Exploring the potential pathogenicity of *Ornithobacterium rhinotracheale* isolates, individually and in association with avian influenza virus infection

Naser Ghodsian*[1], Mansour Banani[1], Shahla Shahsavandi[1], Mohammad Majid Ebrahimi[1]

1. Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization, Karaj, Iran

Corresponding author: Naser Ghodsian

E-mail: N.ghodsian@rvsri.ac.ir

Abstract

Ten field isolates of *Ornithobacterium rhinotracheale* (ORT) were studied for their pathogenicity in specific pathogen-free (SPF) embryonated chicken eggs and 71-day-old chickens. The lethal dose 50 (LD_{50}) of each ORT isolate was determined by intraperitoneal injection of chickens with serial CFU dilutions from the bacterial samples. SPF embryonated eggs were inoculated through the allantoic sac at 11 days of age and monitored daily. The isolates appeared to be non-pathogenic, with only one strain showing low pathogenicity, resulting in a 10% mortality rate within 8 days post-infection. In another experiment, 71-day-old chickens were grouped based on the ORT isolates (1-10) and inoculated intratracheally with bacterial suspensions. No obvious clinical signs were observed in live or necropsied chickens. At 3 days post-inoculation, birds from each group were divided into two equal subgroups. Subgroups (1A-10A) were inoculated with H9N2 avian influenza virus (AIV) and compared with the other subgroup (1B-10B). Within two weeks after inoculation, chickens given ORT+AIV exhibited slight depression, sneezing, and coughing. Histological examination revealed mild to severe hemorrhagic tracheitis, heterophil infiltration in the trachea, pneumonia with necrosis and mononuclear infiltration in the lung, and necrotic sinusitis with mononuclear cell infiltration. Since chickens were less susceptible to ORT infection than embryonated chicken eggs, it is recommended to inoculate embryonated chicken eggs as an alternative method to obtain more accurate pathological data.

Keywords: *Ornithobacterium rhinotracheale*, embryonated egg, pathogenicity, influenza virus

1. Introduction

The global demand for poultry products has increased because producing poultry meat and eggs is more cost-effective than other protein sources. In parallel to the continual rise in poultry products, infectious agents can significantly affect farm performance and, in turn, the poultry industry. Consequently, the primary objective of any production system—whether conventional or sustainable—will always be to minimize the risk and impact of diseases (1). The most effective way to protect against disease on farms is biosecurity, which, together with vaccination and good farm management, form the three components of the prevention and control triangle. Many pathogens responsible for chronic diseases in birds primarily infect the respiratory tract. Respiratory infections pose a significant challenge for the poultry industry, leading to substantial economic losses. The most serious bacterial respiratory pathogens identified in poultry include *Mycoplasma gallisepticum*, *Pasteurella multocida*, *Salmonella* species, *Escherichia coli*, and *Ornithobacterium rhinotracheale* (ORT) (2).

ORT is an emerging bacterial pathogen first identified in the early 1990s and has since been isolated from various domestic and wild bird species worldwide (3). Coughing, sneezing, and nasal discharge followed by severe respiratory distress are the clinical signs of ORT, which is accompanied by a reduction in feed consumption and water intake in the field (4, 5). Infection in broiler chickens leads to purulent airsacculitis, pleuritis, pneumonia, and unilateral or bilateral consolidation of the lungs. Infected young birds may also show purulent brain inflammation and osteomyelitis of the skull bones (6). Isolating ORT in the laboratory can be challenging due to the specific requirements of the bacterium for enriched media and microaerophilic conditions. In addition, other bacteria can mask the detection of ORT, further complicating its isolation and identification (4).

