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Evaluation of the Razi *Ornithobacterium rhinotracheale* Vaccine by Experimental Challenge System Using LaSota Strain

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Abstract

BACKGROUND: *Ornithobacterium rhinotracheale* (ORT) is a remarkable pathogen in the world poultry industry. The vaccine against this agent is used in poultry farms to prevent infection and reduce the incidence of disease.

OBJECTIVES: In the present study, the efficacy of the first Iranian inactivated ORT vaccine produced by the Razi Vaccine and Serum Research Institute was evaluated using the experimental challenge system.

METHODS: Ninety day-old specific-pathogen-free White leghorn chickens were divided randomly into five groups of 18 chickens. The birds were housed in separate specific cages in isolation rooms. At the age of 14 days, the birds of two groups were vaccinated. Afterwards, at the age of 42 days, two groups of unvaccinated chickens and all of the vaccinated subjects were challenged with the LaSota strain of Newcastle Disease Virus (NDV) and ORT. One group of unvaccinated birds was maintained as the negative control. Blood samples were taken from chickens on days 14 (before vaccination) and 42 (before challenge) of the experiment. In addition, blood samples were collected on days 2, 4, 6, 8, 10, and 12 after the challenge (AC). On days 2, 4, 6, 8, 10, and 12 after challenging with ORT, the isolation and molecular detection of the bacteria were performed on samples from the trachea, lungs, air sacs, liver, and spleen.

RESULTS: Following vaccination with the Razi ORT vaccine, the titers of antibody in vaccinated chickens were shown to be significantly higher than those of unvaccinated birds. In vaccinated groups, the ORT was not recovered in cultures from lungs, trachea, and air sacs. In the unvaccinated birds challenged with ORT, bacteria were isolated from lungs, trachea, and air sacs. Using the polymerase chain reaction method, ORT was only detected from samples of lungs, trachea, and air sacs 2 days after challenge (DAC) in vaccinated groups. Meanwhile, ORT was detected in lungs, trachea, and air sacs until 4 days after challenge in unvaccinated birds.

CONCLUSIONS: We concluded that the Razi ORT vaccine was effective in protecting layer chickens against infection with serotype A of the ORT.

KEYWORDS: Challenge, Chicken, *Ornithobacterium rhinotracheale*, Polymerase Chain Reaction, Vaccine

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Introduction

Respiratory tract infections are considered as one of the serious problems in intensive poultry production. Diseases cause economic damage to breeding units due to increased mortality, reduced growth, and elevated slaughterhouse condemnation rates (Chin *et al.*, 2013). Various bacterial pathogens have been identified as the etiological agents of respiratory diseases. *Ornithobacterium rhinotracheale* (ORT) is a recently identified bacterial pathogen associated with respiratory infection in poultry (Thieme *et al.*, 2016) which has been isolated throughout the world (Chin *et al.*, 2013). Currently, ORT infection appears to have become endemic, especially in areas with intensive poultry production and multiple age farms. In Iran, the outbreak of respiratory diseases associated with ORT has also been reported (Mayahi *et al.*, 2016).The sensitivity of ORT to antibiotics is strain-dependent and geography-related (Watteyn *et al.*, 2016). ORT can acquire reduced susceptibility or resistance against antibiotics (Umali *et al.*, 2017). Treatment and control of ORT infections with antibiotics are very difficult due to the varying susceptibility and resistance of this bacterium (Chin *et al.*, 2013). Therefore, the best strategy to prevent Ornitho bacteriosis is believed to be vaccination (Schuijffel *et al.*, 2006). A wide range of vaccines has been developed to control experimental and natural infections associated with ORT (Churria *et al.,* 2012). Sprenger *et al.* (2000) showed that an inactivated ORT vaccine in poultry and turkeys induced protective immunity in birds. Recently, the Razi Vaccine and Serum Research Institute of Iran has produced an inactivated ORT vaccine. The researchers have demonstrated that the experimental infection of chickens with ORT causes minor lesions, while the concurrent presence of other respiratory pathogens with ORT increases the severity of lesions associated with ORT (Ellakany *et al.,* 2018).Consequently, to more accurately evaluate the ORT vaccine produced by the Razi Vaccine and Serum Research Institute, a simultaneous challenge was performed with ORT and the LaSota strain of Newcastle Disease Virus (NDV).

