

## Preparation and Evaluation of Combined Inactivated Duck Vaccine against Salmonellosis, Duck Plague and Duck Hepatitis

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**Abstract:** The present study aimed to investigate the protective effect of a locally prepared combined oil emulsified inactivated vaccine against *Salmonella typhimurium* (ST), Duck Plague (DP) and Duck Hepatitis Virus (DHV) in ducks. Evaluation of such preparation following the quality control tests revealed that, it is stable, free from foreign contaminants, safe and immunogenic. The prepared vaccine was evaluated in 7-days old ducklings and the immune response of vaccinated birds to combined vaccine was estimated by microagglutination test for ST antibodies and serum neutralization test (SNT) for DP and DHV antibodies as well as by challenge test. The results of challenge test showed that vaccinated ducklings were effectively protected against virulent strains of *Salmonella typhimurium*, Duck Plague and Duck hepatitis virus. The prepared vaccine found to be sterile, safe, potent and protecting ducklings against *Salmonella typhimurium*, Duck plague and Duck hepatitis virus infections.

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### 1. Introduction

Infections with *Salmonella typhimurium* (ST), Duck Plague (DP) and Duck Hepatitis Virus (DHV) result in high morbidity and mortality, which cause significant economic loss in the duck industry. It can be difficult to distinguish these pathogens based on clinical signs because these pathogens can cause similar clinical signs and coinfections can occur. Thus, rapid and sensitive detection of these 3 major bacterial and viral pathogens are important in ducks (Scanes, 2007, Gough and McNulty, 2008 and Sandhu and Metwally, 2008).

Salmonellosis is considered to be one of the most important causative agent which infect poultry farms specially that which apply the modern intensive system of rearing and management (Nakamura et al, 1994). Air circulation plays a role in the spread of *Salmonella* contamination in a poultry house environment and in the transmission of the infection within flocks (Gast et al., 2004a).

The long persistence of *Salmonella typhimurium* in the environment of the poultry farms can facilitate extensive airborne dissemination of the pathogen via contaminated dust and aerosols (Davies and Breslin, 2004; Gast et al., 2004a, b). *Salmonella* infections are generally spread through the fecal-oral route; however, transmission can also occur through the respiratory route via dust. Air circulation plays an important role in the spread of *Salmonella* contamination in a poultry

house environment and in the transmission of the infection within flocks.

Many different serotypes of *Salmonella* have been isolated from ducks, most of them have a public health significance, but some include *S. typhimurium*, *S. enteritidis*, *S. gallinarum* and *S. anatum* can cause considerable losses in ducks of less than a few weeks of age (Su et al, 2011). Death has been reported to result from a combination of systemic salmonellosis and diarrhea, the disease being referred to, in the past, as "Keel" disease, so-called because birds remained apparently healthy until they keeled over and died (Higgins, 1996).

Control of Salmonellosis in poultry by immunity, whether acquired or innate, is a possible means of containing the problem. Widespread usage of antibiotics has led to the emergence of multiple antibiotic-resistant bacteria. This problem has increasing requirement for effective vaccines to control this important zoonotic infection (Methner et al, 2006 and Barrow, 2007).

On other side, Duck Plague Virus (DPV), a member of the Alphaherpesvirinae is the causative agent of duck plague (DP), one of most serious infectious diseases of waterfowl (duck, geese, and swans). This disease has caused heavy economic losses in the commercial duck industry due to mortality, condemnations, and decreased egg production (Sandhu and Leibovitz, 2008). Vaccination is a desirable method to prevent DPV infection. The conventional

DPV vaccines are inactivated and attenuated DPV preparations, and they have been shown to be able to confer protection against clinical disease. However, as with all or most herpesvirus, DPV has the ability to establish latent infection which adds difficulties in the control and prevention of the transmission of DPV or the establishment of latency. Thus, a more effective therapeutic vaccine will need to elicit sufficient cell-mediated and humoral immune responses (**Shawky and Schat, 2002**).

Duck viral hepatitis is a peracute viral infection of young ducklings showing rapid spread, high mortality, and typical hemorrhages in the liver. Duck virus hepatitis is characterized by sudden onset and the affected ducklings lie on their side with spasmodic paddling leg movements and heads in opisthotonus position. In duck-producing areas of the world where the disease has been reported, DVH has resulted in significant economic losses in domestic and wild waterfowls due to high mortality and condemnations (**Woolcock, 1991**). It causes high losses close to 100% within 3-4 days in a flock of ducklings up to 2 weeks old (**Toth, 1972 and Gough and McNulty, 2008**). Control of duck viral hepatitis mainly involves strict biosecurity procedures and the immunization of young ducklings or breeder ducks with vaccines. Vaccination has been used as a preventive measure, also for controlling DVH disease outbreaks (**Lenhoff, and Summers, 1994**).

