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Efficacy of locally prepared inactivated combined vaccine of *Salmonella* enteritidis, *Salmonella* typhimurium, Avian influenza (H9N2) and Newcastle disease viruses

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This study was conducted to prepare and evaluate the potency of a tetravalent inactivated vaccine for protection of chickens against *Salmonella* typhimurium, *Salmonella*enteritidis, AI-H9N2 and Newcastle disease virus using Montanide ISA-70 VG as an oil adjuvant. The humeral immune response against *S.* typhimurium and *S.* enteritidis increased quickly by the 1st wpv, where the mean Log_2 antibody titers were (40 and 20) as measured by micro agglutination test and continue after boostering reached (1280 and 640) in monovalent and tetravalent prepared *S.* typhimurium, *S.* enteritidis vaccines on 4th wpb, respectively. *S.* typhimurium and *S.* enteritidis monovalent and tetravalent vaccines induced (83.3%, 80% and 86.3%) protection percent in challenged vaccinated birds, respectively. Also the vaccines stimulated the production of specific antibodies against AI-H9N2 and NDV as fast as 1stwpv. Then specific AI-H9N2 mean Log_2 HI antibody titers were (8.6 and 8.8 Log_2) at the 4th wpv in monovalent and tetravalent AI-H9N2 vaccines, respectively just before boostering, After that the titer increase gradually reaching (9.4 and 9.8 Log_2) at the 4th wpb. NDV mean Log_2 HI antibodies titers at the 4th wpb was (11.2 Log_2) in tetravalent NDV vaccine than monovalent vaccines was (10.6 Log_2). On the other hand, the protection percent for AI-H9N2 and NDV monovalent and tetravalent vaccines reached 100% without any clinical signs observed, while the control+ve unvaccinated group post challenge with AI-H9N2 showed gasping, coughing, nasal and ocular discharge, depression, inappetance, weakness and were relactant to move with 30% mortality. Also, NDV control+ve unvaccinated group showed greenish diarrhia, depression, conjunctivities and nervous manifestations with 90% mortality approving the efficacy of the prepared vaccines. It could be concluded from our study that the tetravalent prepared vaccine can reduce AIV-H9N2, NDV, *S.*typhimurium and *S.* enteritidis infections reducing the load on the poultry sector by reducing stress for the worker and the birds.

Keywords: *S.* enteritidis, *S.* typhimurium, Avian influenza (H9N2) virus, Newcastle disease virus, Efficacy, monovalent, vaccine, tetravalent.

INTRODUCTION

Salmonellosis is an important public health problems and causes large economic losses in poultry industry. *Salmonella* enteritidis and *Salmonella* typhimurium are important cause of food-borne illness. Based on surveillance studies, the main vehicles of *S.* enteritidis and *S.* typhimurium infection include raw meat, eggs and poultry products (Kramarenko et al., 2014, Thung et al., 2016). Due to the frequency of antimicrobial resistance and the number of resistance determinants in Salmonella have raised markedly,

vaccination plays an important role in the overall bio-security system on animal farms, typically chicken farms to prevent Salmonella infections (Jawaleet al.,2012). Vaccination is one of the methods suggested to reduce the burden of *S.* typhimurium in the poultry industry; it is expected to increase the resistance of chickens to infection and to prevent spread of bacterial micro-organisms to human society through the food production chain (Barrow 2007). There are numbers of live and killed *S.* enteritidis vaccines used commercially worldwide with some success. Inactivated *S.* enteritidis vaccine had been developed and can confer partial protection against intestinal colonization, fecal shedding, systemic spread and egg contamination (Khan et al., 2003, Nakamura et al., 2004, Haider et al., 2007, Cima 2010).

Avian influenza virus (AIV) subtype H9N2 have become established in multiple avian species across large geographical areas including Asia, Middle East and Africa (Fusaroet al., 2011). AIV subtype H9N2 is categorized as a low pathogenic avian influenza (LPAI) virus, it can cause serious economic losses in poultry industry including reduced egg production and decreased growth rate. In Egypt, AIV (H9N2) has already detected and sequenced from samples obtained from live birds in 2003 and submitted through NAMMRU3 to SEPRL, USA to complete characterization (Arafaet al., 2012). It was reported in Egypt at first time among broilers and layer breeders by Hussein and El-Azab (2001). Vaccination using inactivated local field strains of H9N2 viruses has been recently implemented.

Newcastle disease virus (NDV) is a non-segmented negative sense RNA single-stranded virus that belongs the Paramyxoviridae family; serotype 1 (APMV-1) can cause huge economic losses in the poultry industry, many countries make great efforts to control the virus (Alexander 2000). Newcastle Disease Virus (NDV) was considered as one of the most devastating poultry infections, owing to its worldwide distribution and economical threat, NDV is one of the most important viral diseases of poultry industry of most countries by causing avian pneumoencephalitis and other fatal diseases (Alexander 2003, OIE 2014). NDVs have been categorized into lentogenic, mesogenic, and velogenic strains according to disease severity in chickens (Alexander 1997, Miller and Koch 2013). The NDV LaSota strain, a naturally low-virulent NDV strain, has been routinely used as a live NDV vaccine during the course of the world (Goldhaft 1980). Vaccination of poultry provides an excellent means to lessen clinical signs of infection caused by virulent Newcastle disease virus (NDV) (Alexander and Senne 2008). Using of a genotype II strain, as a vaccine exemplary (live and inactivated strain) could protect against morbidity and mortality, but did not prevent infection and virus shedding. Recently, NDV vaccines from viruses phylogenetically closer to potential outbreak viruses may deliver better ND control by decreasing virus shedding from infected birds (Miller et al., 2007).