In the past two decades, ORT has been found in commercial broiler farms throughout various regions of Iran. Data from seroprevalence studies, isolations, and molecular identifications indicate that ORT has become endemic in Iran (7-9). This situation highlights the need for additional research to understand the pathogenicity of different isolates and the impact on vaccine strain selection. Determining the pathogenicity of ORT is challenging (10). The pathogenicity of ORT does not depend on strain origin or serotype, making it difficult to assess. This situation underscores the need for further research to understand the pathogenicity of different isolates and their impact on vaccine strain selection. Determining the pathogenicity of ORT will aid the development of an effective vaccine against ORT in

chickens, though it presents challenges. Furthermore, there is no standard model available for studying the pathogenicity of ORT. The current study aimed to evaluate the pathogenicity of ORT farm isolates using two methods: inoculation into chicken embryonated eggs and intratracheal inoculation of chickens, to introduce a more effective candidate vaccine strain.

2. Materials and Methods

2.1. ORT strains

During the period 1998-2009, several clinical ORTs were isolated from commercial poultry farms located in Alborz, Gilan, Mazandaran, and Qazvin provinces. The isolates were identified using microbiological, serological, and molecular assays (8, 9). In this study, total of ten ORT isolates with the accession numbers JF810488, JF810489, JF810491, JF810493, JF810495, JF810498, JF501955, JF501957, JF501958, and JF501961 were included.

2.2. Determination of lethal dose 50 (LD_{50}) of ORT isolates

For each ORT sample, the number of viable bacteria was determined using the standard plate counting method. Serial ten-fold dilutions were prepared in phosphate-buffered saline (PBS, pH 7.2). Aliquots of 100 µl were spread on sheep blood agar, and the plates were incubated at 37°C for 48 h. The count was calculated by multiplying the average number of colonies per plate by the reciprocal of the dilution used, and it was expressed as CFU/ml. Fifty 20-day-old chickens were injected intraperitoneally with 10^0-10^{-4} CFU dilutions of ten ORT isolates and observed daily for 14 days. The LD_{50} was determined using the Reed-Muench method.

2.3. Inoculation chicken embryonated eggs

Twenty specific pathogen-free (SPF) embryonated eggs (Venky's Company, India) were inoculated with 100 µl of 1×10^{10} LD_{50} of an ORT sample via the allantoic sac at 11 days of age. The eggs were then monitored daily for up to 8 days, and on the last day, all eggs were placed at 4°C overnight. Pathogenicity was measured based on the percentage of dead eggs infected by the ORT. A rate of 10-20% was considered low pathogenicity, 21-61% moderate pathogenicity, and over 61% high pathogenicity (11).

2.4. Inoculation of chickens

The animal studies received ethical approval from the Institutional Animal Care and Use Committee at the Razi Vaccine and Serum Research Institute. The study included eleven SPF chicken groups (n=10 per group) at 71 days of age, which were placed in separate cages and received feed and water *ad libitum*.

Chickens in Groups 1-10 were inoculated intratracheally with 1×10^{10} LD_{50} of ORT isolate suspensions using an angiocatheter. Group 11, without ORT inoculation, served as a control. The chickens were carefully inspected twice daily. On 3 days' post-inoculation (PI), two chickens from each

group were autopsied, and gross lesions were recorded to examine pathological changes compared to the control. As no obvious clinical signs were observed in either the live or necropsied chickens, the birds in each experimental group and control group were divided into two equal subgroups to investigate the pathogenesis of the ORT isolates. Chickens (n=4) in each subgroup (1A-10A) and control group (C1) were inoculated with an avian H9N2 influenza virus (AIV; 10^6EID_{50}) via eye drop. Another subgroup (1B-10B) and control group (C2) were housed separately to conduct thorough analyses of these infections. All chickens were clinically examined for two weeks. Blood samples were taken from infected chickens at two weeks PI, and a hemagglutination inhibition (HI) test. The highest serum dilution inhibiting HA activity was defined as the HI endpoint titers.