The present work aimed to prepare a vaccine which provide a protection for ducks against three of the most dangerous diseases, Salmonellosis, duck plague and duck viral hepatitis, affecting them in a dramatic form saving time and efforts.

## 2. Material and Methods

### 1-Salmonella typhimurium:

A local isolate of Salmonella typhimurium isolated from infected ducks was obtained from Vet.Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. This isolate was used for experimental vaccine preparation as well as challenge of vaccinated ducks.

### 2-Viruses:

#### 2.1 Virulent strain of duck plague virus:

It was kindly obtained from the Central Veterinary Laboratory Weybridge, Surrey, UK. It was used for preparation of vaccine and challenge test. It had a titre of  $10^{7.5}$  EID<sub>50</sub>/ml

#### 2.2 Virulent strain of duck hepatitis virus:

It was supplied by Vet.Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt, with a titer of  $10^{8.5}$  EID<sub>50</sub>/ml. It was used for preparation of experimental combined vaccine as well as challenge test, it was isolated and identified by (**El-Koffy, 1997**).

#### 2.3. Cell culture adapted duck plague virus:

Duck plague virus at its 10<sup>th</sup> passage on VERO cell line with a titer of  $10^7$  TCID<sub>50</sub>/ml and used for estimation of (DP) antibodies in sera of ducks using serum neutralization test (**Abd-El-Khaleik, 1997**).

#### 2.4. Cell culture adapted duck hepatitis virus:

Duck hepatitis virus at its 11<sup>th</sup> passage on VERO cell line with a titer of  $10^8$  TCID<sub>50</sub>/ml and used for estimation of (DH) antibodies in sera of ducks using serum neutralization test (**El-Koffy, 1997**)

#### 3-Specific pathogen free embryonated chicken eggs (SPF-ECE):

Nine days old SPF-ECE were obtained from Nile SPF Egg Farm, Koum Osheim, Fayoum, Egypt. These eggs were used for propagation of Duck plague and Duck hepatitis viruses, testing for complete virus inactivation and virus titration for vaccine preparation.

#### 4-Ducks:

One hundred, one-week old Pekin ducks were supplied by Vet. serum and Vaccine Research Institute, Abbasia, Cairo, Egypt, These birds were divided as follow:

\*10 Ducks were used in the safety test of the prepared vaccine.

\* 90 Ducks were used in the potency test.

**5-Mice:** A total of 250 Swiss Albino mice of about 15-20 g body weight supplied by Veterinary Serum and Vaccine Research Institute, were used for determination of LD<sub>50</sub> of Salmonella typhimurium

#### 6- Vaccine preparation:

##### 6.1. Preparation of Salmonella typhimurium (ST) vaccine:

Inactivated Salmonella typhimurium vaccine was prepared according to **Bachmeier, (1994)** The final bacterial suspension was adjusted to contain  $10^{10}$  colony forming units/ml then inactivated by adding 0.3% formalin.

##### 6.2. Preparation of Duck plague(DP) vaccine:

Inactivated DP vaccine was prepared in 9-days old in (SPF-ECE) according to (**Abd-El-Khaleik, 1997**). Virus titration was of  $10^{8.5}$  EID<sub>50</sub>/ml.

##### 6.3. Preparation of Duck hepatitis virus (DHV) vaccine:

Inactivated DHV vaccine was prepared in (SPF-ECE) according to **Toth(1969)**. Virus titration was of  $10^{8.1}$  EID<sub>50</sub>/ml.

Inactivation of either Duck plague or duck hepatitis virus was carried out with 0.2% formalin for 24 hours at 37° C and complete virus inactivation was tested in SPF-ECE through 2 successive passages. Complete virus inactivation was determined by absence of any gross lesions on the inoculated embryo.

##### 6.4. Preparation of combined Salmonella typhimurium, Duck plague and Duck hepatitis virus vaccine(ST-DP-DHV):

It was prepared according to **Stone et al. (1978)** by mixing previously prepared inactivated ST, DP and

DHV vaccines by equal volumes, the adjuvant used consisted of mineral oil, sorbitan monooleate and Tween 80 using double emulsification method (water in oil in water)

### **7- Quality control testing of the prepared experimental vaccine:**

#### **7.1-Sterility test:**

Testing the freedom of the prepared vaccine from foreign contaminants (aerobic and anaerobic bacteria and fungi) was carried out according to **OIE(2013)**

#### **7.2-Safety test:**

Safety of the prepared vaccine were tested according to **OIE (2013)** through inoculation of double dose subcutaneously in each of 10 ducks which kept under daily observation for 14 days.