So the main objective of our study is to prepare and evaluate a combined tetravalent vaccine containing *Salmonella* enteritidis, *Salmonella* typhimurium, inactivated NDV LaSota strain and LPAI-H9N2 viruses in poultry where combined vaccines have the advantage of protecting against more than one disease at the same time, besides, reducing vaccination expenses, decreasing the stress of vaccination for different vaccines.

MATERIALS AND METHODS

Bacteria and viruses strains:

*S.*typhimurium and *S.* enteritidis strain are local strains obtained from Bacterial Sera and Antigen Research Department at Veterinary Serum and Vaccine Research Institute, used for preparation of the vaccine and challenge of test birds.

The local Egyptian AIV-H9N2 strain (A/chicken/ Egypt/ D4692A/ 2012) was provided by the National Research Center (NRC), Enviromental Research Division, Egypt, in the form of infected allantoic fluid with an original titer of $10^{10.5}EID_{50}$/ml (egg infective dose 50/ ml) and 10 Log_2 HAU. The virus was propagated in 9- 11 day old SPF-ECE eggs according to(Garcia et al., 1998) and EID50 was calculated according to Reed and Mennch (1938).

Lentogenic NDV strain LaSota was provided by the Central Veterinary laboratories, New Haw, Weighbridge, Surry, UK in the form of lyophilized infected allantoic fluid with an original titer of $10^{10.5}$ EID_{50}/ml and 10 Log2 HAU. The virus was rehydrated in 1 ml sterile PBS and was used for preparation of the monovalent and tetravalent inactivated vaccines.

Challenge viruses:

AI-H9N2 and NDV isolates were used as challenge virus. They were obtained from strain bank at Central Laboratory for Evaluation of

Veterinary Biologics (CLEVB) and identified by National Laboratory for Quality control of Poultry (NLQP) for AI-H9N2 as A / chicken / EG / 16194V /2016 of accession No KU296207 and for NDV as NDV-B7-RLQP-CH-EG-12 of accession No KM288609. Titer 10^6 EID_{50} / 0.1 ml per bird for both AI-H9N2 and NDV viruses and administered intra-nasal for AI-H9N2 and intra-muscular for NDV.

Specific pathogen free emberyonated chicken eggs (SPF-ECE):

Specific pathogen free embryonated chicken eggs (SPF–ECE) were purchased from the Specific Pathogen Free Egg Project, KomOshim, El-Fayoum Governorate, Egypt. The eggs were incubated at 37°C and 80% humidity until inoculated at 9-11 days of age via allantoic sac route and used for virus propagation, virus titration, and assurance of complete inactivation.

Chickens:

Four hundred and ninety, 1 day-old SPF chickens were obtained from Nile-SPF farm, KomOshimproject , El-Fayoum Governorate, Egypt. The chickens housed in SPF isolator units in specific CLEVB animal care building with water and feed provided *ad-libitum*. At 3 weeks of age, blood samples were collected for serological examination to insure their freedom from maternally derived antibodies against *SE, ST*, AI-H9N2 and NDV. Three hundred and ninety birds used for experimental design and one hundred birds used for safety test.

Preparation of monovalent *Salmonella* enteritidis and *Salmonella*typhimurium vaccines according to Charles et al.,(1994):

*Salmonella*typhimurium and *Salmonella*enteritidis local strains were grown separately on S.S ager for 24 hrs at 37°C. Separate colonies from each type were selected and inoculated on tryptone soya broth and incubated at 37°C for 24 hrs. Then each bacterial suspension was centrifuged at 5000 rpm at 4°C for 30 min. to pellet the bacterial strain. A separate final suspension from each of *S.* typhimurium and *S.* enteritidis was prepared and the bacterial count was adjusted for each type to 10^{10} CFU/0.5ml of final product using total colony count technique. The bacteria were then inactivated by adding 0.3% formalin at 37°C for 24-48 hrs with agitation for 24 hrs. and then cultivated on S. S agar media to assure complete inactivation .

Inactivation of viruses:

Inactivation of AI-H9N2 and NDV (Lasota) viruses was carried out using formalin in a final concentration of 0.1%. The fluid was blended using magnetic stirrer for about 18 to 20 hrs at 25°C to ensure freedom from live virus. Then, sodium bisulfite was added in a final concentration of 2% to stop the action of formalin. Samples from each inactivated virus were tested for complete inactivation by inoculation in 10-day-old SPF ECE for two successive blind passages (OIE, 2014).For completion of inactivation, the prepared vaccines were inoculated in 9-11 days old SPF-ECE (30 eggs for each vaccine) via allantoic cavity. The inoculated eggs were incubated for 7 days post inoculation with daily candling. The allantoic fluids were harvested and examined for HA activity. Negative HA-allantoic fluid from live and dead embryos were pooled separately and re-inoculated for 2nd and 3rd passages to ensure freedom from residual infectivity of any of the viruses.