At the end of the experiment, the trachea, lung, and sinus were removed aseptically. The tissue sections (10 μm thickness) were fixed in 10% buffered formalin and examined histopathologically using hematoxylin and eosin staining. A scoring system ranging from 0 to 3 based on the severity of the lesions was used to evaluate histopathological changes include: 0 = no change; 1 = mild inflammation characterized by infiltration of small numbers of lymphocytes and macrophages; 2 = moderate inflammation characterized by infiltration of moderate numbers of lymphocytes, macrophages, and small numbers of heterophils; 3 = severe inflammation characterized by infiltration of large numbers of lymphocytes, macrophages, and heterophils.

2.5. Statistical analysis

Statistical analysis was taken using the SPSS; version 21. The significant differences were determined using the *t*-student test based on $P < 0.05$.

3. Results

Potential pathogenicity of ten ORTs was assessed in embryonated eggs, showing that six strains did not cause mortality over 8 days PI. By excluding first-day losses, which are not typically caused by bacterial or viral infection but rather by weak embryos or egg handling, only one isolate (Acc. NO. JF810493) displayed a low pathogenic feature with a mortality rate of 10% (Table 1). The observed difference in mortality rate is statistically significant ($P < 0.05$).

Table 1. Pathogenicity of *Ornithobacterium rhinotracheale* isolates based on the mortality rate of chicken embryonated eggs (n=20)

Isolate No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Mortality										

1	-	1*	-	-	-	-	1*	-	-	-
2	-	-	1	-	-	-	-	-	-	-
3	-	1	-	-	-	-	1	-	-	-
4	-	1	-	-	-	-	-	-	-	-
5	-	-	-	-	-	1	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
Sum %	0	10ǂ	5	0	0	5	5	0	0	0

* First day loss eliminated.

ǂ Significant ($P < 0.05$)

To test the pathogenicity of ORT in poultry, 10 week-old SPF chickens were grouped depends on the bacterial strain. The chickens in ORT+AIV subgroups (1A-10A) were inoculated with ORT first and AIV 3-day PI later. No remarkable clinical signs or mortality were observed in the experimental groups. However, within 5 to 7 days' PI, some chickens in 1A-10A subgroups and C2 subgroup exhibited slight depression and showed respiratory signs such as sneezing and coughing. There were no mortalities in the ORT+AIV-infected chickens during the experiment. The H9N2 HI antibody levels were detected in chickens infected with ORT+AIV at the end of experiment. The mean HI titer of the chickens in 1A-10A subgroups and C2 subgroup were approximately 4 $\log_2$, which correlate with early replication of the H9N2 virus in chicken respiratory tract.

On necropsy, no gross pathological changes were observed in the trachea, lung, or sinus tissues of chickens infected with ORT However, the ORT+AIV-infected chickens exhibited mild to severe hemorrhagic tracheitis, as well as infiltration of heterophils in the trachea and foci of pneumonia with necrosis and mild to moderate mononuclear infiltration in the lungs and sinus (Table 2, Figures 1 and 2). Overall, the severity of lesions was higher in the trachea compared to other tissues. The C1 subgroup did not show any clinical or pathological lesions compared to the C2 subgroup. These lesions were not pathognomonic and may be associated with AIV infection, as the increase in the specific AIV HI titer in the C2 and 1A-10A subgroups was solely related to H9N2 infection. The lesions were severe in an ORT isolate (named No.2) causing higher mortality in eggs than the other isolates.

Table 2. Pathological profile of chickens infected with *Ornithobacterium rhinotracheale* isolates and H9N2 avian influenza virus

