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Preparation of a Newly Developed Trivalent *Pasteurella multocida*, *Avibacterium paragallinarum*, and *Ornithobacterium rhinotracheale* Vaccine with an Evaluation of its Protective Efficacy in Chickens

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Abstract

Poultry, mainly chickens, and their white meat represent one of the main, nutritionally valuable, and affordable red meat replacer sources of protein throughout the whole world with special reference to developing countries. A long list of microbial agents especially bacterial pathogens threatens chickens' production cycles. They constitute one of the major problems facing the rapidly expanding poultry industry and are responsible for considerable economic losses. Fowl cholera, infectious coryza, and ornithobacteriosis (ORT) were among the serious bacterial infections that affect the respiratory tract of chickens with a global adverse effect on poultry production. A formalinized whole culture vaccine composed of *Pasteurella multocida* serotypes A5, A8, A9, and D2, *Avibacterium paragallinarum* serotypes A and C, and *Ornithobacterium rhinotracheale* serotype A was prepared. This polyvalent vaccine proved to be safe producing no adverse side effects when injected in chickens. The immunizing efficacy of this vaccine was evaluated in specific pathogen-free (SPF) chickens, which were immunized at 6 weeks of age. The protective efficacy of the vaccine was determined using a challenge test. The developed vaccine was effective in protecting chickens against fowl cholera, infectious coryza, and ornithobacteriosis in chickens against challenge with these pathogens. Vaccinated chickens challenged with virulent *Pasteurella multocida* serotypes A5, A8, A9, and D2 showed protection rates of 86.6%, 93.3%, 93.3%, and 93.3%, respectively, as compared with 100% mortality in the non-vaccinated control. Vaccinated chickens challenged with *Avibacterium paragallinarum* serotypes A and C showed 86.6% and 93.3% protection rates, respectively. Also, the protection rate against challenges with virulent *Ornithobacterium rhinotracheale* serotype A reached 96.6%.

KEYWORDS
Trivalent vaccine, poultry infectious diseases, bacterial respiratory pathogens, economic white meat source, immunizing efficacy, SPF chickens

INTRODUCTION

Poultry represents an important and cheap source of protein throughout the world. The bacterial diseases of poultry, however, constitute one of the major problems facing the rapidly expanding poultry industry and are responsible for considerable economic losses (Sharma, 1999; Bermudez and Stewart, 2008; Cserep, 2008). Fowl cholera, infectious coryza, and ornithobacteriosis were among the serious bacterial infections affecting chickens' respiratory tracts (OIE, 2008). Vaccines and vaccination strategies play an important role in controlling these diseases and overall minimizing the associated economic losses.

Fowl cholera is a contagious disease of domesticated and wild avian species caused by *Pasteurella multocida*. It occurs typically as a fulminating disease with massive bacteremia and high morbidity and mortality rates (Rhoades and Rimler, 1990). Infectious coryza is usually acute, sometimes the chronic, highly infectious disease of chickens caused by the *Avibacterium paragallinarum* (Blackall *et al.*, 2005) and characterized by catarrhal inflammation of the upper respiratory tract, especially nasal and sinus mucosa (Paul McMullin, 2004).

Ornithobacteriosis, or ORT, is an acute highly contagious bacterial disease of birds, which is characterized by respiratory signs such as nasal discharge, sneezing, coughing, and sinusitis but in severe cases is followed by pneumonia, dyspnea, prostration, and mortality (Van Empel and Hafez, 1999). In this respect, vaccination seems to be one of the best methods to prevent the occurrence of the disease (Reid and Blackall, 1983; Blackall and Reid, 1987). Therefore, several trials were conducted to produce bacterin from local bacterial strains either in aluminum hydroxide gel form (Matsumoto and Yamamoto, 1971; Davis *et al.*, 1976; Kume *et al.*, 1880; Zaki, 1985; Reid and Blackall, 1987; Yamaguchi *et al.*, 1988; Mouahid *et al.*, 1991; Fernandez *et al.*, 2005; Kridda *et al.*, 2009; Philemon, 2009) or in mineral oil form (Page *et al.*, 1963; Matsumoto and Yamamoto, 1975; Coetzee *et al.*, 1982; Reid and Blackall, 1983; Blackall and Reid, 1987; Blackall *et al.*, 1992; Jacobs *et al.*, 1992; Terzolo *et al.*, 1997).

