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DIFFERENTIATION BETWEEN VIRAL INFECTED AND VACCINATED CHICKEN BY USE OF TETANUS TOXOID AS AN EXOGENOUS MARKER

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SUMMARY

The present work was carried out to develop a simple and effective method for differentiating infected from vaccinated chickens to improve the control of infectious diseases. The differentiation of vaccinated and infected birds is based on addition of a suitable exogenous marker to the vaccine either during its preparation or mixed with it just before vaccination. This study has evaluated that the use of a tetanus toxoid (TT) marker in chickens vaccinated with Newcastle disease virus (NDV) and avian influenza (AI) vaccines eliciting a strong, protective antibody response and enabling serological discrimination between vaccinated and virus-infected chickens. All chicken groups were seronegative for TT-specific antibodies without vaccination, however post vaccination with three different doses, they elicited high levels of TT-specific antibodies that persisted all over the experiment regardless the used dose. Incorporation of TT with inactivated NDV vaccine during its manufacture elicited strong TT and Newcastle-specific antibody responses. Furthermore, vaccination with combined doses composed of avian influenza vaccine and TT induced high levels of antibodies to both antigens. There was no detectable interference by incorporation of TT in both inactivated NDV and AI vaccines for the viral antigens or TT-seroconversion. Thus TT is recommended as a suitable exogenous marker for avian vaccines. Moreover, testing the antibody response to the marker would confirm approved vaccine use and the antibody responses to the viral antigen would determine levels adequate for protection or indicate recent infection.

INTRODUCTION

Vaccination in poultry has been implemented as a management tool to control disease and to eliminate virus from flocks to prevent virus transmission (Lee and Suarez, 2005). Despite the wide-

spread use of different types of vaccines, ND continues to be a major threat to the poultry industry. Vaccination of chickens, particularly those raised for commercial consumption, is carried out throughout the world. Although effective live or inactivated Newcastle disease (ND) vaccines are currently available, the virus remains as an ongoing threat to commercial flocks. For continuation of successful international poultry trades, introduction of a systematic ND control measure is desirable (Babiuk, 1999).

The ability to identify and selectively delete genes from a pathogen has allowed the development of "marker vaccines" that, combined with suitable diagnostic assays, allow differentiating infected from vaccinated animals (DIVA) by differentiation of antibody responses induced by the vaccine (no antibodies generated to deleted genes) from those induced during infection with the wild-type virus. Such DIVA vaccines and their companion diagnostic tests are now available or in development for several diseases including infectious bovine rhinotracheitis (IBR), pseudorabies, classical swine fever (CSF), and FMD (Meeusen et al., 2007)

Recently intensive vaccination with marker vaccines and stamping-out strategies have been gaining popularity where eradication of specific diseases is of national or international interest (A marker vaccine is a vaccine that, in conjunction with a diagnostic test, enables serological differentiation of vaccinated animals from infected animals. An animal diagnosed as positive for the presence of a field infection has to be eliminated regardless of prior vaccination with a marker vaccine.

A major drawback of all currently used whole-virus-based live and inactivated NDV vaccines is that vaccinated animals cannot be distinguished from infected animals with standard serological tests, such as haemagglutination inhibition (HI) or virus neutralization. An alternative approach for the development of a marker vaccine is the use of ìsubunit vaccines.î but the disadvantage of most subunit vaccines is that they are less effective than whole-virus-based live vaccines (Mebatsion et al., 2002)

On the same manner, using of vaccination in poultry to control avian influenza has been increasing in recent years. Vaccination has been primarily done with killed whole virus-adjuvanted vaccines. Proper vaccination can reduce or prevent clinical signs, reduce virus shedding in infected birds, and increase the resistance to infection. Historically, one limitation of the killed vaccines is that vaccinated birds cannot be differentiated serologically from naturally infected birds using the commonly available diagnostic tests. Therefore, surveillance for avian influenza becomes much more difficult and often results in trade restrictions because of the inability to differentiate infected from vaccinated animals (DIVA).

Several different DIVA strategies have been proposed for avian influenza to overcome this limitation (Suarez, 2005).

As the vaccination programs for the control of avian influenza (AI) in poultry have limitations due to the problem of differentiating between vaccinated and virus-infected birds. Tempe et al., (2005) used NS1, the conserved nonstructural protein of influenza A virus, as a differential diagnostic marker for influenza virus infection. Experimentally infected poultry were evaluated for the ability to induce antibodies reactive to NS1 recombinant protein produced in Escherichia coli or to chemically synthesized NS1 peptides.

