNtRON) were conducted using ready to use kit. The final reaction volume was 20µl including; 5 µl RNA template, 10 µl 2X QRT- PCR master mix solution, 5µl ND detection solution. Thermocycling rRT-PCR conditions were 50°C for 30 min, 95°C for 5 min, followed by 40 cycles at 95°C for 10

sec and 40 sec at 55°C with reading of fluorescence in this step.

Mortality Rate: Mortalities were calculated in all flocks during 38 days of experiment.

Performance: Clinical signs, feed consumption and weight gains were calculated during the period of experiment (38 days).

4. Results

1. Humoral immune response against used vaccines (HI titers). Mean average (MN) and standard deviation (SD) were illustrated in **table (2)** showing HI titers of chicks under study at 10 days old (0-day of vaccination) against both vNDV and AIV-H5 ranged from 1.4 to 1.5. HI mean titers against both vNDV and AIV-H5 at 15 and 21 p.v in the different flocks were as follow:

First flock: ND virus vaccine (NDV) HI mean titer was 3.6 ± 1.061 at 15 days p.v. elevated to 5.2 ± 0.4629 at 21 days

p.v. Regarding H5N1, the recorded HI mean titers were 2.8 ± 1.246 at 15 days p.v. and 4 ± 0.3536 at 21 days p.v.

Second flock: HI mean titers against NDV were 3.8 ± 1.662 at 15 days p.v. and 5.4 ± 0.6848 at 21 days p.v. Concerning H5N1 HI mean titer, it was 3.1 ± 1.446 at 15 days p.v. elevated to 4.3 ± 0.4645 at 21 days p.v.

Third flock: NDV HI titer was 2.2 ± 1.061 at 15 days p.v. and 4.1 ± 0.4332 at 21 days p.v. For H5N1 mean HI titer, the recorded results were 1.8 ± 1.223 at 15 days p.v. and 2.3 ± 0.3303 at 21 days p.v.

Table 2: HI titers of flocks under study at different ages before and post vaccination with NDV and AIV-H5

Vaccine used Day Flocks	0-day of vaccination	NDV		AIV-H5	
	10 day of age	15days p.v	21days p.v	15day p.v	21 day p.v
1 st Flock	1.5 ± 1.662	3.6 ± 1.061	5.2 ± 0.4629	2.8 ± 1.246	4 ± 0.3536
2 nd Flock	1.4 ± 1.061	3.8 ± 1.662	5.4 ± 0.6848	3.1 ± 1.446	4.3 ± 0.4645
3 rd Flock	1.5 ± 0.6848	2.2 ± 1.061	4.1 ± 0.4332	1.8 ± 1.223	2.3 ± 0.3303

SD standard deviation

MN Mean average

2. Interferon-gamma (INF- γ) assay. **Table (3)** presented the average blood INF- γ concentrations in experimental and control broiler chickens. In BEST vaccinated groups (flocks 1 & 2), INF- γ mean levels in the blood were 37.26 ± 128 pg/ml versus 9.675 ± 0.825 in controls. Meanwhile, in bivalent inactivated H5N1-ND vaccinated group (flock 3) the average blood INF- γ concentrations were 26.068 ± 0.896 versus 8.605 ± 0.325 in control group.

3. Interleukin-6 (IL-6) assay. Results of IL-6 concentrations (pg/ml) in the sera of 31 days age flocks vaccinated with the vaccines under the study and measured by ELISA were illustrated in **table(3)** which revealed that the chicken in flocks recorded the highest level of IL-6 (61 pg/ml) followed by the chickens of flock 2 (57 pg/ml) while flock 3 recorded 22 pg/ml. Control groups recorded 16, 12 and 6 pg/ml of IL-6 concentrations in flocks 1, 2 and 3, respectively.