Vaccine formulation (Antigen emulsification):

Four monovalent inactivated vaccines were prepared (monovalent inactivated vaccines each of H9N2, NDV, *S.*typhimurium, and *S.*enteritidis) and a combined tetravalent vaccine containing (*S.*enteritidis, *S.*typhimurium, LaSota strain of NDV and LP H9N2 strain of AIV) was prepared as an oil emulsion vaccine using Montanide™ISA 71 VG adjuvant (SEPPIC) in a ratio of 71 adjuvant: 29 antigen.Merthiolate (Thiomersal) was used in a final concentration of 1:10,000 as a preservative.

Quality control of the prepared vaccines:

The prepared monovalent and tetravalent combined oil emulsion vaccines were tested for sterility, safety, complete inactivation and potency according to the Standard International Protocols as described by the (OIE, 2017).

Sterility test:

The prepared vaccines candidate were tested for sterility (freedom from any bacterial, fungul, Mycoplasma contaminants) by culturing on thioglycolate broth, MacConkey and nutrient agar, incubated at 37°C for 72 hrs., and inoculation of 1 ml of the prepared vaccines on sabaroud maltose agar and incubated at 25°C for 15 days. This procedure was carried out before and after emulsification of the prepared vaccines.

Safety Test:

The prepared vaccines were tested for safety by S/C inoculation of 1ml (double recommended dose) for each prepared vaccine in twenty, 3-week-old SPF chickens, the birds were observed for 21days for any possible local or systemic adverse reaction of each vaccine.

Experimental Design for evaluation of potency test of prepared vaccines:

The three hundred and ninety, 3 week old SPF broiler chickens were divided into 7 groups each of 30 chickens except groups (5 and 6) each of 120 birds, all chickens injected S/C with 0.5 ml of previously prepared vaccines except the control –ve group which left unvaccinated:

Group 1: injected with monovalent inactivated *S.* typhimurium vaccine.

Group 2: Injected with monovalent inactivated *S.* Enteritidisv accine.

Group 3: Injected with monovalent inactivated H9N2 vaccine.

Group 4: Injected with monovalent inactivated NDV vaccine.

Group 5: Injected with combined tetravalent inactivated vaccine.

Group 6: kept as non-vaccinated challenged (control +ve)group.

Group 7: kept as non-vaccinated non challenged (control –ve)group.

On 7 weeks-old (4 WPV), groups 1 – 5 were boostered with the same type of vaccine and the same rout and dose. Blood samples were collected weekly (until 4weeks post boostering) from all groups to check antibody titers against *SE* &*ST* using Microagglutinationtest (MAT) and against AI-H9N2 & NDV using Hemagglutination inhibition test(HI) test. Serum samples were obtained regularly before immunization, weekly for 4 weeks after the 1st vaccination and every week post boostering for 4 weeks., then pooled and stored at -20 °C till used for following up the induced antibodies.

Microagglutination test (MAT):

Antibody titer in vaccinated and unvaccinated chickens was followed up on regular intervals post vaccination applying Micro-agglutination test (MAT) using sonicated antigen, according to the method described by Thaxtonet al., (1975) and Brown et al., (1981).

Hemagglutination test (HA) and Hemagglutination inhibition test (HI):

The hemagglutination (HA) and hemagglutination inhibition (HI) test were carried out following the recommendation ofOIE, (2004) using 4 HAU of homologous antigen (H9N2 AIV, and NDV Lasota strain), to estimate antibody titers in sera of vaccinated and unvaccinated chickens. HI test was carried out to AI-H9N2 vaccine according to Allan and Gough (1976)in a V-bottomed micro titer plate to determine the haemag glutinating titer (expressed as HAU) of virus which was collected from SPF-ECE after propagation. HI test for NDV was carried out using 4 HAU of homologous antigen (AI-H9N2), according to Majujabe and Hitchner (1977), to estimate antibody titers in sera of vaccinated and unvaccinated chickens.

Potency and Challenge test with *S.*typhimurium and *S.* enteritidis:

Birds of groups (1, 2, 5 and 6) were challenged 4 weeks after the booster dose by oral administration of 1ml of broth culture containing 10^8 CFU of each strain *S.*typhimurium, *S.* enteritidis, and combined two strains, respectively (Paivaet al. 2009). The inoculated chickens were observed for one month. The degree of protection was assessed according to the severity of the clinical signs, the mortality and the recovery of the challenge organisms from fecal samples.

Detection of the shedding of *S.*typhimurium and *S.*enteritidis:

One week after the challenge and for 4 weeks, cloacal swabs were collected from each of the infected as well as control group and examined bacteriologically for the presence of *S.*typhimurium and *S.* enteritidis organisms. Each swab was transferred to tetrathionate broth and incubated overnight at 37°C. A loopful from the broth was streaked on S.S agar for Salmonella isolation. Suspected colonies were identified morphologically and biochemically.