#### **7.3-Potency test:**

Combined ST-DP-DHV vaccine was inoculated in a group of 60 ducks of one-week old. The vaccine was inoculated through the subcutaneous route with a dose of 0.5 ml administrated twice with 3 weeks intervals. In addition a group of 30 ducks were kept without vaccination as control. All birds were housed in separate isolates under hygienic measures receiving adequate ration and water. Serum samples were obtained regularly on week intervals to follow up the induced antibody levels up to 12 weeks post the first vaccination.

### **8-Serological evaluation of humoral immune response:**

#### **8.1. Micro-agglutination test:**

This test was carried out to estimate ST antibodies in vaccinated ducks as described by **Thaxton,et al(1970) and Brown,et al.(1981)**.

The geometric mean ST antibodies titer was calculated according to **Brugh(1977)**.

#### **8.2 Serum neutralization test (SNT):**

It was carried out to estimate DP and DHV antibodies in vaccinated ducks by microtechnique according to **Kaleta(1988)**.

#### **8-Challenge test:**

##### **8.1. Challenge with virulent Salmonella typhimurium (ST):**

Vaccinated ducks with combined vaccine and non-vaccinated ducks were challenged with the virulent Salmonella typhimurium using 0.1ml of  $10^8$  colony forming units/ml inoculated intramuscularly according to **Adriaesen et al.(2007)**.

##### **8.2. Challenge with virulent DP virus:**

Vaccinated ducks with combined vaccine and non-vaccinated ducks were challenged with virulent DP virus using 0.1 ml/ containing  $10^6$  EID<sub>50</sub> /bird ( **Abd-El-Khaleik,1997** ).

##### **8.3. Challenge with virulent DHV virus:**

Vaccinated ducks with combined vaccine and non-vaccinated ducks were challenged with virulent

DHV virus using 0.1 ml/ containing  $10^7$  EID<sub>50</sub> /bird (**El-Koffy, 1997**)

**9-Shedding of Salmonella typhimurium(ST) from challenged ducks:** Shedding of S.typhimurium was detected in fecal samples collected from challenged vaccinated and non-vaccinated ducks weekly up to 4 weeks post challenge using Salmonella Shegella medium (**Barrow,2007**).

**10- Recovery of Salmonella typhimurium (ST) from challenged ducks:**

On the 4<sup>th</sup> week post challenge, samples were collected from the heart blood, liver, spleen and caecal junction from vaccinated and non-vaccinated challenged ducks for recovery of the organism using Salmonella Shegella medium.

### **3. Results and Discussion**

Salmonellosis, Duck plague and Duck hepatitis virus infections and their control are still as a subject of interest and usually attract the attention of researcher to know more about the diseases epidemiology and how to control in ducks .Vaccination is still considered one of the major tools for controlling these diseases (**Lenhoff, and Summers,1994 , Methner et al,2006 and Sandhu and Leibovitz,2008**)

During the present work a combined oil emulsified vaccine was successfully prepared against Salmonella typhimurium,Duck plague and duck hepatitis virus (ST-DP-DHV) which found to be stable ,free from foreign contaminants ( aerobic and anaerobic bacteria and fungi ) and safe in vaccinated birds where such birds remained healthy allover the experimental period with slight local reaction at the site of inoculation.These observations agree with the recommendation of **OIE (2013)** .

Microagglutination test was performed to follow up the induced immune response of vaccinated ducks with the prepared combined ST-DP-DHV vaccine. The results demonstrated in table (1) showed that there was a detectable increase in the geometric mean of ST antibody titers in vaccinated ducks from (46) by the 1<sup>st</sup> week and reached value of (1194) on the 11<sup>th</sup> week post vaccination with combined ST-DP-DHV vaccine.

Concerning the protection efficacy of the prepared combined vaccine ,table (2) showed that the protection rate was 90% in ducks vaccinated with combined ST-DP-DHV vaccine , while the control unvaccinated group was unable to withstand the experimental infection with virulent ST strain confirming that the prepared vaccine was effectively potent and hence able to protect ducks against infection .These results came in agree with that reported by **Uytterhoek et al(1989) , Nakamura et al (1994) and Barrow (2007)** who recommended the use of formalized inactivated oil emulsion Salmonella vaccine for protection of chickens against infection .The results

demonstrated in table (3) revealed that ST could be detected in fecal shedding of challenged vaccinated birds at the ratio of 20% on the 1<sup>st</sup> week post challenge from birds vaccinated with combined ST-DP-DHV vaccine and it could not be detected completely by the 4<sup>th</sup> week post challenge, while these ratio were 80% and 33.3% from challenged control birds at 1<sup>st</sup> and 4<sup>th</sup> post challenge, respectively.

On other hand ,table (4) showed that the Salmonella organism could be re-isolated from vaccinated challenged ducks with combined ST-DP-DHV vaccine at ratio of 22.2 to 33.3% from heart blood, liver, spleen and caecal junction on the 4<sup>th</sup> week post challenge ,while these ratio were 75% from control unvaccinated birds .