Materials and Methods

Chickens

In this study, a total of 90 one-day-old specific-pathogen-free (SPF) White Leghorn chickens (Venky's Ltd, India) were randomly divided into five equal groups. The chickens were kept in separate cages. The chickens were housed in the Bird Disease Research Building of the Razi Vaccine and Serum Research Institute. Throughout the experiment, the chickens had unrestricted access to sterile water and disinfected feed. No vaccines or medications were given to the chickens until the end of the test.

Bacteria

The ORT used in the present study with the isolation number of R87-7/1387 (JF 810491) had previously been isolated and identified from poultry farms in different provinces of Iran. The bacteria were kept in the Avian Diseases Department of the Razi Vaccine and Serum Research Institute at -70°C. The bacteria were thawed and cultured on Columbia Agar (Oxoid Ltd, Basingstoke, UK) with 5% sheep blood and was incubated for 48 h at 37°C in an atmosphere with 5% CO_2. Next, the bacterial colonies were transferred into the brain heart infusion (Oxoid Ltd, Basingstoke, UK) and were incubated for 24 h at 37°C. The bacteria were washed twice with phosphate-buffered saline. A suspension containing 1×10^{10} colony forming units (CFU) per 0.5 mL was prepared.

Experiment Design

At the age of 14 days, the chickens in groups V_1 and V_2 were vaccinated by 0.3 mL of Razi ORT vaccine containing 1×10^{7} whole-cell bacteria in a mineral oil adjuvant subcutaneously into the neck. Simultaneously, the birds in groups C_1, C_2, and C_3 were injected with 0.3 mL of sterile physiological serum subcutaneously. At the age of 42 days, according to Table 1, the birds of group V_1 were challenged with

1×10^6 EID_{50}/dose of the LaSota strain of NDV by the ocular route and 1×10^{10} CFU per 0.5 mL of the ORT through the intratracheal route. The chickens of group V_2 were inoculated only with ORT. The chickens of group C_1 were challenged with both intraocular NDV and intratracheal ORT, while the birds in group C_2 were inoculated only with ORT. Group C_3 was maintained as an unchallenged control group. Each bird in group C_3 was given one drop of intraocular sterile distilled water and 0.5 mL of intratracheal sterile physiological saline.

Table 1. Plan of vaccination and challenge of the experimental groups.

Groups	ORT vaccination	Challenge with ORT	Challenge with NDV
V_1	+	+	+
V_2	+	+	-
C_1	-	+	+
C_2	-	+	-
C_3	-	-	-

Sampling

In order to determine the antibody titer in the blood serum of chickens against ORT, at the ages of 14 days (before vaccination) and 42 days (before challenge with ORT), in addition to days 2, 4, 6, 8, 10, and 12 after the challenge with ORT (AC), blood samples were taken from chickens of all experimental groups. On days 2, 4, 6, 8, 10, and 12 AC, three chickens of each group were randomly killed by cervical dislocation. The samples from the trachea, lungs, air sacs, liver, and spleen of those birds were collected immediately and were examined by bacteriological and molecular methods.

Bacteriological Analysis

For microbiological analysis, samples were taken from the lungs, trachea, air sacs, liver, and spleen of chickens under sterile conditions. The steps of culture and biochemical identification of bacteria were completed according to the method of Ghasemipour *et al.* (2019) described previously.

Antibody Detection

The titers of antibodies against ORT were measured by enzyme-linked immunosorbent assay (ELISA).For this purpose, the BioChek ORT Antibody test kit (Netherland) was used. The test was carried out according to the manufacturer's instructions.

DNA Extraction

Bacterial genetic material was extracted using the phenol-chloroform method previously described by Ghasemipour *et al.* (2019).

Primers

Primers used in this study were designed by Van Empel and Hafez (1999). The sequences of primer pairs were OR 16S-F_1 (5′-GAG AAT TAA TTT ACG GAT TAA G-3′) and OR 16S-R_1 (5′-TTC GCT TGG TCT CCG AAG AT-3′). The *16s rRNA* gene is a suitable tool for molecular studies (Montes *et al.*, 2018). These primers amplify a 784 bp fragment on the *16s rRNA* gene of ORT.

PCR

Molecular tracing of the samples was performed using the PCR method. The details of the technique were previously described by Ghasemipour *et al.* (2019).

Statistical Analysis

The data were statistically analyzed by the SPSS software version 22 (SPSS Inc., Chicago, Ill., USA). In order to compare the means between and within the groups, a t-test was applied.