Table 3: INF- γ and IL-6 concentrations in the experimental and control broiler chickens of vaccinated different flocks at 21 day post vaccination

Parameter Flock	INF- γ conc. (pg/ml)		IL-6 conc. (pg/ml)	
	Vaccinated	Control	Vaccinated	Control
1 st Flock			61	16
2 nd Flock	37.26 ± 0.128	9.675 ± 0.825	57	12
3 rd Flock	26.068 ± 0.896	8.605 ± 0.325	22	6

4. Real-Time RT-PCR (rRT-PCR) for detection of AIV-H5 in vaccinated flocks. Results in showed the C_t value of samples collected from all flocks under study. C_t value lower than 38 was considered positive for AIV-H5 under field conditions. Six out of 10 tracheal/cloacal swabs collected from flock 1 showed C_t values of 31, 28, 30, 27.5, 30 and 31 while 4 samples showed 38, 39, 39 and 40 C_t values. Regarding flock 2, 4 out of 10 examined samples showed C_t values lower than 38 (30, 30, 28 and 27.5) while 6 samples were considered negative. Seven out of 10 examined samples of flock 3 showed C_t values lower than 38 (31, 31, 30, 28, 28, 26 and 27.5) and 3 samples C_t values of 38. Control groups in the three flocks (3 samples from each flock) showed the following C_t values 30, 29 and 35 in the first flock; 30, 32 and 35 in the second flock and 33, 27.5 and 35 in the third flock.

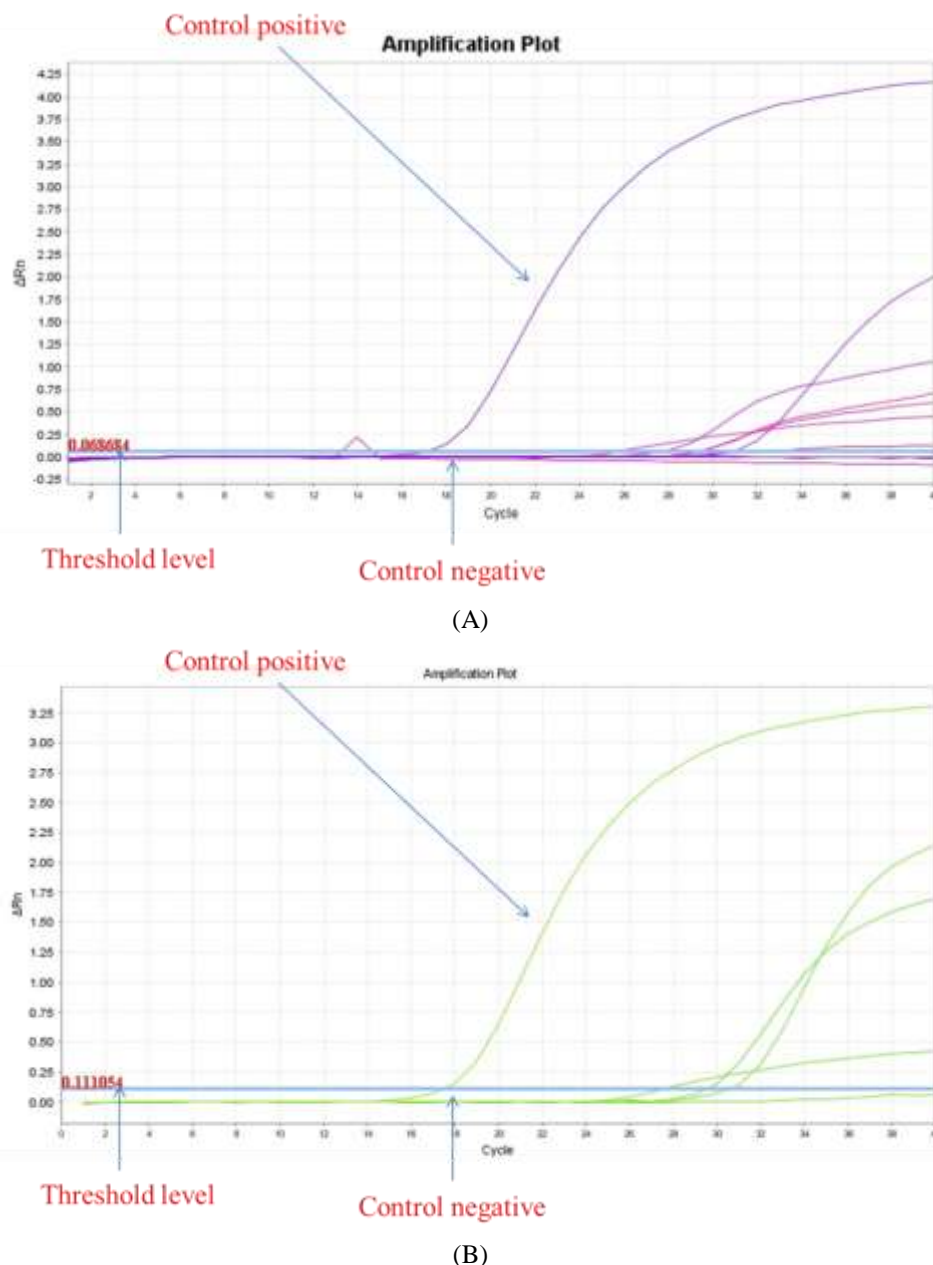


Figure 1: Graph A and B represent some samples that were rRT-PCR tested for detection AIV-H5 infection in flocks under study