Subgroup	Histopathological lesion					
	Trachea	**Score**	**Lung**	**Score**	**Sinus**	**Score**
1A	Severe tracheitis with heterophilic infiltration and severe hyperemia	3	Hyperemia and foci of mononuclear infiltrate of the mucosa of secondary bronchi	2	Sinusitis with necrosis and moderate mononuclear infiltration	2
2A	Severe tracheitis with heterophilic infiltration and severe hyperemia	3	Focal hyperemia and pneumonia with moderate heterophilic infiltration	2	Sinusitis with necrosis and moderate mononuclear infiltration	2
3A	Severe tracheitis with extensive mononuclear infiltrate	3	Small foci of mononuclear infiltration	1	Mild sinusitis	1
4A	Severe tracheitis with mononuclear infiltrate	3	-	0	Sinusitis with necrosis and sparse mononuclear infiltration	1
5A	Severe tracheitis with extensive infiltrates	3	Focal pneumonia with sparse mononuclear infiltrate	1	Sinusitis with necrosis and sparse mononuclear infiltration	1
6A	Severe tracheitis	3	Hyperemia	1	Sinusitis with necrosis and sparse mononuclear infiltration	1
7A	Severe tracheitis	3	Focal pneumonia with sparse mononuclear infiltrate	2	Sinusitis with necrosis and sparse mononuclear infiltration	1
8A	Severe tracheitis	3	Limited foci of mononuclear infiltration	1	Sinusitis with necrosis and sparse mononuclear infiltration	1
9A	Moderate to severe tracheitis	2	Focal pneumonia with sparse mononuclear infiltrate	1	Sinusitis with necrosis and sparse mononuclear infiltration	1
10A	Mild to moderate tracheitis	1	Mild hyperemia	1	Sinusitis with necrosis and sparse mononuclear infiltration	1

4. Discussion

Numerous reports have been published since 1990 concerning poultry respiratory diseases, specifically air sacculitis and acute pneumonia caused by ORT. The consequence of ORT infection varies and is influenced by host factors, environmental conditions, and co-infections with other bacteria and viruses. These situations can exacerbate health issues and lead to significant economic losses. Vaccination against ORT could serve as an effective strategy for prevention and control. The effectiveness of an ORT vaccine depends on its broad antigenic coverage due to the presence of various serotypes and isolates with different pathogenicity (12). As in many countries, ORT isolates were identified from poultry farms across Iran, highlighting the need to monitor their pathogenesis to develop an effective vaccine against different strains.

The ORTs included in this study had previously undergone Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) to evaluate their genetic diversity and relationships. Based on their genetic fingerprint, these ORTs, which were isolated over 11 years from various provinces, exhibited a highly similar molecular profile and belonged to a single genotype A (8). Since the ERIC-PCR profile alone does not fully capture the pathogenic potential of the isolates, we further investigated their pathogenicity by inoculating them into chicken embryonated eggs and chickens through an animal model. When the embryonated eggs were inoculated with the different ORTs, the strains were generally non-pathogenic, except for one isolate that caused a mortality rate of 10% within 8 days PI.

ORT infection does not cause significant clinical signs or gross lesions in young chickens. While, pathogenicity in older chickens is often exacerbated by co-infections with other pathogens (6, 13-15). In line with the egg-inoculating trial, experimental infections with ten ORT isolates in 71-day-old SPF chickens were done resulted in no clinical signs or respiratory lesions, which aligned with expectations. This was insufficient to determine the pathogenicity of the strains. Therefore, half of the birds in each group were subsequently infected with the H9N2 AIV and displayed a typical pneumonia. Both ORT and H9N2 AIV can cause mild respiratory signs, which are exacerbated in co-infection, leading to severe pneumonia, tracheitis, and airsacculitis. In the field, the severity is affected by factors such as age, breed, and immunity level of chickens (2, 3). Under our experimental conditions, none of the ORT isolates showed pathogenic characteristics for SPF chickens and only slight depression and respiratory signs were observed in the ORT+AIV-infected chickens within 5 to 7 days' PI. Early replication of the H9N2 virus in the chicken respiratory tract in the C2 and 1A-10A subgroups

was confirmed by a rise in HI antibody titers. These finding indicate that the mild clinical signs could only be related to co-infection with H9N2 AIV.