Potency and challenge test with NDV and AI-H9N2 (OIE, 2017):

Vaccinated groups (3, 4, 5 and 6)were subjected to challenge test as following:

Group (3): challenged with AI-H9N2 via I/N route at a dose of 0.1 ml/bird.

Group (4): challenged with NDV via I/M route at a dose of 0.1 ml/bird.

Group (5): 2 subgroups were challenged with AI-H9N20.1 ml/ bird I/N. and other subgroup was challenged with NDV 0.1 ml /bird I/M.
Group (6): Unvaccinated control +ve group: 2 subgroups were challenged with AI-H9N20.1 ml/ bird I/N. and other subgroup was challenged with NDV 0.1 ml /bird I/M.
Group (7): Unvaccinated, unchallenged control –ve group.
The challenge test was carried out 4weeks post boostering for all groups. The challenged groups were observed for 10 days post challenge, where clinical signs and mortalities were recorded.

Detection of shedding of AI-H9N2 and NDV viruses:

On 10 days post-challenge, birds from group 3, 4, 5 and 6 were sacrificed, and the samples of trachea and cecal tonsils were collected from each group to detect the AI-H9N2 and NDV. Virus was isolated using QIAampMinElute Virus Spin Kit (Qiagen, Hilden, Germany). RT-PCR was carried out according toShabat et al., (2010). Via Quantinova SYPR green rt-pcr kit (Dusselddorf, Germany).

RESULTS AND DISCUSSION

In this study, the obtained results of sterility tests revealed that all the prepared vaccines candidates (either monovalent or tetravalent) were free from any aerobic, anaerobic, fungal and mycoplasmal contaminants.

Regarding to safety of prepared vaccines reported neither signs of local reactions as abscess formation, irritation nor systemic reaction.

Concerning to completion of inactivation there was no lesion in inoculated embryos that means no live residual virus or salmonella in the prepared vaccine and no signs or lesions were noticed in the inoculated birds as the recommendation of OIE, (2014), showingHA negative results against other than AI-H9N2 and NDV.

Data in tables (1and 2) show that antibody titers against *S.*typhimurium and *S.*enteritidis in groups (1 and 2) respectively, as measured by microagglutination test raised from zero before vaccination to (40) for both groups (1 and 5) vaccinated with monovalent and combined tetravalent*S.*typhimurium vaccines respectively, at 1st week post vaccination (wpv) and increased up to (160 and 320) in 4thwpv in both groups respectively. After boostering, slight reduction in the HI titers were recorded on 1st week post boostering (wpb) indicating partial neutralization. Then return to increase from 2nd wpb till reaching (1280) at 4th week post-boostering in groups (1 and 5). While the mean Log_2 MAT antibody titers against *S.* enteritidis were (20) at 1stwpv in groups (2 and 5), the titers increased up to (160) in both groups (2 and 5) at 4thwpv, till reached (640) in both groups at 4th wpb.

These titers proved protective levels as the chicken challenged with live strains, four weeks post-boostering (table 3) and showed 83.3% and 80% protection against *S.*typhimurium and *S.* enteritidis in monovalent vaccines respectively, compared to 86.3% protection in tetravalent vaccine. These levels are relatively higher than those recorded by other researchers; (Ibrahim et al., 2018 and Salama et al., 2018**)** who recorded a protection of 80% and 82.5%, respectively in trivalent vaccines against *S.*typhimurium and *S.*enteritidis and *S.*Kentuky.

Fecal shedding post challenge was also significantly reduced in the vaccinated chickens compared with those in the unvaccinated suggesting that the vaccine could be effective against *S.*typhimurium and *S.* enteritidis. Table 4 showed that the fecal shedding of*S.*typhimurium in group 1 vaccinated with monovalent *S.*typhimurium vaccine was (10.7%, 7.14% and 3.5%) in 1st, 2nd, 3rd week post challenge, respectively. Also the results of fecal shedding of monovalent *S.* enteritidis vaccine group 2 were (14.28%, 10.7% and 7.4%) in the same times respectively. Shedding disappeared by the 4th week post challenge. Regarding fecal shedding of both *S.*typhimurium and *S.* enteritidis in group 5 vaccinated with tetravalent vaccine, they were 10.7 % and 7.14% in 1st and 2nd week and disappeared completely in 3rd week post challenge. Reducing or controlling intestinal colonization and fecal shedding associated with salmonella infection could be useful in several contexts. As intestinal colonization is the first step in the process by which orally introduced salmonella cells disseminated systemically and are deposited in eggs, any protection against colonization should presumably affect the frequancy of egg contamination. Moreover, the shedding of salmonella into laying houses also fosters horizontal transmission to other hens, so that any reduction in the number of *S.*typhimurium and *S.* enteritidis cells shed in feces should help control the spread of infection within and between houses Gastet al., (1993**).**

Table (1): Mean Log_2 MAT antibody titers against *S.*typhimurium and *S.* enteritidis in sera of chickens groups (1 and 2).