However, the available diagnostic tests as for example either H5N1 or H5N2 vaccinated and naturally infected birds will produce H5-specific antibody, which makes surveillance for avian influenza more difficult so, there is a strong need for differentiating infected from vaccinated animals (Cassandra et al., 2007).

Apparently it's extremely difficult to differentiate between vaccinated bird and bird that is infected as the vaccinated birds were partially protected. So if it's infected it shows less clinical symptoms of disease as well as the vaccination can mask clinical infection, that is, both the vaccinated bird and the infected birds will produce very similar antibody responses (Cassandra et al., 2008). They explained the role of the biological 'tag' that was put into the vaccine by introduced a biomarker TT (tetanus toxoid) into the avian influenza vaccine in chickens and then infect the chickens with a virulent Vietnamese strain of H5N1, all of the chickens survived. There was no clinical illness at all indicating that it didnít interfere with protective efficacy of the vaccine so, TT was completely independent of the virus vaccine strain. This vaccine will called vaccine "tag" and using of this type of vaccine will also enable specific identification of vaccine failure as the marker will indicate that a vaccinated flock, with sick or dead birds shown to be infected, have not been protected by that vaccine.

Tetanus toxoid appears to have been selected as a non-mycobacterial delivery agent. It was used widely as the safe protein antigen to evaluate the systemic antibody response in chickens (Muir et al., 2002), to compare several components of the innate and acquired arms of the immune system in five related, but ecologically diverse, migratory shorebirds (Mendes et al., 2006) and to evaluate the process of induction of the immune response in the chicken gut (Haghighi et al., 2005 and 2006)

Avian species are not susceptible to tetanus toxin and the toxic dose, per gram body weight, is 350,000 times the toxic dose for horses (Hagan and Bruner, 1961). Birds are resistant to tetanus caused by infection with Clostridium tetani, as Cassandra et al., (2007) found that 100% of

chickens were seronegative for antibodies against TT prior TT vaccination in spite of the diversion in age, genetic background, geographical location, and housing. So, tetanus toxoid is a potential candidate for use as exogenous marker in the vaccines.

The main goal of the present study was evaluation of a simple diagnostic test for the differentiation of NDV and AI-vaccinated chicken and infected chicken. This differential test based on the production of antibodies to TT protein in vaccinated birds but not in infected ones.

MATERIALS AND METHODS

1. Vaccines:

1.1. Inactivated Newcastle disease virus vaccine:

The LaSota strain of NDV was propagated into the allantoic sacs of 9-11 day old embryonated specific pathogen-free (SPF) eggs. Inactivation of the harvested allantoic fluid was performed with 0.1% (v/v) formaldehyde (37% formalin) (Liljebjelke et al., 2008). Before inactivation, the NDV suspension had an infectivity titer of $10^{9.5}$ EID_{50}/ ml. Inactivated whole virus with 27 HAU/ ml undiluted allantoic fluid was adjuvanted with oil (liquid paraffin with 10% v/v of Span 80) for use as W/O emulsion. Tetanus toxoid was mixed with the vaccine during its preparation as 4.8 µg TT per dose.

1.2. Inactivated avian influenza virus vaccine:

Inactivated oil emulsified vaccine prepared with AI virus, type A, subtype H5N2, A/chicken/mexico/232/94/CPA strain (Intervet), was purchased from local market. Each 0.5ml contained minimal titer of $10^{8.5}$ EID_{50} / dose and /or a minimum titer of 256 HAU.

2. Antigens:

2.1. Tetanus toxoid (TT) antigen:

A crude preparation of tetanus toxoid (TT), prepared by formalin treatment of Clostridium tetani culture supernatant fractions. Total protein concentration was determined by the Lowry assay (Garvey et al., 1977) to be 33 µg /ml. The selected volumes were 150, 250, 500 µl equal to 4.8, 8 and 16 µg per dose, respectively, which mixed with the vaccine either during its preparation (NDV vaccine) or during the vaccination (AI vaccine).

2.2. NDV antigen:

Four HA units of formalin inactivated NDV LaSota strain was used as test antigen in completing the HI tests.

2.3. Avian influenza antigen:

Avian influenza H5N2 inactivated for HI test produced by Schering -Plough Animal Health Co. A, USA.

3. Chickens:

A total of 120 one-day-old unvaccinated chicks

were procured from a local hatchery. They were brooded together for 7 days, until they were divided into groups each of 10 chicks. Three groups were vaccinated (s/c) with three different doses of TT antigen (4.8 µg, 8 µg and 16 µg per dose). Three groups were vaccinated (s/c) with inactivated NDV vaccine incorporation with the previous doses of TT and another three groups were vaccinated (s/c) with inactivated AI mixed with the same doses of TT prior to injection. The last three groups were considered as control groups, NDV and AI vaccinated control groups and unvaccinated control group which was kept where vaccinated birds were kept.

