Original Article

The Immunogenic Potential of an Inactivated Vaccine Candidate against *Ornithobacterium Rhinotracheale* in SPF Chicken

Ghodsian, N[1*], Shahsavandi, S[1], Ebrahimi, MM[1], Karimi, V[2]

1. *Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization, Karaj, Iran*
2. *Department of Poultry Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*

How to cite this article: **Ghodsian N, Shahsavandi S, Ebrahimi MM, Karimi V. The Immunogenic Potential of an Inactivated Vaccine Candidate against *Ornithobacterium Rhinotracheale* in SPF Chicken. Archives of Razi Institute Journal. 2024;79(4):865-872.** DOI: 10.32592/ARI.2024.79.4.865

Article Info:
Received: 21 January 2024
Accepted: 13 March 2024
Published: 31 August 2024

Corresponding Author's E-Mail:
N.ghodsian@rvsri.ac.ir

ABSTRACT

Ornithobacterium rhinotracheale (ORT) is a gram-negative bacterium that causes respiratory infection in chickens and turkeys. The Co-infection of ORT with other viral or bacterial pathogens results in the development of severe clinical symptoms and significant economic losses. The proportion of ORT strains resistant to the current antibiotics employed in poultry flocks has increased in successive years. Contingent on the source of the isolate. It is recommended that the inactivated whole-cell vaccine (bacterin) be administered against multi-drug resistant strains of ORT that are present on poultry farms. In the present study, a formalin-inactivated bacterin formulated with an oil adjuvant (Montanide ™ ISA 70 VG) was developed based on a local ORT isolate. A prime-boost regimen was employed for the immunization of specific pathogen-free chicken (SPF) groups. Subsequently, the immunogenic potency of the vaccine candidate was evaluated via ELISA and compared with that of a Nobilis® commercial inactivated ORT vaccine.The safety of the Vaccine was studied following the inoculation of a dose that was twice the recommended dose of the prepared bacterin. The commercial inactivated ORT vaccine and the prepared bacterin both elicited the production of induced specific antibodies after three weeks following the initial vaccination, with this response continuing until 16 weeks post-vaccination. The immunization of chickens with the commercial vaccine resulted in a higher level of antibody compared to the experimental vaccine. However, no significant difference (P<0.05) was observed between the treated groups overall. The safety test revealed the absence of any adverse local or systemic reactions were found in chickens throughout the post-vaccination period. The data indicate that the prepared ORT-inactivated vaccine is safe and capable of inducing adequate and long-lasting immune responses in experimental SPF chickens. It is imperative to conduct field trials to ensure the efficacy of this vaccine candidate in preventing ORT infection.

Keywords: *Ornithobacterium rhinotracheale*, 16S rRNA, inactivated vaccine, immune response, ELISA