5. Real-Time RT-PCR (rRT-PCR) for detection of vNDV in vaccinated flocks.

Results showed that all samples collected from all flocks under study were negative vNDVrRT-PCRthus mean the flocks under study not field challenged by vNDV.

6. Mortality Rates in flocks under study.Mortality rate in the first flock was 7% (700 out of 10,000 birds) started from 2 days of age (20-25 chicks daily till 38 days of age) compared with 8.5% in the second flock (from 2-38 days of age) and 22% in the third flock which reached its maximum during the period from 28-33 days of age.

7. Performance in flocks under study.The recorded clinical signs in the flock were slight respiratory manifestations; slight diarrhea and slight flocculation in feed and water consumption. The mean feed consumption was 3.4 kg/bird and the mean weight gain was 2.2 kg/bird. The second flock recorded moderate respiratory signs, diarrhea and ruffled

feathers and the mean feed consumption was 3.35 kg/bird and the mean weight gain was 2.15 kg/bird. The third flock revealed severe respiratory signs, rales, gasping and diarrhea. Severe drop in feed and water consumption from 25-30 days of age was recorded. The mean feed consumption was 3.6 kg/bird and the mean weight gain was 1.95 kg/bird.

5. Discussion

Highly pathogenic Avian Influenza A virus (HPAIV) of subtype H5N1 caused outbreaks in poultry in Asian, European and African countries including Egypt. The sharp increase in the number of outbreaks of AI in poultry occurred and the impact on the poultry industry has increased 100-fold and the implications for human health of AI infections of poultry have been identified, especially as a result of the spread of Asian lineage HPAI H5N1 virus.

Following vaccine administration, the host immune system directs uptake and processing of the antigen. The adaptive immune responses use highly specific antigen receptors on bursal-derive B (humoral immunity) and thymus-derived T (cellular immunity) lymphocytes that are generated by random processes in gene rearrangement. The adaptive immune responses to vaccines results in virus-neutralizing antibodies and AI virus-specific cytotoxic lymphocytes which are responsible for virus recognition and clearance in the host (Swayne and Kapczynski, 2008).

To determine the best vaccination programme which gives the highest antibody titers against the used vaccines, HI test was performed according to (OIE, 2014) with slight modifications for detection of the humoral immune response against AIV-H5 NDV inactivated vaccines.

In Baculovirus Expressed H5-ND inactivated (BEST) vaccinated first and second flocks, the recorded HI mean titers of NDV were 3.6 ± 1.061 at 15 days post vaccination elevated to be 5.2 ± 0.4629 at 21 days post vaccination (1st flock) and 3.8 ± 1.662 and 5.4 ± 0.6848 at 15 and 21 days post vaccination, respectively (2nd flock). H5N1 HI mean titers recorded were 2.8 ± 1.246 and 4 ± 0.3536 at 15 and 21 days post vaccination in the first flock and 3.1 ± 1.446 and 4.3 ± 0.4645 at 15 and 21 days post vaccination, respectively.

The recorded HI titers against Bivalent inactivated H5N1-ND vaccine in the third flock were as follow: 2.2 ± 1.061 and 4.1 ± 0.4332 at 15 and 21 days post vaccination, respectively while H5N1 mean HI titers were 1.8 ± 1.223 and to 2.3 ± 0.3303 , respectively.

In the present study, HI titers of chicks under study at 10 day old (0-day before vaccination) against both vNDV and AIV-H5 which were very low ranging from 1.4 to 1.5 while HI titers against both vNDV and AIV-H5 vaccine in the first and second flocks significantly increased at 15 day post vaccination and reached to the maximum protective level at 21 day post vaccination. These results were supported with that reported by (Pastoret, 1998) where the immunity increases till reach a protective level at 15 day post vaccination and reach the maximum level after 21 days post vaccination. On the other hand, the level of antibody titers against NDV (3rd flock) slowly increased and reach to protective level at 21 days post vaccination while in AIV-H5 antibody titers failed to reach to protective level at 21 day post vaccination.