Blood samples were collected from all chickens groups at 0, 1, 2, 3, 4, 6 weeks post-primary vaccination

4. Challenge test:

NDV vaccinated chickens were challenged 21-days post vaccination intramuscular with a virulent field isolate of velogenic viscerotropic Newcastle disease (VVND) virus. Titer of challenge virus was 10^8 EID_{50} /ml and kindly supplied by the Central Laboratory for Evaluation of Veterinary Biologics, Abassia. Cairo.

5. Haemagglutination -Inhibition (HI) test:

Haemagglutination inhibition (HI) tests to ND and AI were done by standard methods, using 4 haemagglutinating units (HAU) of AIV antigen and NDV antigen (Swayne et al., 1998). End-point titrations were carried out and haemagglutination inhibition titers expressed as log2 of the reciprocal of the highest dilution completely inhibiting the 4 HAU of the antigen.

6. Enzyme linked immunosorbent assay (ELISA): Anti-tetanus antibody responses within chicken serum samples were determined by enzyme-linked immunosorbent assay (ELISA). These assays were performed as described by Hamid et al., (2005).

7. Statistical analysis:

The unpaired Student's t-test assuming unequal variance between means was used to assess statistical significance ($P < 0.05$).

RESULTS AND DISCUSSION

It is reported that the use of an inactivated influenza vaccine containing the marker NA permits differentiating infected from vaccinated animals (DIVA) (Capua et al., 2003; Lee et al., 2004; Liu et al., 2003a). A previous report (Liu et al., 2003b) and avian influenza strain surveillance data have revealed that multiple HA and NA subtypes of avian influenza viruses exist in the domestic poultry, therefore, using NA as a marker to differentiate the infected from the vaccinated birds may not be applicable.

Although the birds are resistant to tetanus caused by infection with Clostridium tetanai, experimental production of TT antibodies in avian species

was studied by Haghighi et al., (2005 and 2006) and Hamid et al., (2005). The combination of using tetanus toxoid as a biological marker to differentiate vaccinated from unvaccinated birds was investigated. Two experiments were performed. The first involved co-delivery of the tetanus toxoid and an inactivated NDV vaccine in mixed preparation, whereas the second experiment investigated the viability of combining TT together with inactivated H5N2 avian influenza vaccine in a single injection. Both vaccines incorporated the use of oil as an adjuvant, which is commonly used in commercial poultry vaccinations.

The presence of antibodies against TT was detected prior to vaccination. All chickens were negative for TT antibodies; however all groups of tetanus toxoid vaccinated chickens successfully produced 100% of anti-TT antibodies, compared to controls, at 2 weeks post vaccination (Table.1). Although the levels of TT antibodies decreased in titer 4 weeks post vaccination, but persisted at 6 weeks post vaccination without significant differences in antibody levels between the groups vaccinated with various doses of TT. These results were agreed with Cassandra et al., (2007) who discussed that the levels of TT-specific antibodies in chickens vaccinated with the dose range of 0.1 mg, 0.3 mg and 1.0 mg of TT were sustained to at least week 53 and indeed, this level of antibody was not significantly different from that observed at week 6 post vaccinations.

The results of chickens groups vaccinated with combined TT and NDV prepared vaccine with three separate doses of TT (4.8 μg, 8 μg and 16 μg) mixed with inactivated whole NDV (Table.1 and Fig.1) showed no significant differences in anti-TT antibodies at any of the time points tested between the group given TT alone compared to the groups given the combined TT and NDV vaccines independent of TT dose. Challenge test with the virulent NDV three weeks post vaccination had no observed effect on the titer of TT. Thus, there was no interference caused by the two antigens in the combined TT and NDV vaccine affecting the antibody response to TT. Furthermore, the group vaccinated with inactivated NDV only did not show any antibodies against TT throughout the study. The HI titers to NDV in chickens vaccinated with the combined NDV and TT vaccines generally increased over the 6 weeks post vaccination (Table.2 and Fig.3). Two weeks post vaccination; the HI titers were significantly higher ($p<0.05$) in the groups given combined vaccines compared with the group given NDV vaccine only. Amongst the vaccinated groups with combined vaccines with different TT doses, there were no significant differences in the HI antibody titers between any of the TT/NDV groups. Post challenge test, the differences in the levels of antibody titers were observed between the vaccinated groups all over the experiment but they considered non significant.