The histological lesions in the trachea, lung, and sinus of ORT+AIV-infected chickens revealed minimal infiltration of polymorphonuclear granulocytes, as shown in Table 2. Similar to AIV, mixed infections of ORT have been reported in association with other avian pathogens, such as infectious bronchitis disease, *Escherichia coli*, *Avibacterium paragallinarum*, and *Mycoplasma* sp (6, 13-16). These can cause clinical signs that vary from respiratory symptoms to mortality and increase the severity of the pathological lesions associated with ORT. Overall, the field-isolated ORT strains were unable to induce mortality in embryonated chicken eggs or noticeable lesions in adult chickens. The pattern observed was consistent across all ORT isolates; however, the isolate that caused 10% mortality in embryonated eggs led to severe pulmonary issues accompanied by heterophilic infiltration in infected birds. A comparison of the clinical signs and respiratory lesions in the ORT+AIV group and the ORT-only group confirms that all isolates exhibited the same pathological features. Additionally, the clinical signs and lesions of ORT isolates observed in this study were not pathognomonic.

The main factors influencing the pathogenicity of ORT are virulence factors, adherence profiles, the presence of various serotypes, and genetic and phenotypic characteristics. The pathogenesis of ORT varies between serotypes and is host-dependent. Serotype A is the most prevalent ORT serotype in chickens, with an incidence of 94%. The prevalence of ORT infection in turkeys is much higher than in broiler chickens, with the most frequently isolated strains belonging to serotypes A, B, D, and E. Research has shown that serotypes C, D, and E exhibit a higher capacity to adhere to a culture of primary chicken tracheal cells than A and B (70% vs. 20%) (17). Phylogenetic analyses of the ORT 16S rRNA gene deposited in GenBank indicated that most isolates from various geographical regions in Iran were homogeneous and clustered together. Iranian ORT isolates are closely related, with a similarity ranging from 98.8% to 100% at a scale of 0.002 substitutions per site. Additionally, evolutionary inference indicates that molecular divergence has influenced the genetic diversity of recent ORT isolates, resulting in the generation of a new clade (18). These isolates may be undergoing positive selection or have originated from a distinct source. To provide a better understanding of the epidemiology of ORT outbreaks, fingerprint analysis using the RAPD-PCR technique has been conducted (19). The analysis has revealed a high similarity among Iranian ORT isolates, with only one isolate showing a diverse pattern out of the 30 ORTs collected between 2000 and 2017 (20). Our pathogenicity results confirmed the findings regarding the serotyping and genetic relatedness of ORT strains isolated from poultry farms in Iran. Since Iranian ORT isolates

belong to serotype A, show high genomic homology, and share similar pathogenicity, selecting a vaccine candidate strain to develop a more effective and safer vaccine is not challenging. However, the ability of a new ORT isolate to elicit a protective immune response compared to the older ones should be evaluated.

In conclusion, embryonated chicken eggs are used as an alternative animal model in various research fields, including pathogenesis, because they provide a relatively inexpensive, accessible, and ethically manageable system compared to more complex animal models. Here, the pathogenicity of each ORT isolate was investigated using two animal models inoculation of embryonated eggs and adult chickens. The relatively lengthy period of SPF chicken trials, which includes rearing and maintaining the chickens until they reach 71 days of age, the requirement for co-infection with another pathogen to induce pathological lesions, the time-consuming process of preparing histological slides, and the difficulty in interpreting pathological features of ORT due to the lack of specific criteria are significant limitations in utilizing live animals. Therefore, inoculating embryonated chicken eggs is recommended as an alternative method to achieve more accurate results in determining pathogenicity.

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Figure 1. Histopathological lesions in the trachea of a chicken infected with *Ornithobacterium rhinotracheale* isolate and H9N2 avian influenza virus, Hematoxylin and eosin stain, 200× magnification).

Figure 2. Histopathological lesions in the lung of a chicken infected with *Ornithobacterium rhinotracheale* isolate and H9N2 avian influenza virus, Hematoxylin and eosin stain, 200× magnification).