1. Introduction

Ornithobacterium rhinotracheale (ORT) is a gram-negative bacterial pathogen that is responsible for a contagious respiratory disease affecting chickens and turkeys worldwide. The infection is mainly characterized by pneumonia, tracheitis, and airsacculitis, which result in growth depression, reduced egg production, and increased mortality (1). The severity of symptoms may vary considerably, depending on the pathogenicity of the strain, environmental factors, the type of secondary infection, and the age of the bird at the time of infection (2). ORT was initially identified in turkey flocks in Germany during the early 1990s and has since become a prevalent pathogen in the broiler population in many countries (2). The 18 identified serotypes of ORT (A-R) have been isolated from domestic and wild birds all over the world (3-5). The first report of an ORT infection in Iran published in 2000, describing the presence of the pathogen in broiler and laying flocks with respiratory symptoms (6). Subsequently,To now several studies have been conducted for the isolation and detection of ORT by using amplification of a DNA fragment within the 16S rRNA region, agar gel precipitation (AGP), as well as enzyme-linked immunosorbent assay (ELISA) (7-13). Seroepidemiological studies indicate that ORT was introduced to the Iranian broiler farms approximately two decades ago, with all field isolates as serotype A. It seems that ORT has become endemic, particularly in regions with intensive poultry production and multiple-age farms. The results of antibiotic sensitivity tests of ORT isolated from different parts of Iran indicate that acquired resistance to antibiotics commonly used in poultry production is within the moderate to high range. As a result, the treatment of ORT infections is becoming increasingly challenging due to the high variability in antibiotic sensitivity and the circulation of multi-drug resistant strains among poultry farms (10, 14, 15). The two primary difficulties in controlling ORT infection are the high prevalence and resistance to antibiotics. Therefore, strategies to overcome these problem are needed. The satisfactory control of ORT infection depends on the implementation of biosafety measures and vaccination strategies, as most isolates have acquired resistance against the regularly used antibiotics (16, 17). Therefore, the use of an efficacious vaccine represents a promising strategy for the control of ORT infection, offering a means to address the associated challenges, including, carcass rejection for consumption, growth retardation and mortality. Despite the development of live live and recombinant vaccines for the control of experimental ORT infection, whole-cell inactivated vaccines (bacterins) have been successfully commercialized and used for the control of the infection in farms (2, 16). Given that only serotype A has currently been identified in Iran, an immunization strategy based on the vaccination of broiler chickens with the homologous serotype can protect chickens from ORT infection. In the present study, we candidate a local ORT isolate was selected as the candidate, and a monovalent inactivated bacterin was prepared in an oil adjuvant. Subsequently, the vaccine's capacity to elicit a protective immune response against ORT was investigated following the vaccination of chickens.

2. Materials and Methods

2.1. Bacterial Strain

In order to prepare the bacterin with the highest protective potential, a total of 25 ORT isolates were subjected to analysis. The bacterial samples were derived from various flocks reared in four distincts provinces: Alborz, Gilan, Mazandaran, and Qazvin (Table 1). Genomic DNA was extracted from each isolate ,and a 784-bp fragment within the 16S rRNA was amplified (18). The PCR product was sequenced in both directions, and the resulting nucleotide sequences were subsequently deposited in GenBank, and assigned accession numbers JF330125, JF810484-87, JF810490-98, and JF501953-59.

2.2. ORT Bacterin Preparation

Based on the results of phylogenetic analysis and evolutionary affiliations of the 16S rRNA gene, the ORT strain (JF810492) was identified as a potential candidate for bacterin preparation. The strain was cultured on Blood agar (Oxoid™ Ltd, Basingstoke, UK) with 5% sheep blood agar at 37°C in a 5% CO_2 incubator for 48 hours. The sterility of the ORT bacterin was evaluated through microbiological analysis for aerobic, microaerophilic, anaerobic, mycoplasma, and mycotic microorganisms in accordance with the World Organization for Animal Health (WOAH) Terrestrial Manual 2023. For the liquid phase of the bacterin, a single colony was inoculated in Brain Heart Infusion Broth (Oxoid™ Ltd.), supplemented with 5% bovine serum (Sigma-Aldrich), and grown at 37°C on a 100-rpm shaker overnight. The concentration of bacteria was estimated by preparing a serial dilution and culturing on Brain Heart Infusion agar (Oxoid™ Ltd.,) medium by using the following formula: the number of colonies × the dilution factor/the volume of the culture plate. Bacterial concentrations were adjusted to 1×10^7CFU/ml in PBS, and treated with formalin at the final concentration of 0.5% for 16 hours with shaking at 37°C. Then the complete inactivation was confirmed by plating on Blood agar overnight at 37°C. Subsequently, complete inactivation ORT antigen was then mixed with Montanide ™ ISA 70 VG (SEPPIC, France) at a ratio of 30:70 to form an emulsion. In this regard the adjuvant was filtered and placed inside a sterile Bécher under a rotary homogenizer (Heidolph Diax900, Germany). The inactivated ORT antigen was gradually incorporated into the adjuvant and homogenized for 6 minutes, with three cycles of two minutes each. The bacterin was prepared with the adjuvant and stored in a sterile vial and stored at 4°C until required for use.