Similar to that found in TT/NDV vaccination re-

sults, combining of AI vaccine together with TT in a single subcutaneous injection showed positive TT antibody levels obtained in all groups after the vaccination and the antibody titers were maintained up to 6 weeks after the initial vaccination (Table.1and Fig.2). There were no significant differences between the different doses, although the 16 µg TT dose consistently provided slightly higher TT titers compared to the 4.8 µg TT dose. In addition, TT-specific antibody levels were not found to be significantly different in chickens up to 6 weeks post vaccination between any of the combined TT and AI vaccine groups and the TT only vaccine groups.

The HI titers in vaccinated chickens were successfully elicited following vaccinations, with all groups achieving high antibody titers (Table.2 and Fig.3). Two weeks post vaccination, the titer of TT/AI vaccinated groups were higher significantly than Al vaccinated control group, indicating that possibility of some synergistic activity was responsible for the higher HI titers obtained in the TT/AI vaccinated groups. Three weeks post vaccination; both groups received 8 and 16 µg TT with AI vaccine differ significantly ($p<0.05$) from the AI vaccine group however 4 weeks post vaccination only the group received 16 µg TT was differ significantly ($p = 0.029$). No interference in the induction of specific antibodies to either TT or Al was observed by administration of both TT and Al vaccines and as expected, the group vaccinated with AI alone did not show TT-specific antibodies.

The obtained results showed that incorporation of influenza vaccine with tetanus toxoid enhanced the serum antibody titers. From the results presented here it is clear that immunization with formulations of NDV or influenza vaccines containing TT yields significantly higher antibody levels when compared with the vaccine alone, which suggested that TT could strengthen the humeral immunity. The humeral immunity is one of the major ways to resist infectious diseases (Suarez and Schultz-Cherry, 2000).

On the other hand, TT can elicit strong antibody responses in chickens regardless of the vaccine and field viral strain, therefore, TT as exogenous marker could have a great potential for use with other strains of influenza used in avian vaccines. Moreover, there is no change to the standard operating procedures for vaccine preparation or vaccination as TT was simply added to the inactivated NDV fluid and adjuvanted by the oil without need to more operating steps. So it can be applicable to other vaccines for pathogens in poultry, such as agents causing infectious bursal disease and infectious bronchitis.

Tetanus toxoid concentration did not play effective role in stimulation of TT-specific antibody responses and these agreed with Cassandra et al (2007) who revealed that birds vaccinated Hydroxide will bind 50-200µg antigen) induced TT

specific antibody responses significantly higher compared to those given 0.3mg TT. However these results gave the chance for optimal diseases control by incorporation of the appropriate concentration of TT with the viral vaccines that is capable of producing a consistently high and persistent antibody response for identification of vaccinated birds, which will be positive to the viral antigen and TT, while infected birds will only be positive to infective virus.

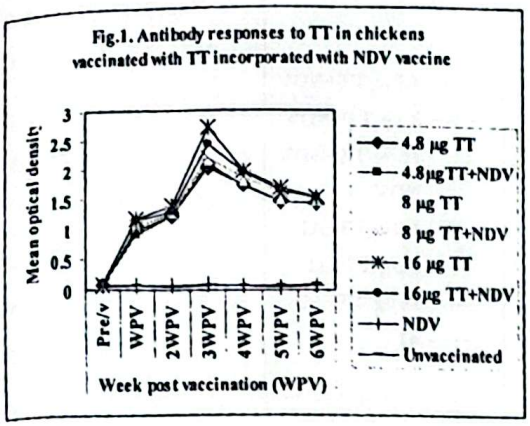
Furthermore, the village birds including ducks and turkeys and in wildlife species, particularly migrating birds are considered an important source of many viruses transmission especially AI and currently available vaccines does not allow the differentiation between infected and vaccinated birds, which interferes with monitoring the diseases status (Middleton et al., 2007). Efficacy of TT as exogenous vaccine marker in chickens could plan to be effective in other village birds like duck, geese and turkey.

The conclusion of this studies revealed that tetanus toxoid is a suitable exogenous vaccine marker for use in poultry vaccines allowing for simple and effective sero-surveillance especially for protection from highly pathogenic avian influenza as the toxoid produced consistent, strong TT antibody responses in chickens that persist in vaccinated birds Also, this exogenous marker will improves control of poultry movements between the farms and markets based on serological evidence of successful vaccination, as birds would need to show positive antibody responses to the TT marker with the adequate antibody levels for protection against viral infection.

Table (1): The TT-specific antibody ELISA results in chickens vaccinated with different doses of tetanus toxoid and TT incorporated with NDV and AI vaccines.

