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

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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 vaccineat 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 oldCubb 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 serumsamples 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

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(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)formeasurement 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- NDvaccine at 10 days of age (0.5ml/bird subcutaneously). Sample collection, localityand 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-γELISA 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 measuredspectrophotometrically 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. ThermocyclingrRT-PCR conditions were 50ºC for 15 min, 95ºC for 15 min, followed by 40 cycles at 95ºC for 15 sec and 30 sec at 54ºC (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. Thermocyclingr RT-PCR conditions were 50ºC for 30 min, 95ºC for 5 min, followed by 40 cycles at 95ºC for 10 sec and 40 sec at 55ºC 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).

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4. Results

1. Humoral immune response against used vaccines (HI titers). Mean average (MN) and standard deviation (SD) were illustrated in **table (2)**showingHI 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.velevated 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.vand 4± 0.3536 at 21 daysp.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 daysp.v. Concerning H5N1 HI mean titer, it was 3.1± 1.446 at 15 daysp.v elevated to 4.3± 0.4645 at 21 daysp.v.

Third flock: NDV HI titer was 2.2±1.061 at 15 days p.v and 4.1± 0.4332 at 21 daysp.v. For H5N1 mean HI titer, the recorded results were 1.8± 1.223 at 15 days p.vand2.3± 0.3303 at 21 daysp.v.

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

Vaccine used	0-day of vaccination	NDV		AIV-H5	
Day / Flocks	10 day of age	15days p.v	21days p.v	15day p.v	21 day p.v
1st Flock	1.5±1.662	3.6±1.061	5.2±0.4629	2.8±1.246	4±0.3536
2nd Flock	1.4±1.061	3.8±1.662	5.4± 0.6848	3.1± 1.446	4.3± 0.4645
3rd 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 were26.068± 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 flocks1recorded the highest level of IL-6(61pg/ml) followed by the chickens of flock 2 (57 pg/ml) while flock 3 recorded 22pg/ml. Control groups recorded 16, 12 and 6 pg/ml of IL-6concentrations 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 21day post vaccination

Parameter	INF-γ conc. (pg/ml)		IL-6 conc. (pg/ml)`	
Flock	Vaccinated	Control	Vaccinated	Control
1st Flock	37.26± 0.128	9.675± 0.825	61	16
2nd Flock			57	12
3rd 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 *Ct* value of samples collected from all flocks under study. *Ct* value lower than 38 was considered positive for AIV-H5under field conditions. Six out of 10 tracheal/cloacal swabs collected from flock 1 showed *Ct* values of 31, 28, 30, 27.5, 30 and 31 while 4 samples showed 38, 39, 39 and 40 *Ct* values. Regarding flock 2, 4 out of 10 examined samples showed *Ct* 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 *Ct* values lower than 38 (31, 31, 30, 28, 28, 26 and 27.5) and 3 samples *Ct* values of 38. Control groups in the three flocks (3 samples from each flock) showed the following *Ct* 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.

