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Original Research Article

EVALUATING THE EFFICACY OF A POLYVALENT INFECTIOUS CORYZA VACCINE AGAINST EMERGING *Avibacterium paragallinarum* SEROTYPES IN EGYPT

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Abstract: Infectious coryza (IC), an acute respiratory disease of chickens, is caused by *Avibacterium paragallinarum* (*A. paragallinarum)*. Herein, we evaluated the efficacy of a polyvalent oil adjuvant IC vaccine against the circulating *A. paragallinarum* field isolates recovered from chickens in Egypt. Thirty two *A. paragallinarum* isolates were recovered from 165 chicken samples (19.39%) then identified by phenotypic and polymerase chain reaction (PCR) assays. Serotyping by hemagglutination inhibition (HI) test revealed the identification of both serotypes A and C. The results of antimicrobial susceptibility testing affirmed that all *A. paragallinarum* isolates were multidrug-resistant (MDR) with multiple antimicrobial resistance (MAR) indices ranged from 0.6 to 0.8. Protection of chickens against *A. paragallinarum* field isolates of serotypes A and C using polyvalent inactivated oil adjuvant vaccine was evaluated followed by bacterial challenge. An experimental study of 90 specific-pathogen-free (SPF) White Leghorn chickens (8-weeks-old) was divided into nine groups; three groups served as non-vaccinated challenged, five groups were used for vaccine evaluation, and the last one was non-vaccinated and non-challenged group. Serum samples were collected regularly during 91 days post 1st vaccination to evaluate the immune response based on the HI antibody titer. The effective protection of the polyvalent inactivated vaccine against the drug resistant field isolates was indicated by the antibody titers that reached the maximum levels at the 5th week post vaccination. Moreover, 80 to 90% survivability percentages were recorded for *A. paragallinarum* serotypes C, and A field isolates, respectively. A strong positive correlation (R = 0.9628, P < 0.001) was found between the mean HI titer and the level of protection against the challenge. Furthermore, low level of bacterial shedding post-vaccination along with bacterial counts below the infectious dose ($2x10^2$ - $6x10^3$ CFU/mL) implying the role of vaccine in decreasing the chance of infection. Evidence from this study confirmed the effective protection of IC vaccine as a proper prophylactic tool against MDR *A. paragallinarum* field isolates of serotypes A and C.

Key words: *A. paragallinarium*; Infectious coryza; Vaccine; Hemagglutination inhibition

Introduction

Avibacterium paragallinarum (*A. paragallinarum*) or *Haemophilus paragallinarum (H. paragallinarum)* is a Gram-negative, non-motile coccobacillus of the family *Pasteurellaceae*. The bacterium is a fastidious slow-growing, needs nicotinamide adenine dinucleotide (NAD, factor V) for the *in vitro* growth (1). It is an important avian pathogen causing infectious coryza (IC); a cosmopolitan highly contagious acute respiratory disease in chickens (2). The disease is associated with a decrease in egg production of up to 40% in layers (3). It is normally acute and spreads rapidly with substantial morbidity of 60-80% in chicken flocks. The mortality may range from 1 to 15% and tends to increase when complicated by other infections (4).

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A. paragallinarum is known to be resistant to various antimicrobials, which has been confirmed to be related to multidrug-resistant plasmids (5).Thus, the antibiogram profile is important for controlling the negative economic impact of the disease on the poultry industry by selecting the most effective antimicrobial agents.

A. paragallinarum was classified into three serovars (i.e. A, B, and C) using hemagglutination inhibition test (HI) (6). The multiple serovars of the bacteria and the absence of cross-protection among them are suspected to be the cause of frequent failures of vaccination programs (7, 8). Inactivated multivalent vaccines are used worldwide for the control of IC, most of them comprising serovars A, B, and C (4).

The locally used SERVAC polyvalent oil adjuvant inactivated vaccine contains three antigens of *A. paragallinarum* representing the three above mentioned serovars. There is a previous trial for its evaluation in a combined vaccine for IC and salmonellosis (9). However, this vaccine has not been tested against drug-resistant field isolates to verify its efficacy. Sometimes the protective efficacy produced by the oil inactivated adjuvant vaccine is very low so, the commercial vaccines are unable to encounter the disease (10)

Due to the scare data about IC in Egypt, this study was designed to: i) determine the prevalence of *A. paragallinarum* in chickens and their emerging serovars; ii) assess the effectiveness of a polyvalent oil adjuvant IC vaccine in chickens by evaluating its efficacy against reference strains and recent circulating virulent field isolates based on HI titer, survival assay post-challenging and standard plate count technique.

Materials and methods

Clinical samples

A total of 165 samples were collected from six chicken farms at various Governorates in Egypt during the period from February 2019 to September 2020. The samples were collected from layer chickens showing conjunctivitis, ocular and nasal discharge, facial swelling and respiratory distress with marked drop in egg production, decrease of feed intake, and average 10% mortality rate. Swab samples were aseptically collected from different sites including infra-orbital sinuses, nasal cavities, trachea, lungs, and air sacs of infected (n= 101) or recently dead (n= 64) birds.