Table 1. The sources and geographic origins of *Ornithobacterium rhinotracheale* isolates

No	Clinical signs	Herd profile	Simultaneously isolated pathogens	Geographic origin
1	Respiratory symptoms	breeder	ILTV	Alborz province-Kordan, Shende
2	Respiratory symptoms, loss of appetite	broiler	H9N2 influenza virus, *E.coli*	Alborz province-Hashtgerd, Najmabad
3	Respiratory symptoms, loss of appetite	broiler	*E.coli*	Alborz province- Mehrshahr, Hossein Abad
4	Ascites, pericarditis, perihepatitis, rickets, paralysis	broiler	*E.coli*	Alborz province- Ardeha
5	Respiratory symptoms, loss of appetite, head swelling, pericarditis	broiler	*E.coli*	Alborz province- Andishe
6	Respiratory symptoms, pneumonia	broiler	-	Alborz province- Kamal Shahr
7	Head swelling, watery discharge	layer	H9N2 influenza virus, *E.coli*	Alborz province- Shahryar
8	Respiratory and digestive symptoms, loss of appetite	layer	H9N2 influenza virus, *E.coli*	Alborz province- kalak
9	Respiratory and digestive symptoms, ascites, pericarditis, swelling of air sac	broiler	*E.coli*	Alborz province- Ghale Chendar
10	Respiratory symptoms, pneumonia	broiler	-	Alborz province- Kamal Shahr
11	Respiratory symptoms	breeder	-	Guilan province
12	Respiratory symptoms	breeder	-	Guilan province
13	Respiratory symptoms	breeder	-	Guilan province
14	Respiratory symptoms	breeder	-	Guilan province
15	Respiratory symptoms	breeder	-	Guilan province
16	Respiratory symptoms	breeder	-	Guilan province
17	Wrinkled neck, watery discharge, head and face swelling, normal egg production	broiler	-	Mazandaran province
18	Slaughterhouse	broiler	H9N2 influenza virus	Qazvin province- Abgarm
19	Slaughterhouse	broiler	-	Qazvin province
20	Slaughterhouse	broiler	-	Qazvin province
21	Slaughterhouse	broiler	-	Qazvin province
22	Slaughterhouse	broiler	-	Qazvin province
23	Slaughterhouse	broiler	-	Qazvin province- Abgarm
24	Slaughterhouse	broiler	-	Qazvin province- Abgarm
25	Slaughterhouse	broiler	-	Qazvin province

2.3. Chicken Immunization Trial

The animal studies were ethically approved by the Institutional Animal Care and Use Committee at the Razi Vaccine and Serum Research Institute. The study involved 30 specific pathogen-free (SPF) chickens (Venky's Company, India) aged 54 days. The birds were included in the study and divided into three equal groups (A, B, and C) and housed in positive pressure stainless steel isolation cabinets. They had access to feed and water *ad libitum*. In the course of the experimental trials, the chickens in Group A were subcutaneously vaccinated with 0.3 ml of the prepared bacterin at the back neck, and were subsequently boosted four weeks later. Chickens in Group B received the Nobilis® commercial inactivated ORT vaccine (Intervet International B. V., The Netherlands) in a similar manner. The active ingredients of this vaccine include inactivated whole cell suspension of ORT serotype A, strain B3263/91 ($1x10^7$ cells),formulated with an adjuvant water-in-oil emulsion. The non-vaccinated control group(Group C)similarly received sterile phosphate-buffered saline (PBS, pH=7.4).

2.4. Specific Antibody Detection

Blood samples were collected from chickens in each group at 2, 3, and 4 weeks post-primary vaccination and then at 2-week intervals until 16 weeks. Serological monitoring was conducted using the BioChek *Ornithobacterium rhinotracheale* Antibody Test kit in ELISA assay (BioCheck, Inc.). In briefly, 100 µl of each diluted serum (1:100), positive and negative controls were added to the coated microplate and incubated for 60 minutes at room temperature. Following a washing step, 100 µl of the conjugate solution was added to each well and incubated for 30 minutes. Subsequently, 100 µl of theTMB substrate solution was added and incubated for 15 minutes at room

temperature in the dark, followed by the addition of100 µl of stop solution (3N H_2SO_4). The absorbance of the sera was measured at 450 nm using a microplate reader, thereby enabling the measurement of the amount of bound antibody. All samples were analyzed in triplicate. A serum/positive (S/P) values greater than the cutoff (titer =1432) were considered positive.