Results

Mortality

We did not observe mortality in any of the experimental groups during the experiment.

Serology

The mean of ELISA ORT titer in different sections of the study is presented in Table 2. Antibodies were not present in the blood of chickens in any of the experimental groups before vaccination. After vaccination, the titer of antibody in vaccinated groups was significantly higher than in unvaccinated groups. After challenging the chickens with ORT, the mean of antibodies in the blood of chickens in vaccinated groups augmented. However, the difference between the mean antibody titers before and after challenge with bacteria in vaccinated groups was not significant (Table 2).

Bacterial Identification

The results of bacteriological isolation are presented in Table 3. Bacteria were not isolated from the liver and spleen of any of the chickens in distinct experimental groups in post-challenge sampling. In vaccinated groups, the ORT was not recovered from the lungs, trachea or air sacs of the birds. However, in the unvaccinated groups challenged with ORT (C_1 and C_2), *O. rhinotracheale* was isolated from lungs, trachea, and air sacs. In group C_1, ORT was isolated from the trachea and air sacs on the second and fourth days after the challenge, while it was isolated from lungs exclusively on the fourth day. In group C_2, the ORT was found in the lungs, trachea, and air sacs on the second day after challenge (AC).

Molecular Detection

To investigate the presence of bacteria after the challenge of chickens in different experimental groups, a PCR test was performed on samples taken from the trachea, lungs, air sacs, liver, and spleen of chickens. Finally, ORT was only detected by PCR in the samples collected from the lungs, trachea, and air sacs of birds on 2 DAC in V_1, V_2, and C_2 groups. In the C_1 group, on days 2 and 4 after challenge with bacteria, ORT was detected in lungs, trachea, and air sacs samples (Table 3).

Table 2. Mean ORT Elisa titer in different phases of the experiment, comparison of mean ORT Elisa titer between groups after challenge, and comparisons of mean ORT Elisa titter before and after challenge within vaccinated groups.

Groups	Mean titer in different phases of the experiment			Groups	Comparison titer between groups after challenge		Groups	Comparisons titer before and after challenge within vaccinated groups	
	BV	BC	AC		t value	sig		t value	sig
V_1	0.00±0.00	12417±1972	14185±1568	V_1 versus V_2	-0.204 ^{ns}	0.839	V_1	-0.702 ^{ns}	0.492
V_2	0.00±0.00	13350±1944	14637±1557	V_1 versus C_1	2.883 **	0.009	V_2	-0.517 ^{ns}	0.611
C_1	0.00±0.00	0.00±0.00	9753±370.4	V_1 versus C_2	2.750*	0.013			
C_2	0.00±0.00	0.00±0.00	9482±449.9	V_2 versus C_1	3.181**	0.005			
C_3	0.00±0.00	0.00±0.00	0.00±0.00	V_2 versus C_2	3.051**	0.006			

BV: before vaccination; BC: before challenge; AC: after challenge.
Ns: not significant, *: significant difference (P≤0.05), **: significant difference (P≤0.01).
V_1 (VAC $^{+}$, ORT $^{+}$, ND $^{+}$), V_2 (VAC $^{+}$, ORT $^{+}$, ND $^{-}$). C_1 (VAC $^{-}$, ORT $^{+}$, ND $^{+}$), C_2 (VAC $^{-}$, ORT $^{+}$, ND $^{-}$), C_3 (VAC $^{-}$, ORT $^{-}$, ND $^{-}$)

Table 3. Results of culture and PCR of the different organs after challenge.

	Days (AC)	2					4					6					8					10					12				
	Groups	V_1	V_2	C_1	C_2	C_3	V_1	V_2	C_1	C_2	C_3	V_1	V_2	C_1	C_2	C_3	V_1	V_2	C_1	C_2	C_3	V_1	V_2	C_1	C_2	C_3	V_1	V_2	C_1	C_2	C_3
Culture	Trachea	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lungs	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AirSacs	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PCR	Trachea	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lungs	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AirSacs	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

AC: after challenge

V_1 (VAC $^+$, ORT $^+$, ND $^+$), V_2 (VAC$^+$, ORT$^+$, ND $^-$), C_1 (VAC $^-$, ORT $^+$, ND $^+$), C_2 (VAC $^-$, ORT $^+$, ND $^-$), C_3 (VAC $^-$, ORT , ND)

Discussion

Nowadays, ORT is considered a promising pathogen of poultry and a serious threat to control practices in the poultry industry (Churria *et al.*, 2012). In profitable poultry production, vaccination plays an important role in herd health. Commercial and experimental bacterins have been the most commonly used vaccines against ORT since 1998.This study demonstrated the efficacy of the first Iranian native inactivated ORT vaccine in layer chickens under a challenging system.