Bacteriological analysis, molecular identification, and serotyping of A. paragallinarum

Isolation of *A. paragallinarum* is difficult because of the fastidious and slow growth pattern of the organism and it could be recovered only during the acute stage of infection (11). Swab samples were inoculated in glycerol-phosphate buffer saline (Oxoid, UK) supplemented with Nicotinamide Adenine Dinucleotide (NAD; Oxoid, UK) at 37°C for 24h with 5% CO_2 (12). Thereafter, a loopful of the enrichment culture was streaked onto chocolate agar (CA; Oxoid, UK) and 5% blood agar (BA; Oxoid, UK) supplemented with NADH (V- factor, 25µg/mL; Oxoid, UK). A *Staphylococcus aureus* strain, kindly obtained from Microbiology Department, Faculty of Veterinary Medicine, Zagazig University, was cross streaked to the plate as a colony feeder. The plates were incubated for 24 h at 37°C with 5% CO_2 then examined for the growth of characteristics colonies.

The isolated organisms were identified on the basis of colonial appearance and Gram staining (13). Furthermore, the suspected isolates were characterized biochemically using the Analytical Profile Index (API) for *Haemophilus* species (API® NH; Biomeriux, France). The bacterial DNA was extracted from fresh isolates using a QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Species specific primer sequences for *HPG-2* gene; F: TGAGGGTAGTCTTGCAC GCGAAT and R: CAAGGTATCGATCGTCT CTCTACT were used for the amplification of 500-bp fragment of *A. paragallinarum* (14). PCR amplification was applied in a MJ, Research PTC-100 programmable thermal cycler (Waltham, USA) using the following conditions: initial denaturation at 95 ºC for 10 min, 35 cycles of denaturation at 95 ºC for 1 min, annealing at 55 °C for 1 min and extension at 72 ºC for 1 min then the final extension was set at 72 ºC for 7 min. Serotyping of *A. paragallinarum* isolates was performed at the Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo, Egypt using the hemagglutination inhibition (HI) test adopting a previously published protocol (15).

Antimicrobial susceptibility testing of A. paragallinarum isolates

The antimicrobial susceptibilities of *A. paragallinarum* isolates were tested on Mueller–Hinton agar media (Oxoid-CM0 337B, Cambridge, UK) supplemented with 5% sterile defibrinated sheep blood using the disc diffusion method (16). A panel of 10 standard antimicrobial discs (Oxoid, Cambridge, UK) within six different antimicrobial categories were examined including penicillins [ampicillin (AM; 10 µg) and amoxicillin (AX;25 µg)], aminoglycosides [gentamicin (CN; 10µg), amikacin (AK; 30µg), and streptomycin (S; 10 µg)], Fluoroquinolone [ciprofloxacin (CIP; 5 µg) and levofloxacin (IVX; 5 µg)], amphenicols [chloramphenicol (C; 30 µg)], sulfonamides [sulfamethoxazole-trimethoprim (SXT; 23.75 /1.25 µg)], and tetracyclines [tetracycline (TET; 30 µg)]. The results were interpreted following the Clinical and Laboratory Standards Institutes (CLSI) recommendations (17) and the instructions of Chukiatsiri et al. (18). The multiple antimicrobial resistance (MAR) indices were calculated as previously reported (19). Multidrug-resistance (resistance to three or more classes of antimicrobial agents) of bacterial isolates was determined as reported elsewhere (20).

Chickens and ethical approval

A total number of 90 Specific-Pathogen-Free (SPF) White Leghorn chickens (8-weeks-old) were purchased from a SPF farm at Fayoum Governorate, Egypt. All birds were ascertained first to be free from IC (organism and antibodies) then divided randomly into nine groups of 10 birds each; three groups were used as non-vaccinated challenged controls (I to III), five groups were used for vaccine evaluation (IV to VIII), while the last group was unvaccinated and non-challenged group (IX). This experiment was performed with the approval of the Animal Welfare and Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University (Approval number ZU-IACUC/2/F/53/2021).

Vaccine

SERVAC polyvalent oil adjuvant IC vaccine was obtained from the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), VSVRI, Abbassia, Cairo, Egypt to be used in the study. It is a formalin inactivated oil emulsion vaccine including antigens of *A. paragallinarum* serovars A, B, and C of doses 2.5 x 10^8, 3.96 x 10^8 and 3.96 x 10^8 colony forming units (CFU)/mL, respectively beside a dose of 1.43 x 10^8 CFU/mL of the local *A. paragallinarum* strain serovar A (http://vsvri.com/index.html).

Challenge bacteria

Reference strains of *A. paragallinarum* (serotypes A, B, and C) were kindly obtained from CLEVB, VSVRI, Abbassia, Cairo, Egypt. Besides, two field *A. paragallinarum* isolates of serotypes A and C recovered from clinically infected layers during the study were used.

Experimental design

Chickens in the vaccine evaluation groups (IV to VIII) were vaccinated at eight-weeks of age and boosted three weeks later before starting egg production. The administration was done slowly by subcutaneous injection of 0.5 mL SERVAC vaccine in the back of the neck. Placebo injection was performed using 0.5 mL phosphate buffer saline (PBS) to the other three non-vaccinated groups (I to III). The challenge was conducted two weeks post administration of the 1st vaccine dose by infra-orbital sinus inoculation of 0.2 mL of overnight broth cultures containing 10^7CFU/mL (21) each of the three reference strains (Groups IV, V, and VI), and the two field *A. paragallinarum* isolates (Groups VII and VIII). Negative control (Group XI) that did not receive vaccination or challenge was included. The experimental design, vaccination and challenging schedule are shown in Figure 1.