In this study, BEST vaccine which was based on a full length H5-coding sequence which derived from an HPAI-H5N1 of Middle East origin of clade 2.2.1 was expressed as a recombinant Baculovirus (Baculo-H5) gave higher HI titers than Bivalent inactivated H5N1-ND vaccine.

In response to questions from CIORAP News, Arafa et al. (2015) prepared a statement about recent H5N1 surveillance findings in Egypt. The General Organization for Veterinary Services (GOVS) and the National Laboratory for Quality Control in Poultry Production, with FAO support, recently stepped up H5N1 and characterized viruses circulating in Upper and Lower Egypt. Genetic sequencing of 52 isolates revealed that the haemagglutinin (H5) genes in virus

continue to evolve but at all the isolates belong to clade 2.2.1. However, the FAO also reported the emergence in 2014 of a new H5N1 cluster with three mutations and this cluster also remains within clade 2.2.1.

The average mean levels of INF- γ in the blood of BEST vaccinated groups were 37.26 ± 128 versus 26.068 ± 0.896 in bivalent inactivated H5N1-ND vaccinated group.

Macrophages respond to a range of different cell products during the innate and acquired immune responses. Of these, INF- γ (originally called Macrophage-activating factor) is among the most important (Schroder et al., 2004). INF- γ is the sole type II INF. It is structurally unrelated to type I INFs, binds to a different receptor, and is encoded by a separate chromosomal locus. Initially, it was believed that CD4⁺ T helper type I (T_H1) lymphocytes, CD8⁺ cytotoxic lymphocytes and Natural killer cells (NK) excessively produced INF- γ (Fischer et al., 2015 and Sachdeva et al., 2015).

On the other hand, the recorded levels of IL-6 were 61 pg/ml in flock 2, 57 pg/ml in flock 2 and 22 pg/ml in Bivalent inactivated H5N1-ND vaccine (flock 3).

Cytokines are secreted proteins involved with cell recruitment and regulation of both innate and adaptive responses. They are essential for an effective host immune response to pathogens. Chicken interleukin-6 (IL-6) has been confirmed to have a role in proinflammatory response (Kaiser et al., 2000). An early stage of inflammation involves secretion of chemokines CXCL12 (interleukin-8) as a chemotaxin for chicken heterophils which are comparable to mammalian neutrophils (Kogut, 2002 and Kaiser et al., 2006).

Real-Time RT-PCR has been reported for the detection of type A influenza virus and AI virus and subsequent identification of certain HA subtypes often H5 and H7 (Munch et al., 2001 and Spackman et al., 2002). Molecular methods offer numerous advantages for AI detection, high sensitivity (Cattoliet et al., 2004) high specificity; scalability; minimization of contact with infectious materials and reasonable cost (Pregliasco et al., 1998).

Six, 4 and 7 out of 10 tracheal/cloacal swab samples showed H5 positive Ct values (lower than 38) in each of flock 1, 2 and 3, respectively.

As one of the major surface glycoproteins of AIV, the haemagglutinin (HA) actually specifies several major functions where it contains the receptors for binding to the host cell surface (Wiley and Skehel, 1987). It also functions to allow fusion of the virus membrane with the host cell membrane and allow release of the genetic information to initiate new virus synthesis (fusion peptide) (Skehel et al., 2001). Cleavage of HA polypeptide is crucial for the exposure of the fusion peptide and the released fusion peptide is absolutely crucial for the initiation of infections.

Despite 60.0% and 40.05 of RT-PCR examined samples in flock 1 and 2, respectively showed H5 virus blood circulation (Ct lower than 38), mortality rates in these flocks were 7.0%

and 8.5%, respectively compared with 22.0% in flock 3. On the other hand, clinical signs suspected to be of AI were slight and mean body weight were 2.2Kg/bird and 2.15Kg/bird in first and second flocks, respectively compared to severe clinical signs and mean body weight of 1.95Kg/bird in the third flock.