Weeks post vaccination	*S.*Typhimurium Group 1	*S.* enteritidis Group 2	Control group
0	0	0	0
1stWpv	40	20	0
2ndWpv	40	40	0
3rdWpv	80	80	0
4thWpv	160	160	0
Boostering			
1st Wpb	80	80	0
2nd Wpb	160	160	0
3rd Wpb	640	320	0
4th Wpb	1280	640	0

Gr. 1: vaccinated with monovalent *S.*typhimurium vaccine.
Gr. 2: vaccinated with monovalent *S.*enteritidisvaccine
Wpv: weeks post vaccination
Wpb: weeks post boostering

Table (2): Mean Log_2 MAT antibody titers against *S.*typhimurium and *S.*enteritidis in sera of chickens group 5.

Weeks post vaccination	*S.* Typhimurium Group. 5	*S.* enteritidis Group. 5	Control group
0	0	0	0
1stWpv	40	20	0
2ndWpv	80	40	0
3rdWpv	160	80	0
4thWpv	320	160	0
Boostering			
1st Wpb	160	80	0
2nd Wpb	320	160	0
3rd Wpb	320	320	0
4th Wpb	1280	640	0

Gr. 5: vaccinated with combined tetravalent vaccine.
Wpv: weeks post vaccination
Wpb: weeks post boostering

Table (3): Efficacy of the prepared vaccines against *S.* typhimurium and *S.* enteritidis in SPF challenged chickens with virulent strains

Group	No. of inoculated chickens	No. of dead and/or diseased	No. of survived chickens	Protection %
Group 1	**30**	**5/30**	**25/30**	**83.3**
Group 2	**30**	**6/30**	**24/30**	**80**
Group 5	**30**	**4/30**	**26/30**	**86.3**
Control group	**30**	**26/30**	**4/30**	**13.3**

Gr. 1: vaccinated with monovalent *S.*typhimurium vaccine.
Gr. 2: vaccinated with monovalent *S.*enteritidisvaccine.
Gr. 5: vaccinated with combined tetravalent vaccine

Although the use of inactivated vaccines to control AI-H9N2 is the most preferable method for vaccination to avoid the antigenic shift and/or drift, several reports have been published noting the continuous evolution of the virus in vaccinated birds (Choi et al., 2004). The most important part of immune response for controlling of AIV is the humeral immunity (Wareingand Tannock, 2001).Our results revealed earlier and high HI titer induced by medium dose (0.5 ml) of the prepared vaccines. Analysis of the results in table (5) showed that chickens in vaccinated groups (3 and 5) showed gradual increasing in the mean Log_2 anti-AI-H9N2 hemagglutination inhibition antibody titers from 1stwpv (3.8 and 4.4Log_2), that increased up to (8.6 and 8.8Log_2), respectively in 4th wpv. After boostering, the mean Log_2 anti- AI-H9N2 reached the highest HI antibody titer at

4th week post boostering in vaccinated chickens (9.4 and 9.8 Log_2) with inactivated monovalent and tetravalent AI-H9N2 vaccines, respectively.

Ultimately, both ND monovalent and tetravalent vaccines prompted significant humeral immune response as measured by HI assay, the specific HI titers increased from the 1st wpv (4.2 Log_2), that increased up to (9.8 and 10.2 Log_2) respectively, in 4th wpv (table 6), which showed the significant increase in ND specific HI titers of groups (4 and 5) as (10.6 and 11.2 Log_2) in 4th wpb, respectively. These results agreed with Zhao et al., (2017**)** who reported that at the 4th wpv with an inactivated bivalent vaccine against the prevalent strains of Newcastle disease and avian influenza H9N2, the peak levels of HI antibodies were detected with a mean HI titre against NDV and H9N2.

On the other hand, the protection percentage obtained on challenge by AI-H9N2 was 100% protection and no signs were observed while the control challenged unvaccinated group with AI-H9N2 showed gasping, coughing, nasal and ocular discharge, depression, inappetance, weakness and were relactant to move and 9/30 were died post- challenge by 30% mortality. Whereas unvaccinated unchallenged birds survived without showing clinical signs. Regarding to NDV protection percent of the prepared vaccines against challenge with NDV was 100% in both monovalent and tetravalent NDV vaccines while unvaccinated challenged birds challenged with pathogenic NDV showed greenish diarrhia, depression, conjunctivities and nervous manifestations and 27/30 were died post-challenge by 90% mortality approving and efficacy of the prepared vaccines. These results agreed with Zhao et al. (2017)who mentioned that all of the unvaccinated chickens challenged with H9N2 and NDV showed 100% mortality rate and high levels of shedding, compared with no mortality and a lower rate of viral shedding in vaccinated groups. Also agreed withLee et al., (2013) who proved that the bivalent vaccine against NDV and HPAI H5N1 induced high titers of both HPAI H5 and NDV-specific antibodies and afforded complete protection against lethal challenge with HPAI H5 and NDV. To evaluate the efficacy of the prepared vaccines it must reduce the shedding of bacterial and viral componants compared with group that have challenged with virulent strains only (positive control groups). The virus shedding from the infected chickens consider the main source of virus maintenance in the enviroment as well as magnifying the disease problem (Hussein et al., 2014 and Miller et al., 2009). Thus one of the most important role of the viral vaccines is reduction of the virus shedding which positively correlated to vaccine efficacy (Kapczynskiet al., 2015). Likewise, Table (7 and 8) showed that in our study the prepared tetravalent vaccine induced reduction in both AI-H9N2 and ND challenge viruses shedding compared to the control unvaccinated group and mono-vaccinated groups. Whereas, the NDV shedding were detected by rRT–PCR from both cloacal and tracheal samples where the cloacal shedding were prevented by monovalent NDV vaccine as well as tetravalent AI-H9N2 vaccine the results come in parallel with OIE, (2014) and El-Naggaret al., (2017), while in tracheal shedding the CT of the bivalent vaccine (CT=36) was more later that of monovalent vaccine (CT=32) as shown in table (7). On the other hand the AI-H9N2 shedding give similar results as NDV as the CT values delayed from 35 in monovaccinated group to 38 in tetra-vaccinated one.