In the present challenge trial, contrary to the high rate of mortality reported by Pan *et al.* (2012), even in the unvaccinated groups that were challenged with ORT and ND, no mortality was observed in chickens. The mortality rate due to ORT is strongly related to the predisposing factors, the virulence of bacteria, and susceptibility of birds. Experimental infections in chickens have shown that the severity of the disease induced by ORT varies between strains (Chin *et al.*, 2013). A difference in pathogenicity between the strains of ORT has also been reported by Van Veen *et al.*(2000). Moreover, White Leghorn chickens used in this study were found to be less susceptible to ORT infection than broilers and turkeys (Van Veen *et al.*, 2000). Variety in mortality rate may be attributed to the high virulence of bacteria, vaccination, inadequate breeding management conditions, including high bird density per unit area, poor ventilation, and the presence of other pathogens in farms exacerbating any disease that has been brought about. Nevertheless, it seems that the presence of infectious and non-infectious agents can act as an intensifier of ORT pathogenicity. Low bacterial pathogenicity, controlled breeding conditions, and low susceptibility of Leghorn chickens might be due to the lack of mortality in the present investigation.

No antibody titer was detected against ORT in the current study before vaccination in none of the experimental groups, before the challenge in unvaccinated birds, and the birds of the negative control group, which might be due to the use of SPF chickens and the breeding of birds in separate cages and separate rooms. After vaccination and after challenge, the titer of antibody against ORT in challenged vaccinated groups (V_1 and V_2) was significantly higher than challenged unvaccinated groups (C_1 and C_2).

The main reason for vaccinating poultry is to induce high levels of antibody to protect birds in the face of disease challenges. The results of the present study indicated the effective role of the vaccine in inducing antibody titers against ORT, which was consistent with the results of Schuijffel *et al.* (2006) and Erganis *et al.*

(2010). Several researchers have developed and used bacterins for the control of ORT infection outbreaks in commercial poultry under experimental and recorded conditions (Schuijffel *et al.*, 2006; Erganis *et al.*, 2010). However, an important issue in using bacterial vaccines is that different bacterial serotypes may not have much antigenic similarity with each other leading to inadequate immunity after usage in birds. Serotype A of ORT is the most prevalent serotype in chickens (Chin *et al.*, 2013). Consequently, this serotype was chosen for the inactivated Razi ORT vaccine. This bacterial serotype was isolated from poultry farms with respiratory diseases in different provinces of Iran.

On days 2, 4, 6, 8, 10, and 12 AC, bacteriological and molecular detection were performed on the samples of lungs, air sacs, trachea, liver, and spleen. In none of the experiment groups, ORT was detected by culture and PCR from the liver and spleen, which may be related to the low pathogenicity of the bacterium. This was in agreement with the study by Hegazy *et al.* (2016) and Umali *et al.* (2017) who did not isolate ORT from the heart and liver. Researchers have shown that in natural and experimental infections, ORT has been isolated from the trachea and lungs of infected birds (De la Rosa *et al.*, 2018; Gavrilovic *et al.*, 2016; Hauck *et al.,* 2015). In the present investigation, ORT was isolated from respiratory organs in unvaccinated groups on 2 and 4 DAC, while in vaccinated birds ORT was not isolated even on 2 DAC. Lack of bacterial isolation after challenge in vaccinated groups could be attributed to the influential role of the Razi ORT vaccine in preventing infection. In unvaccinated chickens challenged with ORT and NDV, the bacteria were detected by culture and PCR on 2 and 4 DAC. However, in birds challenged exclusively with ORT, the bacteria were isolated on 2 DAC. The NDV appears to have caused a longer bacterial infection in the respiratory organs. Van Empel *et al.* (1999) demonstrated that ORT is isolated from the trachea and air sacs of poultry only until the second day after exposure to the bacterium. On the other hand, in birds that were first exposed to the virus and then to the bacterium, the bacterium was isolated from the lungs and air sacs of infected birds for up to ten days after a lung infection. Furthermore, the lack of bacterial isolation in the unvaccinated birds challenged with ORT and ND and unvaccinated birds challenged only with ORT on 6, 8, 10, and 12 DAC can be due to the low pathogenicity of the bacterium and the removal of the bacteria by the immune system of the birds. The bacteria were detected in the PCR of the samples of lungs, trachea, and air sacs on 2 DAC. The ORT can normally be isolated by culture only at an early stage of the infection and attempts to recover the organism at a later stage often fail (Chin *et al.,* 2013). The reason for the positive results of PCR on 2 DAC in vaccinated groups despite the negative result of culture could be the high sensitivity of specific primers in the identification of bacterial genetic material. According to the serologic, microbiological, and molecular results presented in this study, the Razi ORT vaccine can effectively protect chickens against infection with serotype A of the bacterium.