As the effective prevention of these diseases depends upon the use of inactivated vaccines. The use of combined vaccines has the advantage of protection against more than one disease at the same time, besides reducing vaccination expenses, the number of vaccinations performed, and saving time. Therefore, the main objective of this study was to develop an inactivated combined adjuvanted, trivalent vaccine from the most virulent

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and locally prevalent serotypes of *Pasteurella multocida*, *Avibacterium paragallinarum*, and *Ornithobacterium rhinotracheale*, and to evaluate its immunizing and protective efficacy in chickens.

MATERIALS AND METHODS

Ethics declarations

Approval for animal experiments

The current study is reported per (Animal Research: Reporting of In-Vivo Experiments-ARRIVE) guidelines. The guidelines of the (Institutional Animal Care and Use Committee-IACUC of the Faculty of Veterinary Medicine, Cairo University) were completely followed during any procedures involving animal use through the current conducted study. The IACUC approval number of the current study (vet CU 03162023716).

No anesthesia or euthanasia protocols were used with the animal involved during this study as all animal-dependent methodological procedures were considered as no to low pain-causing procedures that ethically can be done on a conscious alive animal.

Study design and experimental groups' categorization

Experimental design can be easily summarized and described in Fig. 1, in the current study, 210 specific pathogen-free (SPF) chickens were used to evaluate the efficacy of the prepared trivalent vaccine. Those SPF were divided into 3 groups; Group (A): This group was used as a non-vaccinated control group and was divided into four subgroups which were 1, 2, 3, and 4 subgroups containing 10, 20, 40, and 10 SPF chickens, respectively. The subgroups 3, 2, and 1 were used for challenge tests with fowl cholera, infectious coryza, and ornithobacteriosis, respectively. The SPF chickens in subgroup 4, however, were used for determination of the developed vaccine safety. Group (B): This group was divided into two subgroups 5 and 6. The subgroup 5 consisted of 90 SPF chickens that were used for evaluation of the vaccine efficacy, and three weeks after immunization all the chickens in this subgroup received a booster dose. After further 3 weeks from the booster dose the immunized chickens were challenged with virulent fowl cholera serovars (15 chickens/each serovars) and with virulent *Ornithobacterium rhinotracheale* (30 chickens). While subgroup 6 was used for the challenge of immunized chickens at 4 weeks post-immunization with *Avibacterium paragallinarum* serotypes A and C (15 chickens/each serotype). Group (C): This group contained 10 SPF chickens that were injected with double the recommended dose of the developed vaccine for determination of its safety.

Obtaining the bacterial seed strains and serovars required for both vaccine preparation and further evaluation stages

Pasteurella multocida serotypes A5, A8, A9, and D2 were obtained from the Aerobic Bacterial Vaccines Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. *Avibacterium paragallinarum* serotypes A and C that were supplied from Intervet International B.V. Boxmeer, Holland. *Ornithobacterium rhinotracheale* serotype A was obtained from the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. These strains were used for the preparation of vaccines, production of antigens, and as challenge bacteria.

Bacteria propagation in specific pathogen-free embryonated chicken eggs (SPF-ECEs)

SPF-ECEs (5-7 days old), obtained from Kom Ushim farm for SPF-ECEs, El-Fayoum, Egypt, were used for propagation of *Avibacterium paragallinarum* for the challenge bacterial antigen preparation (Shivachandra *et al*., 2006). Brain heart infusion (BHI) broth (OXOID) was used for the propagation of *Pasteurella multocida*, and *Ornithobacterium rhinotracheale*, while tryptose phosphate broth (OXOID) was used during the cultivation of *Avibacterium paragallinarum* for haem-agglutinating antigen preparation.

