

## Molecular characterization of *Ornithobacterium rhinotracheale* isolated from broiler chicken flocks in Iran

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**Abstract:** The present study was designed to isolate *Ornithobacterium rhinotracheale* in broiler flocks in Iran and characterize it using biochemical, polymerase chain reaction assays, and phylogenetic analyses. A total of 150 tracheal swabs were collected from broiler chickens in slaughterhouses. Additionally, 150 lung and 150 tracheal tissue samples were taken from dead birds of broiler flocks that were developing symptoms of respiratory diseases. For PCR assay DNA was extracted from ORT isolates and also from tissue samples. ORT was isolated from 1 out of 150 swabs samples and 3 out of 300 total tissue samples. Four ORT isolates together with 7 DNA extractions from tissue samples generated amplification products of 784 bp after the PCR with specific primers. Finally, it was confirmed that the sequence analysis of 16S rRNA and ORT isolates is close to the isolates from GenBank with identity ranging from 98% to 100%.

**Key words:** *Ornithobacterium rhinotracheale*, phylogenetic analyses

### Introduction

*Ornithobacterium rhinotracheale* (ORT) is a pathogen best known for causing respiratory tract infections, such as airsacculitis and pneumonia, in birds all over the world. ORT can be a primary or secondary etiological agent depending on strain virulence, adverse environmental factors, the immune state of the flock, and the presence of other infectious agents (1). The pathogen may cause systemic diseases such as hepatitis, joint lesions, and cerebrovascular pathology or could lead to economic losses due to growth retardation and the rejection of carcasses for consumption (2).

The diagnosis of ORT infection is based on isolation, identification, serology, and polymerase chain reaction studies. ORT is a difficult bacterium to culture. It grows slowly and needs special growth conditions and so attempts at isolation are often negative and plates are overgrown by other bacteria. Infections with ORT can be treated with antibiotics successfully; however, the bacterium rapidly develops resistance to antibiotics. The outbreak of respiratory disease associated with ORT has been reported in the USA, France, the Netherlands, Belgium, Spain, Germany, Hungary, Israel, Korea, Japan, Taiwan, Turkey, and South Africa (1-7).

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In Iran, ORT infection was reported by Banani et al. for the first time (8). Consequently, the serological evidence from poultry flocks indicates that ORT occurs in different regions of the country (9). The aim of the present study was to isolate ORT in broiler flocks in Iran and characterize the organism using biochemical, PCR assays, and phylogenetic analyses.

## Materials and methods

**Sample collection and ORT isolation:** A total of 150 tracheal swabs from different commercial broiler flocks, with or without respiratory signs, were randomly collected from slaughterhouses that were located in the north, west, and center of Iran. In the same regions, a total of 150 lung and 150 tracheal tissue samples were taken from dead birds of 10 broiler flocks that were developing symptoms of respiratory diseases.

In the study, for PCR assay DNA was extracted both from ORT isolates and from tissue samples. While half of each tissue sample was used for ORT isolation the second half was kept at  $-80^{\circ}\text{C}$  for DNA extraction. Samples were streaked from the frozen stock onto 5% sheep blood agar with  $10\ \mu\text{g}/\text{mL}$  of gentamicin. Plates were incubated in a moist chamber with 7.5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$  for 24-48 h. The pinpoint, circular, small opaque to grayish and non-hemolytic colonies with 1-3 mm diameter, were selected (4). Colonies with characteristics of ORT were used by various identification methods such as staining by Gram's method, biochemical identification tests, and finally genetically identified by polymerase chain reaction (PCR) and DNA sequencing (3,10). Suspected ORT isolates were stored in brain heart infusion (BHI) with 30% glycerol at  $-80^{\circ}\text{C}$ .

**Biochemical identification:** Biochemical characterization was performed with oxidase, catalase, growth on MacConkey agar, triple sugar iron agar, indole, urea, lysine decarboxylase, nitrate, gelatinase, motility, and carbohydrate fermentation tests such as sucrose, manitol, glucose, galactose, lactose, and maltose (3).

### Polymerase chain reaction

#### a-DNA extraction from isolates

For DNA extraction individual colonies were suspended in  $300\ \mu\text{L}$  of pure water and heated at  $100^{\circ}\text{C}$

for 10 min and then were centrifuged for 10 min at 11,600 rpm. The supernatant fluid was used for DNA extraction and frozen at  $-20^{\circ}\text{C}$  until further use (1,10).

#### b- DNA extraction from lung and trachea tissue samples

Bacterial DNA was extracted from lung and tracheal tissue by using the DNeasy Tissue kits (Qiagen GmbH, Hiden, Germany) according to the manufacturer's instructions. In this assay, 25 mg of lung or tracheal tissue was placed into small pieces in a 1.5 mL microcentrifuge tube and  $180\ \mu\text{L}$  of ATL buffer was added. Then  $20\ \mu\text{L}$  of proteinase K was added and mixed by vortexing and then incubated at  $55^{\circ}\text{C}$  until the tissue was completely lysed. This was followed by adding  $200\ \mu\text{L}$  of all buffer thoroughly by pipetting to yield a homogeneous solution. The solution was again incubated at  $70^{\circ}\text{C}$  for 10 min and then received  $200\ \mu\text{L}$  of ethanol (96%-100%) and was mixed by vortexing. The mixture was pipeted into a DNeasy Mini Spin Column that was placed in a 2 mL collection tube and was centrifuged at 8000 rpm for 1 min. The DNeasy Mini Spin Column was replaced with a new collection tube and  $500\ \mu\text{L}$  of AW1 buffer was added and centrifuged as mentioned above. The DNeasy Mini Spin Column was again replaced with the new tube and  $500\ \mu\text{L}$  of AW2 buffer was added and centrifuged for 3 min at 14,000 rpm. At the last stage, the DNeasy Mini Spin Column was transferred to another tube and  $200\ \mu\text{L}$  of AE buffer was pipeted directly onto the DNeasy membrane and then centrifuged for 1 min at 8000 rpm. The supernatant fluid was used for DNA extraction.