Blood sampling followed by serum separation was collected regularly during the 91 days post vaccination period as following; on day 0 (before vaccination), 0 day (after the first vaccination), 14 day (after the first vaccination, 0 day after challenge) and day 0, 14, 42, 70 (after booster vaccination) as shown on vaccination and challenging schedule in Figure 1. Serum samples were subsequently used for serological evaluation of the antibody responses of the experimental groups using the HI protocol, which was conducted using four HA antigen units and 1% glutaraldehyde-fixed chicken red blood cells (GA-RBCs), as previously described (22). Positive control serum for the HI test was kindly obtained from the CLEVB, VSVRI, Abbassia, Cairo, Egypt. Also, serum

from SPF chickens was used as the negative control. The titers were expressed as the reciprocal of the highest dilution of a serum sample that showed complete inhibition of the hemagglutinating activity (22).

Survival assay

After challenge, the chickens were examined daily for IC clinical signs, and the mortalities were recorded during the 77 days post-challenge period (23). Survivability was defined as the absence of mortality of IC i.e. calculate the number of chickens still alive

Shedding and quantification of A. paragallinarum in the experimental groups

Re-isolation of bacterial isolates after challenge test was performed to determine the evidence of *A. paragallinarum* shedding (24). Infraorbital sinus swabs from all groups at the 7th till 77th days post-challenge were streaked onto blood agar media and crossed with a *S. aureus* feeder strain. After incubation for 24 h at 37°C with 5% CO_2, the satellite growth in the plates was examined. The bacterial re-isolation from swabs detects the positivity of infection. The shedding percentage was calculated as number of infected samples/total number of samples. The re-isolated bacteria were serotyped by HI test to confirm the shedding of the same challenged serotypes. Quantification of *A. paragallinarum* was done using the plate count technique as documented previously (25). The results were expressed as CFU per mL, and their $\log_{10}$ values were then estimated.

Statistical model and analysis procedure

Hemagglutination inhibition antibody titers were analyzed using a general linear model procedure (two way analysis of variance) (26). The following model was applied:

$$Yijk = \mu + Gi + T_J + GT_K + e_{ijK}$$

Where, Yij = Observations, μ = Overall mean, G = effect of ith group (i, 1 to 8), T= effect of jth day (i, 0 to 91), GT effect of interaction between group and times, e_{ij} = random error. The statistical significance was accepted at $P < 0.05$. All results of HI antibody titers and *A. paragallinarum* counts were expressed as least square means. Shapiro-Wilk test was conducted in order to check for normality (27). The association between different groups and each of survivability and shedding rates was detected by Chi-Square test (χ^2). Graph pad prism software was used to calculate the protection percentage in all groups by Kaplan-Meier survival analysis (28).

Results

Prevalence of A. paragallinarum in layer chickens

The overall prevalence of *A. paragallinarum* in layer chickens was 19.39% (32/165). Fourteen out of 32 (43.75%) isolates were recovered from dead birds, whereas 18 (56.25%) isolates were obtained from live cases. Only two isolates were isolated from vaccinated birds (6.25%), and six isolates were recovered from diseased birds with a history of antibiotic treatment (18.75%). Characteristic tiny dewdrop colonies were observed adjacent to the feeder colonies (*S. aureus*) forming typical satellite growth patterns. On chocolate agar, smooth translucent dewdrop colonies were observed. In Gram staining, *A. paragallinarum* were observed as Gram-negative small rods or coccobacilli, polar staining, non-motile and non-sporulated bacteria. Typical isolates were biochemically confirmed as *A. paragallinarum* by API® NH. The species identification of the isolates was further confirmed by the PCR detection of *HPG-2* gene, which produced the predicted size of 500 bp amplicons. Serotyping of *A. paragallinarum* isolates by HI test revealed that all isolates were categorized into either serogroup A (28/32; 87.5%) or C (4/32; 12.5%) (Table 1).

Antimicrobial susceptibilities of A. paragallinarum

The *in vitro* antimicrobial susceptibilities of 32 *A. paragallinarum* isolates against 10 antimicrobial agents within different six antimicrobial categories are summarized in Table 2. The results revealed that all isolates from both serotypes were resistant to amoxicillin, ampicillin, amikacin, gentamycin, streptomycin, and chloramphenicol. Moreover, the overall levels of resistance recorded for ciprofloxacin, and tetracycline was 75% and 25%, respectively. In which 24 and four isolates from *A. paragallinarum* serotype A were resistant and intermediate susceptible to ciprofloxacin, respectively, while the four isolates of serotype C were intermediate susceptible to ciprofloxacin. All isolates of *A. paragallinarum* serotype

C were susceptible to tetracycline, while eight and nine isolates from serotype A were resistant and intermediate susceptible to tetracycline, respectively.

As presented in Table 1, the antibiogram analysis revealed that all *A. paragallinarum* isolates were MDR with MAR indices ranged from 0.6 to 0.8 and demonstrated three distinct resistance patterns. The resistance pattern AM, AX, AK, CN, S, C, CIP was the most prevalent among the analyzed isolates (n= 16/32; 50%).