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Conflict of Interest

The authors declared no conflict of interest.

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مجله طب دامی ایران، ۱۳۹۹، دوره ۱۴، شماره ۴، ۴۰۳-۴۱۱

ارزیابی کارآیی واکسن *اورنیتوباکتریوم رینوتراکئال* رازی در سیستم چالش تجربی با استفاده از سویه لاسوتا

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زمینه مطالعه: *اورنیتوباکتریوم رینوتراکئال* یک عامل بیماری‌زای مهم تنفسی در صنعت پرورش طیور است. واکسیناسیون یک ضرورت اجتناب‌ناپذیر برای جلوگیری از عفونت و تکثیر عوامل بیماری‌زا و کاهش میزان بروز و مرگ‌ومیر در پرورش طیور محسوب می‌شود.

هدف: در مطالعه حاضر کارآیی نخستین واکسن ایرانی غیر فعال *اورنیتوباکتریوم تراکئال* در یک فرایند چالش تجربی مورد ارزیابی قرارگرفت.

روش کار: تعداد ۹۰ قطعه جوجه یک روزه عاری از عوامل بیماری زای خاص از نژاد لگهورن سفید به طور تصادفی به ۵ گروه ۱۸ قطعه‌ای تقسیم شدند. پرندگان هر گروه در قفس‌های مخصوص به صورت مجزا و در اطاق‌های ایزوله نگهداری شدند. جوجه های دو گروه در ۱۴ روزه‌گی واکسینه شدند. در ۴۲ روزه‌گی، چالش جوجه‌های گروه‌های واکسینه‌شده و جوجه‌های دو گروه غیر واکسینه با *اورنیتوباکتریوم رینوتراکیال* و ویروس لاسوتای بیماری نیوکاسل انجام شد. پرندگان یک گروه غیر واکسینه بدون هیچ چالشی به‌عنوان گروه کنترل منفی در نظر گرفته شد. نمونهٔ خون در سن ۱۴ روزه گی (قبل از واکسیناسیون)، ۴۲ روزه‌گی (قبل از چالش)، و روزهای ، ۴، ۶، ۸، ۱۰ و۱۲ بعد از چالش برای آزمایش‌های سرم شناسی گرفته شد. نمونه‌های نای، ریه، کیسه‌های هوایی، کبد و طحال نیز در روزهای ۲، ۴، ۶، ۸، ۱۰ و۱۲ بعد از چالش، تهیه و با روش‌های باکتری شناسی و مولکولی ارزیابی شدند.

نتایج: پس از واکسیناسیون با واکسن *اورنیتوباکتریوم رینوتراکئال* رازی، تیتر آنتی‌بادی در جوجه‌های واکسینه‌شده به‌طور معنی‌داری بالاتر از جوجه‌های غیر واکسینه شد. درگروه‌های واکسینه، باکتری به‌وسیله کشت از اندام‌های تنفسی (ریه، کیسه‌های هوایی و نای) جدا نگردید. در گروه‌های غیر واکسینه چالش شده، باکتری از اندام‌های تنفسی در روزهای دوم و چهارم پس از چالش جداسازی و رد یابی شد. در گروه‌های واکسینه باکتری تنها در روز دوم پس از چالش، به‌وسیلهٔ روش مولکولی از اندام‌های تنفسی ردیابی شد.

نتیجه‌گیری نهایی: نتایج این پژوهش بیانگر نقش موثر واکسن *اورنیتوباکتریوم رینوتراکئال* رازی علیه عفونت با سروتیپ A *اورنیتوباکتریوم رینوتراکئال* در جوجه‌های تخم‌گذار بود.

واژه‌های کلیدی: *اورنیتوباکتریوم رینوتراکئال*، واکسن، چالش، واکنش زنجیره‌ای پلیمراز، جوجه

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