In this study the performance of adjuvant prepared vaccines evaluated by detecting the level and duration of humoral immune response. Those parameters were investigated for each of the prepared vaccines by monitoring antibodies in the sera collected from vaccinated groups for 4 weeks post boostering.

Polyvalent vaccine strategies increase reactivity for many pathogens including, but not limited to, influenza (Crevar and Ross, 2008) and (Fiore et al. 2010) although polyvalent vaccine formulations clearly expand the breadth of a single vaccine formulation, the reactivity is still limited to the individual components. Vaccines and other alternative products are central to the future success of animal agriculture because they can help minimize the need for antibiotics by preventing and controlling infectious diseases in animal populations.

It could be concluded from our study that the tetravalent prepared vaccine can reduce *S.*typhimurium *S.* enteritidis, AI-H9N2 and NDV infections reducing the load on the poultry sector by reducing stress for the worker and the birds.

Table (4): Fecal shedding of *Salmonella* typhimurium and *Salmonella* enteritidis from challenged chickens:

Group	No. of birds positive for isolation/total number of living birds			
	1st week	2nd week	3rd week	4th week
Group 1	3/28(10.7%)	2/28(7.14%)	1/28(3.5%)	0/28(0%)
Group 2	4/28(14.28%)	3/28(10.7%)	2/27(7.4%)	0/27(0%)
Group 5	3/28(10.7%)	2/28(7.14%)	0/28(0%)	0/28(0%)
Control group	7/15(46.6%)	5/10(50%)	2/6(33.3%)	1/3(33.3%)

Table (5): Mean Log_2HI (H9N2) antibody titers in vaccinated chickens with inactivated monovalent and tetravalent combined vaccines.

Weeks post Vaccination	Mean Log_2 HI titers in chickens vaccinated with		
	Inactivated monovalent (H9N2) vaccin (Group 3)	Tetravalent Combined vaccine (Group 5)	Control
1stWpv	3.8	4.4	Less than 2
2ndWpv	6.4	6.6	Less than 2
3rdWpv	7.8	8.2	Less than 2
4thWpv	8.6	8.8	Less than 2
	Boostering		
1st Wpb	8.4	8.2	Less than 2
2nd Wpb	8.8	8.8	Less than 2
3rd Wpb	9.0	9.2	Less than 2
4th Wpb	9.4	9.8	Less than 2

Gr. 3: vaccinated with monovalent AI-H9N2 vaccine.
Gr. 5: vaccinated with combined tetravalent vaccine.
Wpv: weeks post vaccination
Wpb: weeks post boostering

Table (6): Mean Log_2HI (NDV) antibody titers in vaccinated chickens with inactivated monovalent and tetravalent combined vaccines.

Weeks post Vaccination	Mean Log_2 HI titers in vaccinated chickens with		
	Inactivated monovalent (NDV) vaccine (Group 4)	Tetravalent Combined vaccine (Group 5)	Control
1stWpv	4.2	4.2	Less than 2
2ndWpv	6.2	6.8	Less than 2
3rdWpv	8.4	8.2	Less than 2
4thWpv	9.8	10.2	Less than 2
	Boostering		
1st Wpb	9.8	10.0	Less than 2
2nd Wpb	10.0	10.6	Less than 2
3rd Wpb	10.4	10.8	Less than 2
4th Wpb	10.6	11.2	Less than 2

Gr. 4: vaccinated with monovalent NDV vaccine.
Gr. 5: vaccinated with combined tetravalent vaccine.
Wpv: weeks post vaccination
Wpb: weeks post boostering

Table (7): NDV results of Real time PCR for qPCR reaction.

Organs / Groups	Cloacal shedding	Trachea
Group 7	No amplification detected	No amplification detected
Group 3	No amplification detected	+ve at CT 20
Group 4	No amplification detected	+ve at CT 32
Group 5	No amplification detected	+ve at CT 36
Group 6	+ve at CT 24	+ve at CT 20

Group 3: vaccinated with monovalent H9N2 vaccine
Group 4: vaccinated with monovalent NDV vaccine
Group 5: vaccinated with combined tetravalent vaccine
Group 6: +ve control group
Group 7: -ve control group

Table (8): H9N2 results of Real time PCR for qPCR reaction.