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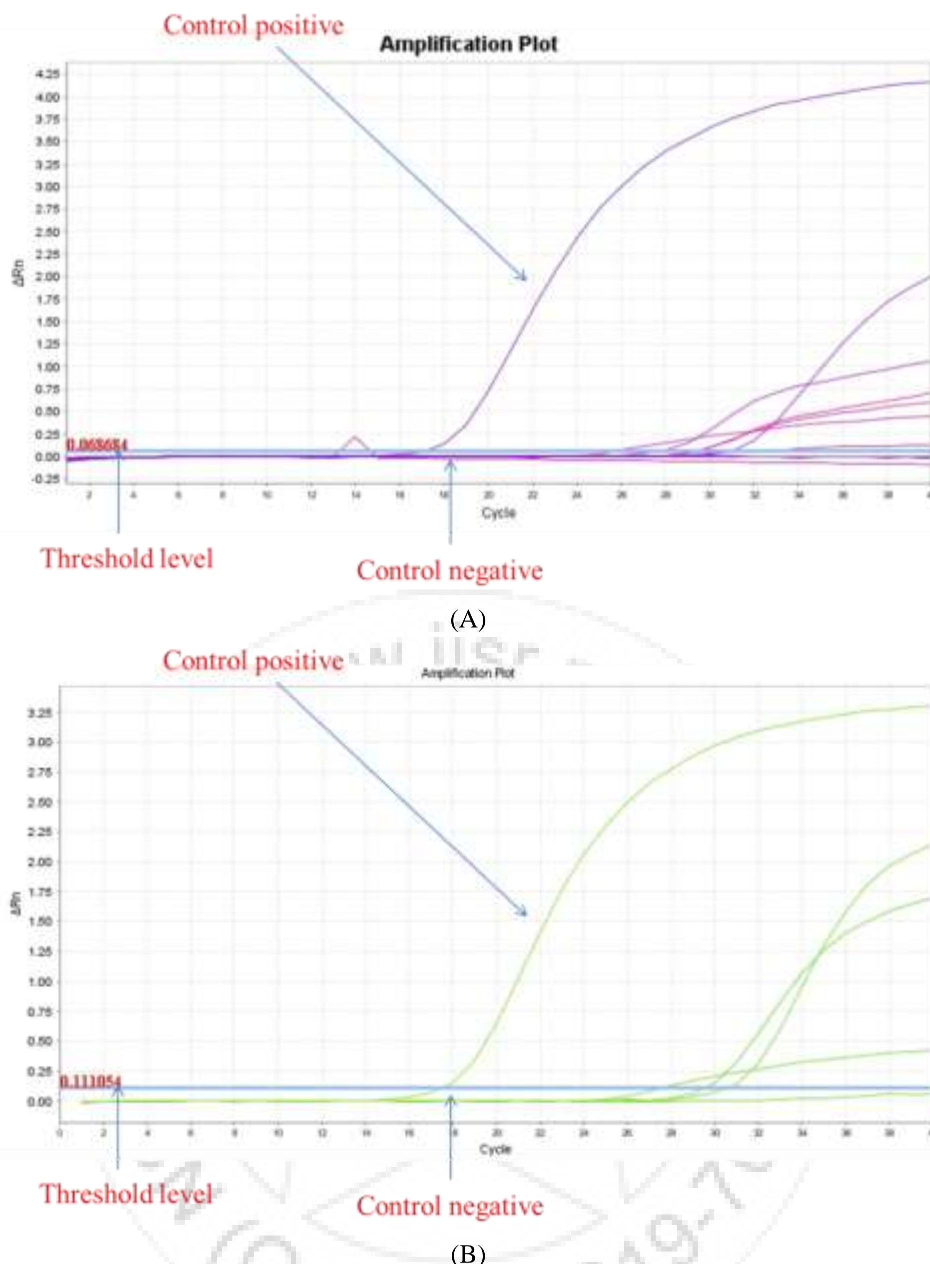

Figure 1: Graph A and B represent some samples that were rRT-PCR tested for detection AIV-H5 infection in flocks under study

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 daysof 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.

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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 15days post vaccination elevated to be 5.2±0.4629 at 21days post vaccination (1^{st}flock) and3.8±1.662 and 5.4± 0.6848 at 15and 21dayspost vaccination, respectively (2^{nd}flock).H5N1 HI mean titers recorded were 2.8± 1.246 and 4± 0.3536 at15and 21days post vaccination in the first flock and 3.1± 1.446 and 4.3± 0.4645 at 15and 21dayspost 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 whileH5N1 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 21days post vaccination. On the other hand, the level of antibody titers against NDV (3rd flock) slowly increased and reach to protective level at 21 dayspost 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± 128versus26.068± 0.896in 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 IIINF. 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 andSachdeva*et al.*, 2015**).

On the other hand, the recorded levels of IL-6 were 61pg/ml in flock 2, 57pg/mlin flock 2 and 22pg/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 CXCLi2 (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 andSpackman*et al.*, 2002**). Molecular methods offer numerous advantages for AI detection, high sensitivity **(Cattoli*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 polyprotein 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%

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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 applicationcan be an effective tool within a comprehensive control programmewhich 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.

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