2.5. Safety Test

Ten healthy 6-week-old SPF chickens were inoculated with twice the recommended dose of the prepared bacterin and observed for any possible local or systemic adverse reactions during 21 days.

2.6. Statistical Analysis

A Repeated-Measures ANOVA (SPSS Inc., USA) was used to ascertain the significance within the experimental groups. $P<0.05$ was considered statistically significant.

3. Results

The indigenous ORT bacterin was selected based on the results of microbiological data and phylogenetic analysis of 16S rRNA genome sequences. The selected isolate was cultured on a rich culture medium and tested for sterility. The inoculated culture media were examined and no fungal contamination or growth of any bacteria other than the ORT strain used as a vaccine was detected. The antigen component of the ORT vaccine candidate strain was estimated and then the adjusted concentration of 1×10^7CFU/ml was completely inactivated. This was followed by formulation using an oil adjuvant. The collected serum samples were processed to determine the presence of specific antibodies against ORT. The results demonstrated that the developed bacterin, similar to the commercial vaccine, elicited the production of detectable IgG antibodies within a three-week period following the initial vaccination. The generation of antibodies was continued until 16 weeks post-vaccination, as shown in Figure 1. The serum samples obtained from the negative control group exhibited no anti-ORT antibody activity. The antibody levels of group B , which received the commercial vaccine, were significantly higher ($P<0.05$) than those of group A during the 2nd to 4th weeks post-vaccination. However, this pattern was changed after the booster administration. The value increased in chickens that received the experimental bacterin until it reached its peak tat the 8th-week post-vaccination. The variations in the serum titer of chickens in group B exhibited less variation than that of group A, reaching its peak in the 10th-week post-vaccination. From the peak until the conclusion of the trial period, the quantity of antibody remained largely unchanged in the commercial vaccine,while it decreased more rapidly in the prepared bacterin, although it remained within the positive range. The positivity rate remained for up to 12 weeks following the booster dose for both vaccines. Nevertheless, at the 16t-weeks post-vaccination or the end of the trial, a notable disparity in the antibody titer difference between the two groups was observed at $P<0.05$. In accordance withthe ELISA kit manufacturer's instructions, a titer of 8000 is considered to be protective. Although the rise in antibody titer in the commercial vaccine occurred more rapidly, both vaccines demonstrated a protective titer greater than 8000 until the conclusion of the experiment.