Organs / Groups	Cloacal shedding	Trachea
Group 7	No amplification detected	No amplification detected
Group 3	No amplification detected	+ve at CT 35
Group 4	No amplification detected	+ve at CT 34
Group 5	No amplification detected	+ve at CT 38
Group 6	No amplification detected	+ve at CT 30

Group 3: vaccinated with monovalent H9N2 vaccine
Group 4: vaccinated with monovalent NDV vaccine
Group 5: vaccinated with combined tetravalent vaccine
Group 6: +ve control group
Group 7: -ve control group

CONCLUSION

It could be concluded from our study that the tetravalent prepared vaccine can reduce S. typhimurium, S. enteritidis, AI-H9N2 and NDV infections reducing the load on the poultry sector by reducing stress for the worker and the birds.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

ASM designed the concept of the review article. ASM and WRA wrote the manuscript. All the authors designed and performleed the experiments and reviewed the manuscript. All authors read and approved the final version.

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REFERENCES

Alexander DJ, 1997. Newcastle disease and other avian paramyxoviridae infections. In: Calnek

BW, Barnes HJ, Beared CW, McDougald LR, Saif YM, editors. Diseases of Poultry. Iowa: Iowa State University Press. Pp: 541–569.

Alexander DJ, 2000. Newcastle disease and other avian paramyxoviruses. Rev. Sci. Tech., 19: 443–462.

Alexander DJ, 2003. Report on avian influenza in the Eastern Hemisphere during 1997-2002. Avian Dis., 47: 792-797.

Alexander DJ, and Senne DA. 2008. Newcastle Disease Virus and other Avian

Allan WH and Cough RE, 1976. A comparison between the haemagglutination inhibition and complement fixation tests for Newcastle disease. Vet. Sci. 20: 101-103.

Arafa A, Hagag N, Erfan A, Mahdy W, El-Hussieny M, Adel A and Nasef S, 2012. Complete genome characterization of avian influenza virus subtype H9N2 from a commercial quail flock in Egypt. Virus Genes, 45: 283-294.

Barrow PA, 2007. *Salmonella* infections: Immune and non-immune protection with vaccines. Avian Pathol., 36 (1): 1–13.

Brown SL, Kiln FT and Jones WL 1981. Safranin O stained antigen microagglutination test for detectionofbrucella antibodies. J. Clin. Microbiol., 13: 398–400.

Charles SD, HussainI, Choi K V,Nagaraja, and SivanandanV,1994.Adjuvanted subunit vaccines for the control of Salmonella Enteritidis infection in turkeys. Am. J. Vet. Res., 55: 636–642.

Choi YK, Ozaki H, Webby RJ, Webster RG, Peiris JS, Poon L, Butt C, Leung YHC and Guan Y, 2004. Continuing Evolution of H9N2 Influenza Viruses in Southeastern China. J. Virol., 78: 8609-8614.

Cima G, 2010. Vaccine use up since *Salmonella* outbreak. J. Amer. Vet. Med. Associ., 237 (12): 1356–1357.

Crevar CJ and Ross TM. 2008. Elicitation of protective immune responses using a bivalent H5N1 VLP vaccine. Virol. J., 5(131): 131.

El Naggar HM, Madkour MS and Hussein HA. 2017. Preparation of mucosal nanoparticles and polymer-based inactivated vaccine for Newcastle disease and H9N2 AI viruses, Veterinary World, 10(2): 187-193.

Fiore AE, Timothy MU, Karen B, Lyn F, James AS, Flavia Z, Analia B, Ariel P, Oscar T and Elis C. 2010. Prevention and control of influenza with vaccines. Recommendations of the Advisory Committee on Immunization Practices (ACIP), 59 (RR08): 1-62.

Fusaro A, Monne I, Salviato A *et al*. 2011. Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. J Virol., 85: 8413-8421.

Garcia A, Johnson H, Srivastava AD, Wehr RD, and Webster GR 1998.Efficacy of inactivated H5N2 influenza vaccine against lethal A/chicken/quereta.v/19195 infection. Avian Dis., 42: 248-256.

Gast RK, Stone HD and Holt PS. 1993. Evaluation of the efficacy of oil-emulsion bacterins for reducing fecal shedding of *S*. enteritidis by laying hens. Avian Dis., 37 (4): 1085–1091.

Goldhaft TM. 1980. Historical note on the origin of the LaSota strain of Newcastle disease virus. Avian Dis., Apr-Jun; 24(2): 297–301.

Haider MG, Rahman MM, Hossain MM, Rashid M, Sufian MA, Islam MM. and Haque AF. 2007. Production of formalin killed fowl typhoid vaccine using local isolates of *Salmonella* gallinarum in Bangladesh. Bangladesh J. Vet. Med., 5 (1 & 2): 33–38.

Hussein HA and El Azab A. 2001.Evidence for the presence of H6 and H9 among broiler and layer breeders in Egypt.Proce. XII. Int. Cong. World. Vet. Poultry Assoc. Cairo-Egypt, pp: 227-235.

Hussein HA, Emara MM and Rohaim MA. 2014. Molecular characterization of newcastle disease virus genotype VIId in avian influenza H5N1 infected broiler flock in Egypt. International Journal of virology, 10(1): 46-54.