The selected laboratory animals to be used in the different vaccine preparation and evaluation stages

Experimental chickens

The involved chickens were used and obtained from SPF stocks from Kom Ushim, El-Fayoum, Egypt. All involved chickens were housed in positive-pressure stainless steel isolation cabinets at the central laboratory for evaluation of veterinary biologicals (CLEVB) with continuous light exposure and an ad-libitum

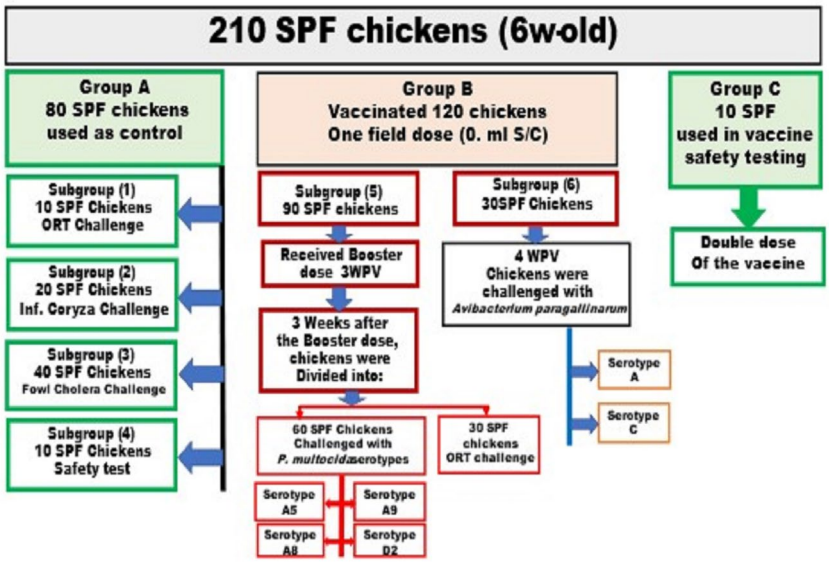

Fig. 1. Flowchart to illustrate the overall SPF chicken grouping and subgrouping throughout the whole study.

feeding system throughout the whole study period.

Experimental Boskat rabbits

Two months old male Boskat rabbits with an average weight of 2.0 kg were used for the serial passage of *Pasteurella multocida* and preparation of hyper-immune sera.

Vaccine preparation

Preparation of the bacterial components of the vaccine

Pasteurella multocida (serotypes, A5, A8, A9, and D2), *Avibacterium paragallinarum* serotypes A and C, and *Ornithobacterium rhinotracheale* serotype A were grown separately in the culture media specific for each strain. The concentration of each *Pasteurella multocida* serotype (A5, A8, A9, and D2) culture was adjusted to $2X10^9$ CFU/ml as the final concentration (Singer and Malkinson, 1979), while the concentration of *Avibacterium paragallinarum* serotypes A and C was adjusted to $3X10^8$ CFU/ml as final concentrations (Mohamed, 1996). The concentration of *Ornithobacterium rhinotracheale* serotype A culture was adjusted to $2X10^9$ CFU/ml (Erganis *et al*., 2011) as the final concentration.

Vaccine inactivation with formalin (0.1%)

Formalin as an inactivating agent was obtained from BDH Limited Co., Poole, England. It was used in the form of a formaldehyde solution of 37% and was added to bacterial suspension for inactivation in 0.1% of the final concentration. Formalin was added to inactivate each bacterial culture separately (Hassan *et al*., 1992). The mixed antigens were absorbed with mineral oil and MONTANIDE ISA-70 at a ratio of 30:70, respectively (Stone *et al*., 1978).

Determination of the sterility, safety, potency, and stability of the prepared vaccine

It was done according to the British Veterinary Codex (1970), European Pharmacopoeia 1997, Egyptian standard regulation for evaluation of veterinary biologics 2009, and OIE Guidelines 2008 as follows;

Sterility testing

It was done by inoculating samples of the prepared vaccine on different bacterial and fungal media plates to determine if the prepared vaccine samples were free from bacterial, fungal, and Mycoplasma contamination or not.