Avian influenza (AI) vaccines and their field application can be an effective tool within a comprehensive control programme which should include the following: biosecurity, education on how to prevent AI, diagnostics and surveillance to detect disease and infection, and elimination of AI virus infected poultry (Swayne, 2004).

While many cell types are involved with establishing an immune response, vaccination may or may not produce protective immunity. Factors that can contribute to immunity failure are numerous and include a lack of vaccine antigenic similarity to the field strain, over whelming dose of challenge, or insufficient vaccine antigen content to induce a protective immunological response.

Cell mediated immunity (CMI) is specific immunity mediated by T-lymphocytes (T cells) and has been suggested to be an important factor in the development of protection in chickens vaccinated against viral diseases (Seo and Webster, 2001). The early reactions of the innate immune system use germ-line encoded receptors, known as pattern recognition receptors (PRRs), which recognize evolutionary conserved molecular markers of infectious microbes known as PAMPs (pathogen-associated molecular patterns) (Fearon and Locksley, 1996).

BEST vaccine suggested being an important factor in activation of innate immunity which has been reflected on lowering mortalities, lowering clinical signs of disease and increasing body weight gain.

References

- [1] Arafa, A.S.; Naguib, M.M.; Luttermann, C.; Selim, A.A.; Kilany, W.H.; Hagag, N.; Samy, A.; Abdelhalim, A.; Hassan, M.K.; Abdelwhab, E.M.; Makonnen, Y.; Dauphin, G.; Lubroth, J.; Mettenleiter, T.C.; Beer, M.; Grund, C. and Harder, T.C. (2015): Emergence of a novel cluster of influenza A(H5N1) virus clade 2.2.1.2 with putative human health impact in Egypt, 2014/15. *Euro.Surveill.*, 20:2-8.
- [2] Capua, I. and Alexander, D.J. (2009): Avian influenza and Newcastle disease. A field and Laboratory Manual. Foreword by Joseph Domenech and Bernard Vallat. Chapter 4: Emergency response on suspicion of an Avian influenza or Newcastle disease outbreak. Springer-verlag, Italia, p.31.
- [3] Cattoli, G.; Drago, A.; Maniero, S.; Toffan, A.; Bertoli, E.; Fassina, S.; Terregino, C.; Robbi, C.; Vicenzoni, G. and Capua, I. (2004): Comparison of three rapid detection systems for type A influenza virus on tracheal swabs of experimentally and naturally infected birds. *Avian Pathol.*, 33:432-437.
- [4] Fearon, D.T and Locksley, R.M. (1996): The instructive role of innate immunity in the acquired immune response. *Sci.*, 272(5258):50-53.
- [5] Fischer, U.; Koppang, E.O. and Nakanishi, T. (2013): Teleost T and NK cell immunity. *Fish Shellfish Immunol.*, 35(2):197-206.
- [6] Food and Agriculture Organization (FAO), (2015): FAO notes mutations in H5N1 samples from Egypt's poultry. CIDRAP News: <http://www.cidrap.umn.edu/ongoing-programs/news-publishing/news-publishing-staff>. Jan 7, 2015.
- [7] Helle, M.; Boeijs, L.; de Groot, E.; de Vos, A. and Aarden, L. (1991): Sensitive ELISA for interleukin-6. Detection of IL-6 in biological fluids: Synovial fluids and sera. *J. Immunol. Methods* 138:47-56.
- [8] Kaiser, P.; Rothwell, L.; Galyov, E.E.; Barrow, P.A.; Burnside, J. and Wigley, P. (2000): Differential cytokine expression in avian cells in response to invasion by *Salmonella Typhimurium* and *Salmonella Gallinarum*. *Microbiol.*, 146:3217-3226.
- [9] Kaiser, M.G.; Cheeseman, J.H.; Kaiser, P. and Lamont, S.J. (2006): Cytokine expression in chicken peripheral blood mononuclear cells after in vitro exposure to *Salmonella enterica* serovar Enteritidis. *Poult. Sci.*, 85:1907-1911.
- [10] Kalyuzhny, A.E. (2005): Chemistry and biology of the ELISPOT assay. *Methods Mol. Biol.*, 302:15-31. PMID 15937343.
- [11] Karakolev, R.; Gospodinova, K.; Sotirov, L.; Nikolov, D.; Angelov, A. and Koynarski, Ts. (2015): Blood serum interferon- alpha and - gamma concentrations in broiler chickens treated with the immunomodulator Helpankar. *Int. Curr. Microbiol. App. Sci.*, 4(10):296-299.
- [12] Kogut, M.H. (2002): Dynamics of a protective avian inflammatory response: The role of an IL-8-like cytokine in the recruitment of heterophils to the site of organ invasion by *Salmonella* Enteritidis. *Comp. Immunol. Microbiol. Infet. Dis.*, 25:159-172.
- [13] Munch, M.; Nielsen, L.P.; Handberg, K.J. and Jorgensen, P.H. (2001): Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription-PCR and PCR-ELISA. *Arch. Virol.*, 146:87-97.
- [14] Office International des Épidémiologies (OIE), (2014): Chapter 2.3. 4 (Avian influenza) in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals www.oie.int.
- [15] Pastoret, P.P.; Griebel, P.; Bazin, H. and Govaerts, A. (1998): Handbook of Veterinary Immunology.
- [16] Pregliasco, F.; Mensi, C.; Camorali, L. and Anselmi, G. (1998): Comparison of RT-PCR with other diagnostic assays for rapid detection of influenza viruses. *J. Med. Virol.*, 56:168-173.
- [17] Rath, N.C.; Huff, W.E.; Bayyari, G.R. and Balog, J.M. (1995): Identification of Transforming Growth Factor-β and Interleukin-6 in Chicken Ascites Fluid. *Avian Dis.*, 39(2):382-389.
- [18] Sachdeva, M.; Chawla, Y.K. and Arora, S.K. (2015): Immunology of hepatocellular carcinoma. *World J. Hepatol.*, 7(17):2080-2090.
- [19] Seo, S.H. and Webster, R.G. (2001): Cross-reactive, cell-mediated immunity and protection of chickens from lethal H5N1 influenza virus infection in Hong Kong poultry markets. *J. Virol.*, 75(6):2516-2525.
- [20] Schroder, K.; Hertzog, P.J.; Ravasi, T. and Hume, D.A. (2004): Interferon-γ: An overview of signals,