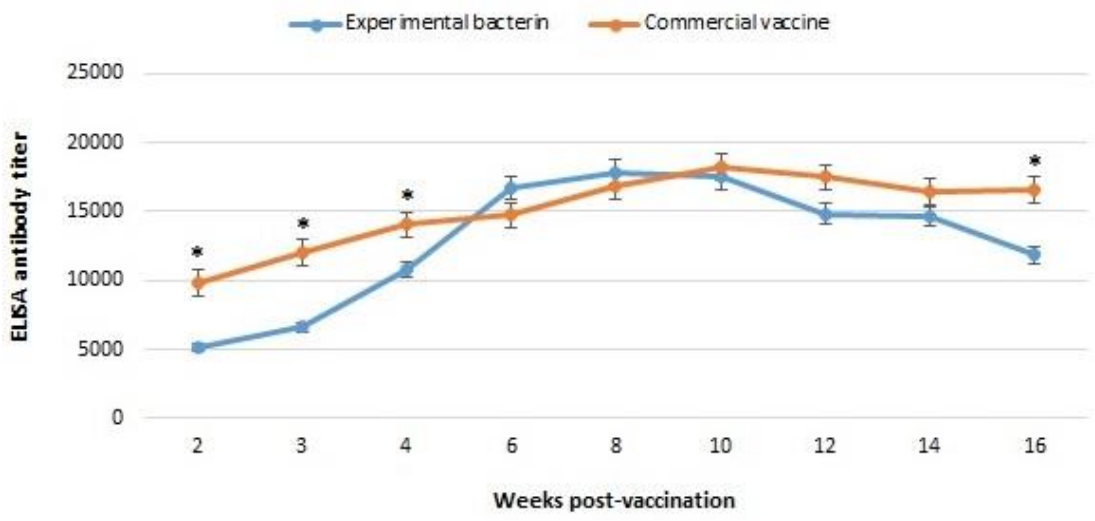

Figure 1. Specific antibody levels against *Ornithobacterium rhinotracheale* in vaccinated chickens at interval times post-vaccination. Chickens received the prim dose in the first week and the booster dose in the fourth week. The significant differences ($P<0.05$) are shown in *.

The statistical analysis revealed that the antibody response kinetics of the vaccinated chickens following the booster injection were comparable, with 100% positivity for and antibodies. The statistical analysis revealed that vaccination with the prepared bacterin significantly influenced the ORT-specific antibody response. Although, the mean value of the antibody levels differed statistically significantly from that of the commercial vaccine before the booster (P <0.05) the discrepancies in ORT antibody were examined by comparing the mean values for both vaccinated groups with the "Tests of Between-Subjects Effects" (Table 2). Overall, no statistically significant difference (P<0.05) was observed between the chicken groups in terms of antibody induction. The "Sig" represents the P value of the study. In accordance with the data analysis presented in Table 2, the Sig value less than 0.05 indicates a statistically significant effect. Conversely, the value i greater than 0.05 indicates indicates no statistically significant effect. The safety study on the prepared ORT bacterin demonstrated that vaccinated chickens did not exhibit any adverse local or systemic reactions throughout the post-vaccination period.

4. Discussion

Poultry products represent a significant source of protein globally, particularly in developing countries. A multitude of viral and bacterial pathogens threatens the poultry production process. One of the major challenges currently facing the poultry industry is the emergenceofmulti-drug-resistantbacterial pathogens, which are responsible for considerable economic losses. Vaccines can be employed to mitigate the adverse effects of the disease or to safeguard the flock against the infection. Previously, vaccines based on inactivated bacterins have been developed and demonstrated to elicit protective immunity in broilers and turkeys against ORT (18, 19). Furthermore, the administration of booster immunizations to breeders has been proposed as a means of ensuring the continued presence of high levels of maternal antibodies in the progeny throughout the entirety of the laying period. The major problem with the homologous bacterin is that it may not offer strong and broad cross-protection against more than 18 serotypes (A–R) of ORT Consequently, an alternative strategy is necessary to safeguard poultry production against distinct serotype infections (16, 17). The Development of the subunit recombinant ORT vaccines has been targeted to provide a high cross-protection for all serotypes, thereby contributing to the improvement of current vaccines (16, 17, 19). In the laboratory trials, the vaccination of broilers with a multicomponent subunit vaccine containing the eight recombinant proteins resulted in high-level protection against ORT challenge with both homologous and heterologous serotypes. Nevertheless, the efficacy of vaccine in field conditions has not been studied. The ability of live vaccines to induce a cross-protective immune response against ORT serotypes has been the subject of debate, with the hypothesis that they are generally of higher quality than inactivated vaccines. With regard to the development of a live vaccine against poultry pathogens, the avirulent ORT strain represents a potential candidate. At this time, the process of genetic engineering is still difficult due to the limited knowledge about the molecular pathogenesis of ORT serotypes and the host immune response to the infection. In general, the vaccination of broilers with adjuvanted inactivated bacterin has been observed to induce high and long-lasting responses against ORT, as well as a one- log rise in the mean antibody titers after booster vaccination (20). Based on the identification and characterization studies regarding ORT in Iran (6, 8-10, 12, 14), only serotype A has been identified from ORT infection cases in Iran. Consequently, vaccination of poultry