Post-vaccinal hemagglutination inhibition titer

Hemagglutination inhibition titer was estimated during the 91 days post-vaccination in all experimental groups, following the administration of first and booster doses. The results of this immunization are given in Table 3 and Figure 2. Both vaccinated and non-vaccinated groups showed negative titers prior to the vaccination. The HI test on day zero before vaccination showed that the antibody titers of all chickens were very low. Also, no antibodies were detected in the non-vaccinated groups (I to III) throughout the duration of experiment. Immunized chickens (Groups IV to VIII) were able to generate a positive titer two weeks post first vaccination, while the HI antibody titer reached its peak two weeks post boostering. Based on the HI test results, the vaccine was able to produce good antibody titers that significantly differed among all vaccinated groups. HI antibody titers in the vaccinated groups slightly decreased at the 56th day after reaching its peak at the 35th day post vaccination with significantly difference ($P < 0.05$).

Survival assay and determination of the vaccine efficacy

As shown in Table 3, the HI antibody titer and survival assay were used to detect the level of developed immune response and the degree of protection after challenge. The HI titer is a denotative only for the good immune response after vaccination but, it is not an indicative whether the vaccinated groups confer protection against the challenging bacteria or not.

The results revealed that challenged chickens two weeks post first immunization were able to generate a significantly higher HI titer one week post challenge compared with unvaccinated birds, and this HI titer reached its peak three weeks after challenge then the titer was slightly decreased.

Of note, *A. paragallinarum* serotype A (the reference and field isolates) challenging the vaccinated groups had higher HI titer compared with serotypes B and C.

The birds were monitored for survival over 77 days post challenge. The survival percentages were calculated from the daily protection rates of the vaccinated and non-vaccinated groups (Table 3 and Figure 3). Following infra-orbital sinus challenge of the vaccinated groups, only 0 to 10% mortality rates were observed. On the other hand, 30, 40 and 50% of the challenged unvaccinated groups died. The vaccinated groups showed a protection rate ranging from 80- 100% at the 3rd week post challenge. On the other hand, the unvaccinated groups showed only 40-50%.

Overall, 70, 50, and 60% of the infected birds succumbed to infection then dead in G1, G2, and G3 unvaccinated groups, respectively. The mortality rate in the vaccinated birds of groups G4-G8 challenged with the reference and field isolates were significantly lower than those of the unvaccinated groups reaching only 10-30%. Thus, statistical analysis showed significant differences between vaccinated and unvaccinated groups in survival rates at all days post challenge, while the non-significant differences ($P > 0.05$) were detected between times within challenged groups.

Correlation between the survival rate and HI titers of chickens after challenge

However, the HI titer reached its peak three weeks after challenge then slightly decreased, the survival rate remained constant three weeks post challenge in the vaccinated groups. The mean HI titer and survival % have a strong positive correlation as shown in Figure 4 ($R = 0.9628$, $P < 0.001$). The mean HI titer of the vaccinated G4 and G7 (89.862 and 82.192, respectively) corresponds to a 90% survival rate. The HI titer in the vaccinated chickens of G6 and G8 (81.644 and 62.33, respectively) corresponded to survival rates 80%. The HI titer 54.306 in the vaccinated chickens of G5 corresponded to a 70% survival rate. However, the HI titer between 2-2.4 corresponded to survival rates of 30–50% were observed in the unvaccinated chickens.

Table 1: Characteristics, serotypes, and antibiotypes of *A. paragallinarium* isolated from laying chickens

Isolate No.	Bird case	Vaccine/antibiotic received	Serotype	Resistance pattern	MAR index
1	Live	-/ -	A	AM,AX,AK,CN,S,C,CIP	0.7
2	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
22	Live	+/-	A	AM,AX,AK,CN,S,C,CIP, TE	0.8
23	Dead	-/+	A	AM,AX,AK,CN,S,C,CIP, TE	0.8
5	Dead	-/-	C	AM,AX,AK,CN,S,C	0.6
3	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
4	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
6	Dead	-/-	C	AM,AX,AK,CN,S,C	0.6
7	Dead	-/-	C	AM,AX,AK,CN,S,C	0.6
8	Dead	-/-	C	AM,AX,AK,CN,S,C	0.6
9	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
10	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
11	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
31	Dead	-/-	A	AM, AX, AK, CN, S, C	0.6
32	Dead	-/-	A	AM, AX, AK, CN, S, C	0.6
12	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
13	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
30	Dead	-/-	A	AM, AX, AK, CN, S, C	0.6
14	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
24	Dead	-/+	A	AM,AX,AK,CN,S,C,CIP, TE	0.8
25	Dead	-/+	A	AM,AX,AK,CN,S,C,CIP, TE	0.8
15	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
16	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
17	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
21	Live	+/-	A	AM,AX,AK,CN,S,C,CIP, TE	0.8
18	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
19	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
27	Dead	-/+	A	AM,AX,AK,CN,S,C,CIP, TE	0.8
20	Live	-/-	A	AM,AX,AK,CN,S,C,CIP	0.7
26	Dead	-/+	A	AM,AX,AK,CN,S,C,CIP, TE	0.8
28	Dead	-/+	A	AM,AX,AK,CN,S,C,CIP, TE	0.8
29	Dead	-/-	A	AM, AX, AK, CN, S, C	0.6