Safety testing

Ten healthy 6 weeks old SPF chickens were inoculated with twice the normal recommended dose of the prepared vaccine. The birds were observed for any possible local systemic adverse reactions for 21 days.

Potency testing

Samples of completed vaccines should be tested for potency in hamsters or guinea pigs. Potency is usually measured by the vaccine's ability to prevent the death of the animal when challenged with between 10 and 10,000 LD50 (50% lethal dose). A frequently used protocol is to inject 1/40 rabbit dose of the vaccine into each of ten healthy mice no more than 3 months old. After 15–30 days, each vaccinated mouse, and each of ten unvaccinated mice of the same age, is injected intraperitoneally with a suitable quantity of a virulent culture of bacteria of each species used to make the vaccine. In the case of trivalent or polyvalent vaccines, each bacterial species is tested separately. For the vaccine to pass the test, at least 8/10 of the vaccinated animals should remain in good health for 14 days after the death of the controls.

Stability testing

When stored under the prescribed conditions for long periods (6-12 months).

Evaluation of the prepared vaccine's effectiveness and the induced immunity duration

Duration of immunity should be determined in the animal species for which the vaccine is intended using natural routes of challenge. Duration of immunity should not be estimated based on the duration of ELISA titers in vaccinated animals as protection against clinical disease may be present with very low titers. Vaccinal immunity should persist for at least 6 months or longer depending on the label claim.

Evaluation of the immunizing and protective efficacy of the prepared vaccine

Six weeks old SPF chickens were injected subcutaneously with 0.5ml of the prepared vaccine. As shown in the experimental design some chickens in group B were boosted at 3 weeks post-vaccination with another field dose of the vaccine (subgroup 5). This subgroup was challenged at 3 weeks after the booster dose with the virulent serovars of *Pasteurella multocida* and *Ornithobacterium rhinotracheale* pathogens. While chickens in (subgroup 6) remained without booster doses and were challenged at 4 weeks post-immunization with virulent serovars of *Avibacterium paragallinarum* to determine the immunizing potential of the *Avibacterium paragallinarum* component of the tested trivalent vaccine. The following bacterial culture doses were used for the challenge test of the vaccinated groups and non-vaccinated control chicken groups;

Pasteurella multocida group was challenged with a dose of $2x10^2$ CFU/0.5ml of *Pasteurella multocida* virulent serovars via the intramuscular route (Egyptian standard regulation for evaluation of veterinary biologics, 2009).

Ornithobacterium rhinotracheale group was challenged with $2x10^9$ CFU/0.5ml of *Ornithobacterium rhinotracheale* strain via spraying on the nose and eye (Erganis *et al*., 2011).

Avibacterium paragallinarum group was challenged with virulent *Avibacterium paragallinarum* serotypes (A and C) with approximately 10^7 CFU/ 0.5ml via infraorbital sinuses as well as via nostril route at a dose of 0.2 ml (Mohamed, 1996; Egyptian standard regulation for evaluation of veterinary biologics, 2009).

The challenged vaccinated and control group, chickens were kept under observation for 14 days (OIE, 2008; Erganis *et al*., 2011). All challenged birds were observed daily after the challenge and the morbidity and mortality rates were recorded for each group till the end of the observation period to measure the protection rate. Swabs from the nostrils of challenged and control chickens were cultured for the re-isolation of bacterial pathogens used in the challenge.

Statistical analysis

All data collection, tabulation, manipulation, and statistical analysis and representation were done using basic the Microsoft Excel features.

RESULTS

Sterility, safety, potency, duration of immunity, and stability evaluation

Vaccine samples that have been streaked on enriched media plates (brain heart infusion and blood agar plates), then incubated for 48 hours at 37°C showed no growth after the incubation period which is a sterility sign. The birds are observed for 14 days post-vaccination and should show no adverse local or systemic effects attributable to the vaccine which was the safety sign. The vaccinated animals remained in good health for 14 days after the death of the controls which insures the vaccine potency. Vaccinal immunity persisted for at least 6 months post-primary vaccine dose administration indicating the minimal vaccine-based immunity duration. Finally, the stored vaccines are expected to retain their potency for 1–2 years. Stability was assessed by determining potency after storage at 2–5°C, room temperature) 25-27°C), and 35–37°C.