Table 2. Results of "Tests of Between-Subjects Effects" for antibody levels against *Ornithobacterium rhinotracheale* in vaccinated chickens

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	2471389935.804	1	2471389935.804	1331.684	.000
s1	5794450.795	1	5794450.795	3.122	.103
Error	22270049.868	12	1855837.489		

with an inactivated vaccine may be sufficient in areas where only one serotype is prevalent. In this study, a formalin-inactivated bacterin was formulated based on a local isolate with the objective of developing an effective vaccine against the homologous serotype of ORT infection. In light of the aforementioned considerations, this comparative study was undertaken to demonstrate the benefits of using a local isolate in the development of an inactivated vaccine against ORT. Our study showed that a whole-cell vaccine inactivated by formalin has the potential to prevent ORT infection. The vaccine candidate elicited a rapid immune response in chickens, with a high rate of antibody positivity. Despite the induction of an appropriate immune response, the pattern of antibody eliciting differed in the two groups of chickens, especially before the booster injection. Following the administration of two doses of ORT bacterin, with an average interval of four weeks between doses, the antibody positivity rate was 100%. In chickens that received the prepared bacterin, the antibody levels were expressed at a higher level only after the booster administration. Furthermore, post-peak antibody titers were decreased more than the Nobilis® vaccine. In general, the differences in effect can be attributed to various factors, including the bacterial strains, growth conditions, bacterial concentration per dose, and the specific inactivation process employed. In the process of inactivating a pathogen the purpose of developing a vaccine, two major goals should be addressed: complete inactivation of the pathogen and antigen integrity (22). Following the inactivation of ORT with formalin, no colonies were observed following the plating the culture on Blood agar, indicating that bacteria were completely inactivated. A second challenge is ensuring the integrity of bacterial cells. Modifications to antigen conservation and integrity result in variations in the induction of immune response and the duration of immunity. Accordingly, in order to obtain of high quality, the limitations associated with the inactivation process should be addressed. One of the challenges is that the inactivators typically result in damage to bacterial DNA/protein synthesis or impairment of cellular integrity. Bacterins, which are used in veterinary medicine, are usually produced through the incubation of bacterial culture with chemicals such as formalin. This chemical modifies amino acids by the addition of reactive carbonyl groups, thereby inactivating and inactivates organisms by crosslinking their macromolecules (23). Despite the extensive use of formalin in the production of bacterins for many decades, there are drawbacks in usage due to its negative impact on proteins. The cross-linking between formalin and bacterial surface antigens may result in changes in the antigenic epitopes and immunogenic properties of the vaccine. Conversely, intact inactivated bacteria may be employed for immunization purposes (24). The current study evaluated the immunogenicity of the ORT bacterin, which was manufactured using a local isolate. The results of this study indicate that imply that the formalin-treated bacterin elicited proper humoral immune responses, mainly following the booster dose regimen. However, the delay in creating an immune response and the relatively rapid decline in antibody titer compared to the commercial vaccine represent significant challenges that must be addressed. For the development of an inactivated vaccine capable of inducing a protective immune response, the quality of the antigen is of high importance. Crosslinkers, such as formalin, have the potential to influence bacterial proteins and degrade their antigenic structure. Therefore, in the event that replacing the inactivator is not feasible, setting up a controlling system to predict the quality of the resulting antigen should be a priority in future studies.

Acknowledgment

This work was supported by Razi Vaccine and Serum Research Institute under grant number 12-18-18-9460-94002.

Authors' Contribution

N.G (the corresponding author) designed the study, carried out the analysis and interpretation of data with contributions from all co-authors; S.S drafted the manuscript and all authors read and approved the final the manuscript.

Ethics

The animal studies were ethically approved by the Institutional Animal Care and Use Committee at the Razi Vaccine and Serum Research Institute.

Conflict of Interest

The authors report no declarations of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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