AM, ampicillin; AX, amoxicillin; AK, amikacin; CN, gentamicin; S, streptomycin; CIP, ciprofloxacin; LVX, levofloxacin; TE, tetracycline; SXT, sulfamethoxazole-trimethoprim; C, chloramphenicol; MAR, multiple antibiotic resistance; -, negative; +, positive

Table 2: Antimicrobial resistance of *A. paragallinarum* serovars recovered from laying chickens

AMA	*A. paragallinarium* (n= 32)		MAR index
	Serovar A (n= 28)	Serovar C (n = 4)	
AM	28 (100%)	4 (100%)	0.1
AX	28 (100%)	4 (100%)	0.1
AK	28 (100%)	4 (100%)	0.1
CN	28 (100%)	4 (100%)	0.1
S	28 (100%)	4 (100%)	0.1
CIP	24 (85.7%)	0 (0%)	0.075
LVX	0 (0)	0 (0)	0.0
TE	8 (28.5%)	0 (0)	0.025
SXT	0 (0)	0 (0)	0.0
C	28 (100%)	4 (100%)	0.1

AMA, antimicrobial agent; AM, ampicillin; AX, amoxicillin; AK, amikacin; CN, gentamicin; S, streptomycin; CIP, ciprofloxacin; LVX, levofloxacin; TE, tetracycline; SXT, sulfamethoxazole-trimethoprim; C, chloramphenicol.
Data represented by No, (%).

Table 3: Hemagglutination inhibition titers means of experimental groups post vaccination and survivability% post challenge

Experimental groups	HI antibody titer (DPV)						*p*-value	Survivability % (DPC)					*p*-value
	0	14	21	35	63	91		0	7	21	49	77	
G1	1	2	2	3	1	3	--	100	70	40	30	30	0.001
G2	2	1	3	3	1	3	--	100	50	50	50	50	0.001
G3	3	2	1	3	2	1	--	100	60	40	40	40	0.001
G4	3	35.3	81.3	127.5	107.5	98.3	0.001	100	90	90	90	90	0.928
G5	2	21.3	57.8	71.4	65.4	55.6	0.003	100	90	80	70	70	0.081
G6	1	29.3	65.3	114.5	104.5	91.5	0.001	100	90	80	80	80	0.445
G7	4	34.5	71.8	117.5	98.5	90.3	0.001	100	100	90	90	90	0.865
G8	2	20.3	53.3	85.3	81.3	70.3	0.001	100	100	80	80	80	0.243
***p*-value**	--	0.017	0.005	0.001	0.001	0.001	0.001‡	--	0.001	0.001	0.001	0.001	0.001‡

DPV, day post vaccination; DPC, day post challenge; G, group

Table 4: Shedding % and quantification of *A. paragallinarum* along the experimental groups at the 7th, 21st, 49th and 77th day post challenge

Experimental group	Shedding %	Serotype	*A. paragallinarum* counts							
			7th		21st		49th		77th	
			CFU/mL	Log_{10} value	CFU/mL	Log_{10} value	CFU/mL	Log_{10} value	CFU/mL	Log_{10} value
G1	100	A	2×10^6	6.30	4.9×10^8	8.6901	4.9×10^6	6.69	4.6×10^3	3.663
G2	100	B	3×10^5	5.477	6×10^8	8.7781	6×10^6	6.778	3.2×10^3	3.505
G3	75	C	2×10^6	6.30	8.9×10^7	7.9493	8.9×10^5	5.949	5×10^3	3.699
G4	11.11	A	2×10^3	3.30	2×10^2	2.3010	0.0		0.0	
G5	12.5	B	1×10^4	4	5.6×10^3	3.7481	0.0		0.0	
G6	12.5	C	3×10^4	4.477	4.2×10^3	3.6232	0.0		0.0	
G7	11.11	A	4×10^4	4.60	6×10^3	3.7781	0.0		0.0	
G8	12.5	C	4×10^3	3.60	3×10^2	2.47712	0.0		0.0	
***p*-value**	0.001	--		0.012		0.001		0.048		0.124