Criteria for evaluation of the prepared vaccines

Signs, mortalities, gross lesions, and protection rate

Typical respiratory symptoms (nasal discharge, sneezing, conjunctivitis, swelling of the sinuses, and facial edema) were noted in all the chickens in the experimental groups. (Yamamoto, 1980). Those chickens who displayed no symptoms clinically or at necropsy were regarded as protected.

Bacterial re-isolation

Swabs from birds with symptoms, deceased birds, and birds that had been necropsied were taken from the infraorbital sinuses in order to re-isolate the challenge bacterium. (Rimler *et al*., 1975). Each swab sample was streaked with 0.0025% NAD onto CMI agar and cultured for 48 hours at 37°C in a CO2 incubator. If birds showed no development on cultures, they were regarded as protected.

Protection efficacy of the prepared inactivated trivalent vaccine in chickens challenged with virulent Pasteurella multocida serotypes A5, A8, A9, and D2

The data presented in Table 1, indicated that chicken groups challenged with virulent *Pasteurella multocida* serotypes A5, A8, A9, and D2 serovars showed protection rates of 86.6%, 93.3%, 93.3%, and 93.3%, respectively. In the non-vaccinated control group 100 % of challenged chickens died within 3-4 days. Re-isolation of *Pasteurella multocida* serotypes A5, 8, A9, and D2 was successful from control died chickens otherwise healthy vaccinated chickens showed negative results.

Protection efficacy of the prepared trivalent inactivated vaccine in chickens challenged with Avibacterium paragallinarum serotypes A and C

In immunized chicken groups challenged with *Avibacterium paragallinarum* serotypes A and C, the protection rates reached to 86.6% and 93.3%, respectively. Chickens in the control non-vaccinated group that received the same challenge dose, 100 % of challenged chickens became diseased within a time of 3-4 days Table 2. Re-isolation of *Avibacterium paragallinarum* serotypes A and C was successful from control diseased chickens otherwise healthy vaccinated chickens showed negative results.

Challenge test in immunized and non-vaccinated chickens challenged with Ornithobacterium rhinotracheale serotype (A)

As shown in Table 3, vaccinated chicken groups challenged with *Ornithobacterium rhinotracheale* serotype A showed a protection rate of 96.6%. In the control non-vaccinated group that received the same challenge dose, 90 % of challenged chickens were diseased. Re-isolation of *Ornithobacterium rhinotracheale*

Table 1. Protection efficacy of the prepared vaccine in vaccinated chickens after challenge with virulent *Pasteurella multocida* serotypes (A5, A8, A9, and D2).

Groups	*Pasteurella multocida* serotypes	Number of birds /challenges	Mortality rate	Protection%
Vaccinated	A5	15	2/15	86.60%
	A8	15	1/15	93.30%
	A9	15	1/15	93.30%
	D2	15	1/15	93.30%
Control	A5, A8, A9, and D2	10/each serotype	10/10	0%

Table 2. Protection rates in vaccinated chickens by challenged with *Avibacterium paragallinarum* serotypes (A and C).

Groups	Serotype	Number of birds/challenges	Morbidity rate	Protection%
Vaccinated	A	15	2/15	86.60%
	C	15	1/15	93.30%
Control	A and C	10/each serotype	10/10	0%

Table 3. protection rates in chickens vaccinated with the prepared inactivated polyvalent vaccine after challenge with *Ornithobacterium rhinotracheale* serotype A.

Groups	Serotype	Number of birds/challenge	Morbidity rate	Protection%
Vaccinated	A	30	1/30	96.60%
Control	A	10	9/10	10%

serotype A was successful from non-vaccinated control diseased chickens, otherwise healthy vaccinated chickens showed negative results.