Vaccine	Mean optical density values / week post vaccination (WPV)						
	Pre/v*	WPV	2WPV	3WPV**	4WPV	5WPV	6WPV
4.8 µg TT	0.064	0.981	1.225	2.013	1.742	1.456	1.427
4.8µgTT+NDV	0.057	0.897	1.209	2.106	1.716	1.463	1.429
4.8µgTT + AI	0.09	0.971	1.226	2.032	1.696	1.486	1.475
8 µg TT	0.075	1.061	1.255	2.154	1.792	1.475	1.453
8 µg TT+NDV	0.098	1.014	1.258	2.189	1.858	1.616	1.514
8 µg TT + AI	0.056	1.089	1.248	2.167	1.831	1.449	1.402
16 µg TT	0.071	1.174	1.373	2.707	1.983	1.696	1.522
16µg TT+NDV	0.086	1.144	1.289	2.437	1.960	1.643	1.521
16µg TT + AI	0.060	1.172	1.427	2.402	2.040	1.714	1.653
NDV	0.079	0.068	0.067	0.091	0.060	0.049	0.070
AI	0.051	0.051	0.055	0.056	0.099	0.070	0.079
Unvaccinated	0.058	0.062	0.054	0.080	0.077	0.074	0.088

*= pre-vaccination samples **= Challenge test of NDV vaccinated groups



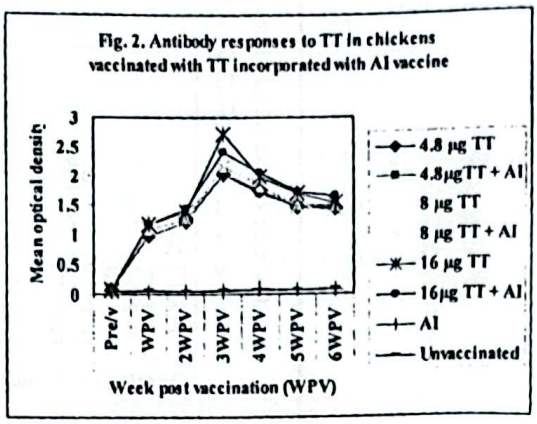
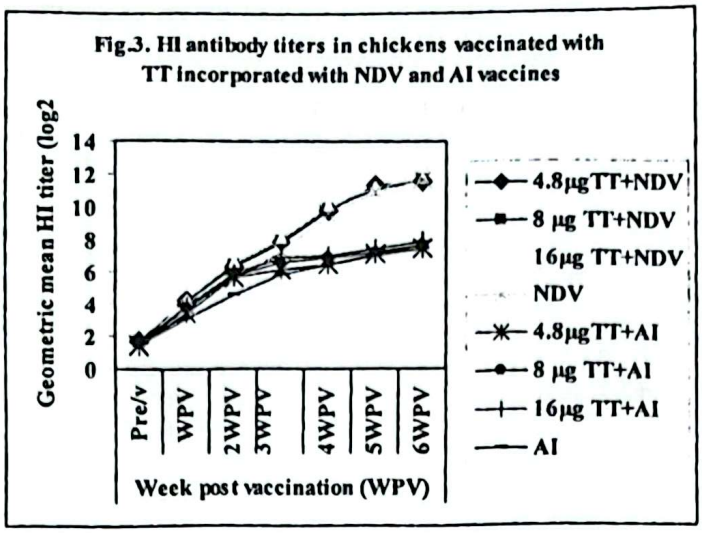


Table (2): Haemagglutination inhibition (HI) antibody responses of vaccinated chickens with different doses of TT incorporated with both NDV and AI vaccines.

Vaccine	Geometric mean HI titer (log_2) / week post vaccination (WPV)						
	Pre/v*	WPV	2WPV	3WPV**	4WPV	5WPV	6WPV
4.8µgTT+NDV	< 2	4.17	6.29P	7.78P	9.76	11.32	11.54
8 µg TT+NDV	<2	4.27	6.4P	7.88P	9.88	11.08	11.7
16µg TT+NDV	< 2	4.38	6.64P	8.1P	10.09	11.26	11.74
NDV	< 2	3.94	5.53	7.21	9.54	10.87	11.65
4.8µgTT+AI	< 2	3.4	5.63P	6.14	6.5	7.07	7.5
8 µg TT+AI	< 2	3.75	5.75P	6.6P	6.86	7.21	7.63
16µg TT+AI	< 2	3.88	5.9P	6.9P	7P	7.5	7.88
AI	< 2	3.13	4.6	5.8	6.5	6.98	7.5

*= pre-vaccination samples **= Challenge test of NDV vaccinated groups

P = values differ significantly (P<0.05) with group received the vaccine without TT.



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