G, Group; CFU, colony forming unit

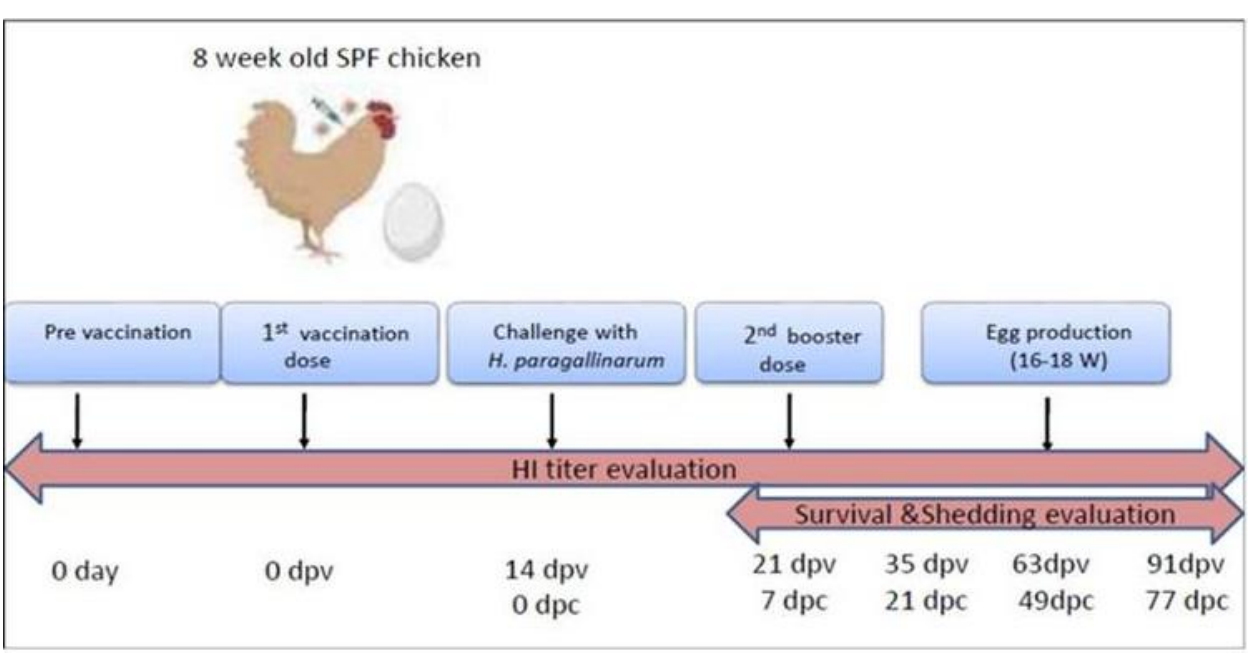

Figure 1: The experimental design for IC vaccine trial in layer chickens. Sera collection was performed during 91days post vaccination. The value of vaccination was measured by monitoring antibody production post vaccination in the regularly collected sera by HI titer, survival rate of chickens post challenging, and shedding of bacterial isolates post challenge

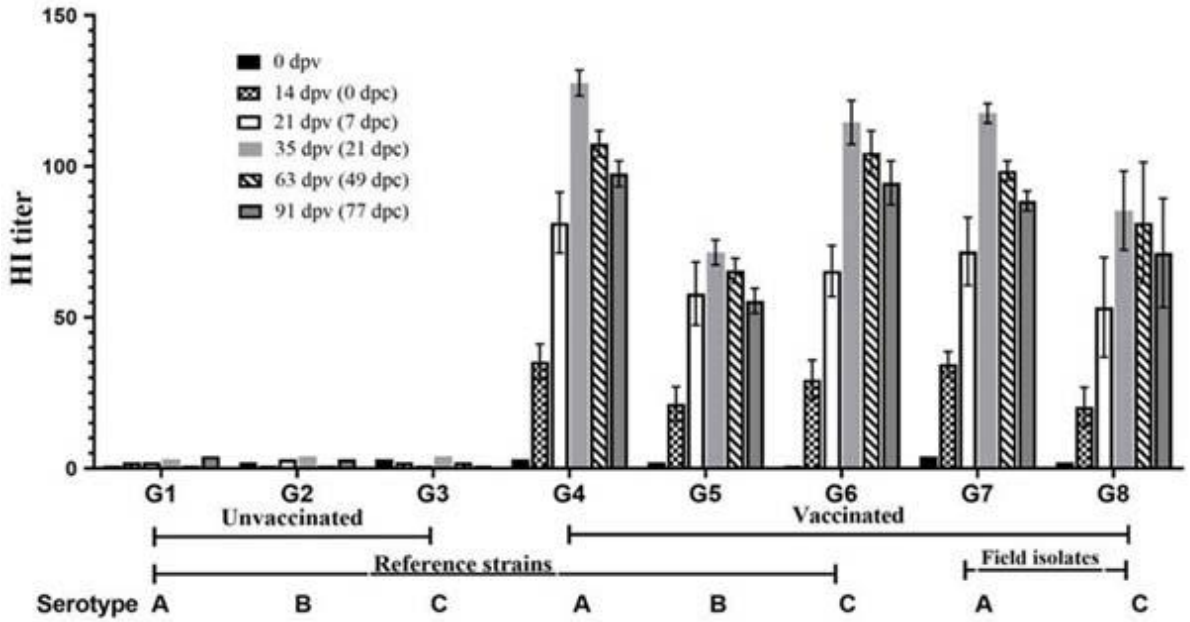

Figure 2: Hemagglutination inhibition (HI) antibody titer of chickens in vaccinated groups (G4-G8) in comparison to unvaccinated ones (G1-G3). The groups (G1-G6) challenged with reference strains resembling different serotypes, while G7 and G8 challenged with field isolates of *A. paragallinarium* serotypes C and A, respectively. Different column indicated the day of serum tested post vaccination, and post challenge. The HI titer reached its peak at day 35th post vaccination then gradually decreased