DISCUSSION

Fowl cholera, infectious coryza, and ornithobacteriosis are standing, either as a separate sole cause or complexed causation, clearly behind severe clinical respiratory distress, cough, sneezing, and sinusitis in chickens. The major economic losses associated with these bacterial infection results from the rejection of carcasses for consumption, growth retardation, and mortality (OIE, 2008). The control of *Pasteurella multocida*, *Avibacterium paragallinarum*, and *Ornithobacterium rhinotracheale* infections in chickens through vaccine have been previously discussed (Gergis *et al*., 1992; Kalaydari *et al*., 2004; Jabbri and Moazani, 2005; Erganis *et al*., 2010). Control of these diseases is very important and can be achieved by using various innovative combating vaccination strategies (Gergis *et al*., 1992).

Preparation of combined inactivated vaccines is one of the tools for control of some bacterial respiratory diseases such as fowl cholera, infectious coryza, and ornithobacteriosis (Bisschop *et al*., 2004; Kalaydari *et al*., 2004; Schuijffel *et al*., 2006; Erganis *et al*., 2010). Inactivated vaccine adjuvanted with MONTANIDE ISA-70 proved to induce long duration of immunity and high protective antibody titer (Amal *et al*., 2001; Dungu *et al*., 2009; Ismail *et al*., 2013).

Safety and sterility testing of the prepared vaccine was carried out and proved that this vaccine had no adverse reactions and was free from bacterial and fungal contaminants. The use of formalin as an inactivating agent together with the application of all safety procedures during the vaccine preparation stands behind these results.

The challenge under strict conditions may be also used to predict flock exposure-response and can add considerable significance value obtained with the sera collected from the same chickens (Stone, 1988).

Data presented in Table 1, showed that all chickens immunized with the prepared vaccine were protected from infection with *Pasteurella multocida* serotypes A5, A8, A9, and, D2 when challenged at the third-week post-injection of the booster dose with a protection rate of 86.6%, 93.3%, 93.3%, and 93.3 %, respectively. This agreed with those reported by Chute *et al*. (1962) and Shafi (1995) which proved that *Pasteurella multocida* trivalent vaccine can give a high mean protection rate for controlling fowl cholera in Egypt.

Also, protection rates of 83.3% and 93.3% were recorded in immunized chickens after challenge with virulent *Avibacterium paragallinarum* serotypes A and C as shown in Table 2. These results agreed with those reported by Glisson (1998); Mouahid *et al*. (1991) and Nakamura *et al*. 1994) who found that chickens vaccinated with *Avibacterium paragallinarum* autogenous bacterin showed a very broad immune response and protection against *Avibacterium paragallinarum* infection.

Data represented in Table 3, showed the protection rate recorded in chickens immunized with the prepared vaccine against challenge with virulent *Ornithobacterium rhinotracheale* serotype A (96.6%) agreed with those reported by other authors (Schuijffel *et al*., 2006; Murthy *et al*., 2007) who proved that vaccination of broiler at 6th of age and followed by booster dose can effectively protect against *Ornithobacterium rhinotracheale* infection. It has been reported that natural protection against ORT infection is largely based on the development of a humoral immune response (Schuijffel *et al*., 2006).

Also, it is interesting that no re-isolation of *Pasteurella multocida*, *Avibacterium paragallinarum* and *Ornithobacterium rhinotracheale* was recorded from respiratory and internal organs in vaccinated chickens. While the re-isolation of these bacterial strains was successful in controlling non-vaccinated chickens.

CONCLUSION

Finally, it has been recorded through the present work that the use of a trivalent vaccine against the virulent serotypes of the following pathogens *Pasteurella multocida*, *Avibacterium paragallinarum*, and *Ornithobacterium rhinotracheale* combined with the use of a potent adjuvant, MONTANIDE ISA-70, induced strong humoral immune responses and protective immunity in the vaccinated chickens. Thus, this vaccine can be considered as a promising, efficient, and strategic confrontation approach to control the respiratory manifestations caused by these contagious pathogens.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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