These results agreed with **Uytterroek et al (1989) and Timms et al (1990)** who found that Salmonella vaccine protects against experimental challenge with shedding of the organism on the same period with declined rate post challenge with indication that the highest incidence of the organism is that in the caecal junction.

Regarding the immune response of the vaccinated ducks to combined ST-DP-DHV vaccine exhibited good levels of specific DP and DHV antibodies as estimated by SNT test.

These antibodies recorded their peak by the 4<sup>th</sup> to 10<sup>th</sup> week post vaccination with combined ST-DP-DHV vaccine. Such results come in agreement with those reported by **Nawath and Ayollo (1981) and Mervat et al (1999)** who mentioned that viral vaccines of poultry did not interfere with the immune

response of birds to bacterial vaccines ,if both were given in combined form . Such finding showed that there was no antagonizing effect among ST,DP and DHV antigens on the immune response of ducks against each other.

Table (5) showed the antibody titers of SNT that was carried out on serum samples obtained from vaccinated ducks with combined ST-DP-DHV vaccine exhibited a good levels of specific antibodies against DPV by the 1<sup>st</sup> week(4) and reached to the maximum value (128) on the 4<sup>th</sup> till 12<sup>th</sup> week post vaccination which induced a complete protection against challenge virulent DPV as shown in table (6) .These results were parallel to those reported by **Samia and Sandhu(1997)** who mentioned that inactivated duck plague vaccine was effective in enhancing protection in ducks against virulent duck plague virus.

On other hand,the detected DHV antibodies in vaccinated ducks were within the protective levels as shown in table (5) where they showed protection percentage of 100%,while unvaccinated control birds did not withstand the virulent virus as shown in table (6) with combined ST-DP-DHV vaccine against virulent DHV virus confirmed by the findings of **Woolcock and Crighton(1979) and Fan et al(1993)** who said that oil inactivated DHV vaccine gave complete protection against virulent virus.

So, it could be concluded that the combined experimentally prepared Salmonella typhimurium ,Duck plague and Duck hepatitis virus vaccine was of good quality and able to protect ducks effectively without any antagonizing effect among them .

Table (1): Salmonella typhimurium (ST) antibody titers in ducks sera as measured by microagglutination test

Type of vaccine	Geometric mean of ST antibody titers/weeks post vaccination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Combined ST-DP-DHV vaccine	9	46	86	98	320	453	557	680	788	905	1114	1194	1194
Control	5	6	7	8	7	8	9	10	11	10	12	11	11

Table (2): Protective efficacy of the prepared vaccine against challenge with virulent Salmonella typhimurium

Type of vaccine	No of challenged ducks	No of survived ducks	Protection %
Combined ST-DP-DHV vaccine	20	18/20	90%
Control	10	3/10	30%

Table (3): Fecal shedding of Salmonella typhimurium (ST) from challenged ducks

Type of vaccine	No of positive shedding/ survived birds on weeks post challenge			
	1WPC	2WPC	3WPC	4WPC
Combined ST-DP-DHV vaccine	4/20(20%)	3/18(16.7%)	0/18 (0%)	0/18 (0%)
Control	8/10(80%)	4/6(66.7%)	2/4(50%)	1/ 3(33.3%)

\*WPC= week post challenge

Table (4): Reisolation of *S. typhimurium* (ST) from different organs of vaccinated and control ducks 4 weeks post challenge

Type of vaccine	No. of birds positive for isolation / Total No. of living birds			
	Heart blood	Liver	Spleen	Caecal junction
Combined ST-DP-DHV vaccine	4/18(22.2%)	4/18(22.2%)	5/18(27.8%)	6/18(33.3%)
Control	2/3(75%)	2/3(75%)	2/3(75%)	2/3(75%)

Table (5): Duck plague (DP) and Duck hepatitis (DH) serum neutralizing antibody titers in ducks

Type of vaccine	Type of virus	DP and DH neutralizing antibody titer /weeks post vaccination									
		0	1	2	3	4	6	8	10	11	12
Combined ST-DP-DHV vaccine	DPV	0	4	8	32	128	128	128	128	128	128
	DHV	0	4	8	64	128	128	128	128	128	128
Control	DPV	0	0	0	0	0	0	0	0	0	0
	DHV	0	0	0	0	0	0	0	0	0	0

Antibody titer= the reciprocals of the final serum dilution which neutralized and inhibit the CPC of 100 TCID<sub>50</sub> of the used virus

Table (6): Protective efficacy of the prepared vaccine against challenge with virulent DPV and DHV viruses

Type of vaccine	Type of virus	No of challenged ducks	No of survived ducks	Protection %
Combined ST-DP-DHV vaccine	DPV	20	20	100%
	DHV	20	20	100%
Control	DPV	10	0	0%
	DHV	10	0	0%

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