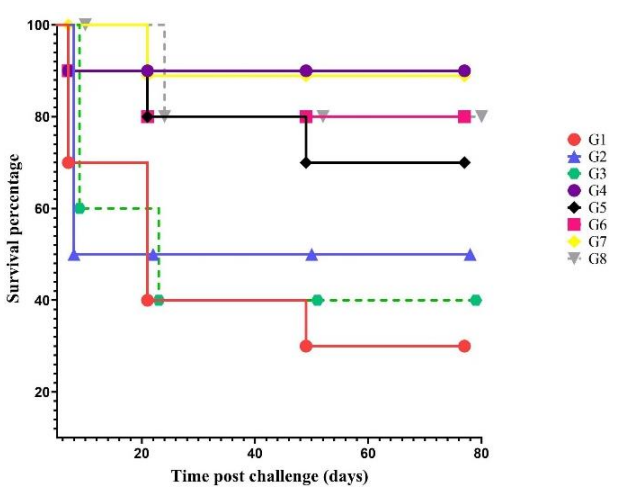

Figure 3: Kaplan-Meier survival analysis of chickens vaccinated by IC vaccine that was assessed before and after *A. paragallinarum* challenge during 77 days post challenge. The different groups showed as different colored shape. The vaccinated groups showed a protection rate ranging from 80- 100% at the 3rd week post challenge. The unvaccinated groups showed only 40-50%. Overall, the vaccinated groups showed a protection rate ranging from 70- 90%, while, the unvaccinated groups showed only 30-50%

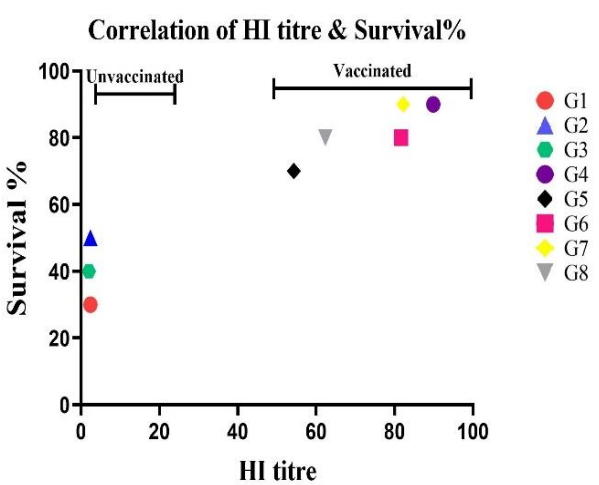

Figure 4: The correlation between chicken survivability (%) (Y-axis) versus mean hemagglutination inhibition (HI) titer (X-axis). Each different colored shape represented each different group (vaccinated- unvaccinated). A strong positive correlation is detected between survival (%) and mean HI titre (R= 0.9628)

Shedding and quantification of A. paragallinarum in challenged chickens

Samples collected from infra-orbital sinuses were cultured at the 7th-77thday post challenge for determining the evidence of *A. paragallinarum* shedding (Table 4). The re-isolation rates for *A. paragallinarum* serotypes A and C in the vaccinated groups were ranged from 11.11-12.5%. In contrast, the unvaccinated groups had an isolation range of 75-100%. Bacterial strains were not isolated from the negative control group. Furthermore, the challenging strains were not re-isolated from vaccinated groups at 49 and 77 days post challenge, while continually detected in the non-vaccinated groups with 100% *A. paragallinarum* re-isolation rate.

Moreover, samples from infra-orbital sinuses were subjected to standard plate count technique for counting of *A. paragallinarum* isolates (Table 4). The results showed that *A. paragallinarum* counts in the swabs of the unvaccinated groups were high ($8.9x10^7$-$4.9x10^8$ CFU/mL) (Log_{10} = 7.9493-8.6901) at the 21stday post challenge, which was above the infectious dose. These birds experienced dullness, inactivity, ruffled feather, decreased body weight, oculonasal discharge, closed eyes, and increased tracheal exudates. The bacterial count slightly decreased in the swabs at 49th-77thday post challenge with slight decrease of oculonasal discharge then signs relieve was noted at 77th day post challenge. In the vaccinated groups, shedding of few numbers of bacteria occurred at 21st day post challenge below the range of infectious dose ($2x10^2$ - $6x10^3$ CFU/mL) (Log_{10} = 2.3010 - 3.7781) then no bacteria was detected at 49th-77th day post challenge. No bacteria were detected from the negative control group. For most time intervals, there were significant ($P < 0.05$) differences in shedding percentage and *A. paragallinarum* counts between vaccinated and unvaccinated groups.

Discussion

Respiratory tract infections are of great importance in poultry industry (29-33). Great economic losses such as poor growth performance in growing birds and 10-40% reduction in egg production in layers are associated with IC due to *A. paragallinarum* infection (3). Determining the prevalence and antimicrobial resistance patterns of *A. paragallinarum* is important for predicting emerging changes to avoid vaccine failure especially due to the lack of cross-protection among serovars with whole-cell-inactivated vaccines (34, 35).

Here, the prevalence of *A. paragallinarum* recovered from layer chickens was 19.39%. While previous studies reported varying prevalence rates of the bacteria in chickens ranging from 7.5% to 72.2% (26, 37).

Different *A. paragallinarum* serotypes have been reported in different regions of the world (4). In the present study, we identified *A. paragallinarum* serotypes A and C by HI test that confirms the existence of different serotypes causing IC in Egypt. *A. paragallinarum* serovars A, B, and C were isolated previously in Egypt with more prominence of both serotypse A and C (36-38).

Very limited data on antibiotic susceptibility profile of *A. paragallinarum* is available as the organism is quite difficult to be isolated. As shown in our results, *A. paragallinarum* isolates showed absolute resistance to ampicillin, amoxicillin, amikacin, gentamicin, and streptomycin, which has been confirmed to be related to MDR plasmids (5, 39). However, the microorganism showed 100% susceptibility to sulfamethoxazole-trimethoprim, which is on contrary to a Korean study which documented a full resistance of the recovered isolates to sulfamethoxazole-trimethoprim (40).