Ibrahim HM, Sayed RHandShereen AM. 2018. Efficacy of a locally prepared inactivated trivalent vaccine against salmonellosis in poultry. Inter. J. Vet. Sci., 7(2): 82-87.

Jawale CV, Chaudhari AA, Jeon BW, Nandre RM and Lee JH. 2012. Characterization of a novel inactivated *Salmonella enterica*serovarenteritidis vaccine candidate generated using a modified cI857/λPR/gene E expression system. Infection and Immunity, 80: 1502-1509.

Kapczynski DR, Esaki M, Dorsey KM, Jiang H, Jackwood M and Gardin M. 2015. Vaccine protection of chickens against antigenically diverse H5 highly pathogenic avian influenza isolates with a live HVT vector vaccine expressing the influenza hemagglutinin gene derived from a clade 2.2 avian influenza virus. Vaccine, 33: 1197- 1205.

Khan MI, Fadl AA and Venkitanarayanan KS.

2003. Reducing colonization of *S*. enteritidis in chickens by targeting outer membrane proteins. J. Appl. Microbiol., 95: 142–145.

Kramarenko T, Nurmoja I, Kärssin A, Meremäe K, Hörman A and Roasto M. 2014. The prevalence and serovar diversity of *Salmonella* in various food products in Estonia. Food Control, 42: 43-47.

Lee DH, Park JK, Kwon JH, Yuk SS, Erdene-Ochir TO.*et al*. 2013. Efficacy of single dose of a bivalent vaccine containing inactivated Newcastle disease virus and Reassortant highly pathogenic avian influenza H5N1 virus against lethal HPA1 and NDV infection in chickens. PLOS ONE 8(3): e58186, DOI: 10.1371/ Journal. Pone. 0058186.

Majujabe KA and Hitchner SB. 1977.Antibody response to strain combination of Newcastle disease virus measured by haemagglutination inhibition test. Avian Dis., 21: 576-584.

Miller PJ and Koch G. 2013.Newcastle disease, other avian paramyxoviruses and avian metapneumovirus infections. In: Swayne DE, Glisson J, McDougald LR, Nolan LK, Suarez DL and Nair V, editors. Diseases of Poultry, Wiley-Blackwell, Hoboken, NJ. 13: 89-138.

Miller PJ, Estenez C, YuQ, Suarez DL and King DJ. 2009. Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. Avian Dis., 53: 39-49.

Miller PJ, King DJ, Afonso CL, Suarez DL. 2007. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine, 25(41): 7238–46.

Nakamura M, Nagata T, Okamura S, Takehara K and Holt PS. 2004.The effect of killed *Salmonella* enteritidis vaccine prior to induced molting on the shedding of *S*. enteritidis in laying hens. Avian Dis., 48: 183–188.

OIE manual 2004. Highly pathogenic avian influenza chapter: 2-71.

OIE., 2014. Avian Influenza OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Ch. 2.3.4. OIE, Paris, France.

OIE., 2017. *Terrestrial Animal Health Code.* Chapter: 6.5. Prevention, Detection and Control of Salmonella in poultry.

Paiva JB, Penha FRAC, Arguello YMS, *et al*. 2009. Efficacy of several *Salmonella* vaccination programs against experimental challenge with *Salmonella gallinarum* in commercial browen layer and broiler breeder hens. Brazil J. of Poult. Sci., 11(1): 65−72.

Paramyxoviruses. In: Swoyne DE, Glisson JR, Pearson JE, Reed WM, Jackwood MW, and Woolcock PR. A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 5[th]edn. American Association of Avian Pathologists, Kennett Square, Penn. Pp: 135-141.

Reed LJ and Muench H. 1938.A simple method of estimating fifty percent endpoint. The American Journal of Hygiene 27: 493–497.

Salama SS, Gamal FEZ, Gadallah F, EmanSoliman, Nourhan Nagy 2018. Estimation of protective indices in chicken vaccinated with single and booster doses of trivalent salmonella vaccine. J. Bacteriol. Mycol. Open Access. 6(3): 200–203.

Shabat MB, Meir R, Haddas R, Lapin E, Shkoda I, Raibstein . 2010. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. Journal of virological methods, 168 (1-2): 72-77.

Thaxton P, Wyatt RD, and Hamilton PB. 1975. The effect of environmental temperature on paratyphoid infection in the neonatal chicken. Poult. Sci., 53:88–94.

Thung TY, MahyudinN,Basri DF, Wan Mohamed Radzi, *et al*. 2016. Prevalence and antibiotic resistance of *Salmonella* enteritidis and *Salmonella* typhimurium in raw chicken meat at retail markets in Malaysia. Poultry Science 95: 1888-1893.

Wareing MD and. Tannock GA, 2001.Live attenuated vaccines against influenza; an historical review. Vaccine, 19: 3320-3330.

Zhao J, YangH, XuH, Ma Z and Zhang G. 2017. Efficacy of an inactivated bivalent vaccine against the prevalent strains of Newcastle disease and H9N2 avian influenza.Virology Journal.14:56 DOI 10.1186/s12985-017-0723-7.