- mechanisms and functions. *J. Leukocyte Biol.*, 75:163-189.
- [21] Skehel, J.J.; Cross, K.; Steinhauer, D. and Wiley, D.C.(2001): Influenza fusion peptides. *Biochemical Society Transactions*, 29(Pt 4): 623-626.
- [22] Slomka, M.J.; Pavlidis, T.; Banks, J.; Shell, W.; McNally, A.; Essen, S. and Brown, I.H. (2007): Validated H5 Eurasian real-time reverse transcriptase-polymerase chain reaction and its application in H5N1 outbreaks in 2005-2006. *Avian Dis.*, 51:373-377.
- [23] Smith, G.J.D.; Fan, X.H.; Wang, J.; Li, K.S.; Qin, K.; Zhang, J.X.; Vijaykrishna, D.; Cheung, C.L.; Huang, K.; Rayner, J.M.; Peiris, J.S.; Chen, H.; Webster, R.G. and Guan, Y. (2006): Emergence and predominance of an H5N1 influenza variant in China. *Proc. Natl. Acad. Sci., U.S.A.*, 103:16936-16941.
- [24] Spackman, E.; Senne, D.A.; Myers, T.J.; Bulaga, L.L.; Garber, L.P.; Perdue, M.L.; Lohman, K.; Daum, L.T. and 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. *J. Clin. Microbiol.*, 40:3256-3260.
- [25] Swayne, D.E. (2004): Application of new vaccine technologies for the control of transboundary diseases. *Developments in Biologicals*, 119:219-228.
- [26] Swayne, D.E. and Kapczynski, D.R.(2008): Avian influenza (Swayne, D.E., 2008). Chapter 19: Vaccines, vaccination, and immunology for Avian influenza viruses in poultry.
- [27] Taha, M.M.; Al., A.M. and Nassif, S.A. (2006): Isolation and molecular characterization of highly pathogenic H5N1 avian influenza virus in Egypt, Early 2006. *Proc. 3rd Inter. Conf. Vet. Res., Div. NRC, Cairo, Egypt*, pp:173-180.
- [28] Wiley, D.C. and Skehel, J.J. (1987): The structure and function of hemagglutinin membrane glycoprotein of influenza virus. *Annual Review of Biochemistry*, 56:365-394.