On the other hand, the overall levels of resistance for ciprofloxacin, and tetracycline were 75 and 25%, respectively. Whereas a previous study revealed maximum resistance rates of *A. paragallinarum* against both antimicrobials (41).

A. paragallinarum serotype C isolates (n = 4) were intermediate susceptible to ciprofloxacin but susceptible to tetracycline. While a previous study stated that 18.1% and 2.7% of serotype C were resistant to tetracycline and ciprofloxacin, respectively (41).

An earlier report had studied the efficacy of a locally prepared vaccine against IC in poultry (9) but little is known about the efficacy of our vaccine, whether it serves a good protection against both reference and field isolates with different serotypes. Hence, the present study appears to be the first investigation on comparing the protective efficacy of IC vaccine against drug resistant field isolates, and reference ones, evaluating the efficacy of the vaccine against different challenging serotypes, and the

quantification of re-isolated bacteria in the shedding.

One of the main objectives of our study was to investigate the duration of protection. Therefore, the chickens were kept until beginning the period of egg production (16 to 18 weeks). The chickens used in the experiment were vaccinated at eight weeks of age, and boosted three weeks later before starting egg production. The dynamics of humeral immune response were analyzed in the vaccinated, unvaccinated, and control groups for determination of HI titers in pre- and post-vaccination and post challenge.

Immunized chickens were able to generate a positive HI titer two weeks post first vaccination, and this titer reached its peak two weeks post boostering (5^{th} week post first vaccination). This is continent with previous studies, which demonstrated that over 90% of vaccinated chickens have titers more than five HI (42) and antibody titers reached the maximum levels at the 6^{th} week post vaccination in chickens receiving a combined vaccine against *A. paragallinarum* and *Salmonella* Enteritidis (9)

A. paragallinarum serovar A is more pathogenic than other serotypes and may lead to higher HI titers (43). Herein, *A. paragallinarum* serotype A (reference and field isolates) challenging the vaccinated groups had higher HI titer compared with serotypes B, and C. HI titer cannot be used as the only evidence to determine that vaccination confers protection or not. The challenge test is the gold standard to determine the immunological response in chickens that have been vaccinated (42). The efficacy of the vaccine is known based on both the immune status and challenge test.

However, previous studies recorded a good level of protection when challenging after administration of a second dose of the vaccine (35, 44). In the present study, we evaluated the effect of challenging in much earlier age. All chickens were challenged two weeks after administration of the first dose of vaccine.

The virulence of different challenging serotypes could be assessed through the monitoring of mortality percentage in the unvaccinated control groups. 70, 50 and 60% of birds succumbed to infection and dead when challenged with serotypes A, B, and C, respectively. The serotype A strain demonstrated the highest virulence, followed by the C and the B strains. The high death recorded with serovar A is consistent with previous result showed that serovar A is more pathogenic than serovars B and C (43). The less virulence of serovar B has been recorded previously (23).

The levels of protection obtained when different vaccinated groups of birds were challenged with field, and reference serovars A, B, C ranged from 80 to 90%. Good levels of protection (80 to 90%, respectively) were recorded for *A. paragallinarum* serotypes C and serotype A field isolates, which strengthen the value of vaccination for the control of IC in Egypt. This is in accordance with a previous study in which the protection rates against *A. paragallinarum* serovars A, B, and C were 80%, 90%, and 70% in vaccinated chickens (9).

The highest level of protection was previously detected while boostering of the vaccine, which extended up to 56 weeks post-vaccination (8). The vaccine generated an unchanged level of protection after three weeks of challenging while the HI titer started to decrease after reaching its peak. In a previous study, Sato and Shifrine (45) reported a lack of correlation between agglutination titer and immunity to challenge. However, we observed a strong positive correlation between the mean HI titer and the level of protection against the challenge.

Shedding post-vaccination is less likely in vaccines containing inactivated *A. paragallinarum* strains than with modified-live vaccines. We found the evidence of *A. paragallinarum* shedding at three weeks post challenge with re-isolation rates for *A. paragallinarum* serotypes A and C ranged from 11.11 to 12.5% in vaccinated groups with low bacterial count in shedding below the infectious dose. In contrast, the non-vaccinated groups had high re-isolation rates with instances of constant infection compared with the vaccinated groups, implying a role of the vaccine in decreasing the period of infection (40). This is in accordance with a previous study showing 17.8% and 81.1% shedding percentage in inactivated vaccine and control group, respectively (46).

Conclusion

The present study appears to be the first investigation on the protective efficacy of IC vaccine against drug resistant *A. paragallinarum* field isolates, and reference strains, evaluating the

efficacy of the polyvalent oil adjuvant inactivated vaccine against different challenging serotypes and quantification of re-isolated bacteria in the shedding.

Authors declare no conflict of interest.

E.Y.E. and N.K.A. contributed equally in the conception and design of the study. M.A. carried out the classical microbiological techniques and experimental studies. N.K.A. and A.H.S. participated in the analysis and interpretation of data and wrote the initial draft of the manuscript; A.A.H. participated in the design of the study and in the analysis and interpretation of data. All authors revised the manuscript critically for important intellectual content and gave the final approval of the version to be published.

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