In the PCR assay, for positive control 2 mL of the inactivated ORT vaccine (Nobilis ORT, Intervet) was centrifuged. The pellet was washed with 2 mL of pure water and centrifuged. The pellet of bacterial cells was resuspended and used for DNA extraction.

**PCR assay:** The PCR assay was performed with the DNA Thermo Cycler (TC-512, England). Primers used in our study were those reported by van Empe and Hafez, (1), OR16S-F1 ( $5' - \text{GAG AAT TAA TTT ACG GAT TAA G}$ ) and OR16S-R1 ( $5' - \text{TTC GCT TGG TCT CCG AAG AT}$ ), which amplify a 784-bp fragment within the 16S ribosomal RNA. The

reactions were performed in a final volume of 25  $\mu$ L containing 5  $\mu$ L of template DNA, 2.5  $\mu$ L of reaction buffer, 1  $\mu$ L of deoxynucleoside triphosphates (dNTPs), 1  $\mu$ L of  $MgCl_2$ , 0.3  $\mu$ L of *Taq* DNA polymerase, 13.2  $\mu$ L of distilled water, and 1  $\mu$ L of each primer for ORT. Initial denaturation was at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 60 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 7 min. The inactivated vaccine and distilled water were used as the positive and negative controls in all tests.

DNA sequencing: PCR product from the isolated-ORT was sequenced in both directions by an automatic sequencer using a commercial sequencing facility ([www.primm.it](http://www.primm.it)). The obtained sequences were compared with the sequences of isolates of GenBank like DQ195252, DQ195242, U87100, U87101, U87102, U87103, U87104, U87105, and U87106.

## Results

Isolation of ORT: A total of 150 tracheal swab samples were obtained from broiler chicken flocks, with or without respiratory symptoms, located in the regions of the west, north, and center of Iran. ORT was isolated from only 1 out of 150 surviving chickens. Additionally, a total of 150 lung and tracheal tissue samples were taken from dead birds. It was indicated that ORT was isolated from 3 of the 150 lung tissues (2%), whereas no ORT bacteria were isolated from tracheal tissues. Gram-negative, pleomorphic, rod-shaped bacteria were observed in all isolates (Figure 1).

Biochemical properties of 4 ORT isolates were identified and presented in the Table. It is interesting that acid production from glucose, galactose, and lactose, which were taken from 3 out of 4 isolates, were positive, while only 1 isolate, produced from maltose, was positive for acid production. In the present study, all isolates were positive to oxidase and urea but negative to catalase, triple sugar iron agar, MacConkey agar, lysine decarboxylase, nitrate, gelatinase, and motility.

Table. The results of biochemical properties of ORT isolates.

Tests	Isolates			
	Swabs	Tissue 1	Tissue 2	Tissue 3
Oxidase	+	+	+	+
Catalase	-	-	-	-
Triple sugar iron agar	-	-	-	-
MacConkey	-	-	-	-
Indole	-	-	-	-
Urea	+	+	+	+
Lysine decarboxylase	-	-	-	-
Nitrate	-	-	-	-
Gelatinase	-	-	-	-
Motility	-	-	-	-
Sucrose	-	-	-	-
Manitol	-	-	-	-
Glucose	+	+	+	-
Galactose	+	+	+	-
Lactose	+	+	+	-
Maltose	-	+	-	-

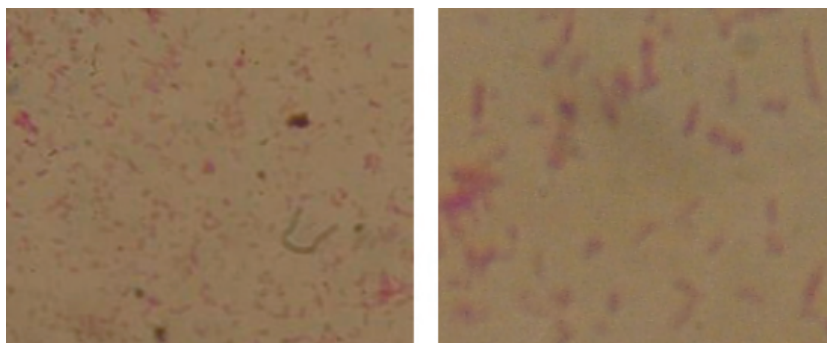


Figure 1. Photographs of gram-stained isolate showing characteristics of pleomorphism.

PCR: All 4 ORT isolates and positive control DNA extracted from ORT vaccine were positive in PCR. All positive PCR amplicons were predicted to be the size of 784 bp. Nonetheless, the ORT PCR assay did not detect the distilled water as a negative control (Figure 2, lanes 2-7). Additionally, in the present assay, PCR produced 784-bp amplicons from DNA extracted from lung and tracheal tissues from dead birds with symptoms of respiratory diseases. Surprisingly, at this stage, 1 out of 150 tracheal (1.5%) and 6 out of 150 lung (4%) tissues were positive in PCR (Figure 2, lanes 8-14).

DNA sequencing: The DNA sequences were obtained and were deposited under GenBank Accession nr: EU730706. The sequences of 16S rRNA of ORT isolated in this study and other sequences obtained from GenBank were analyzed and the sequences of our isolates showed the identity to be ranging from 98% to 100% from GenBank.

## Discussion

ORT is spread horizontally by direct and indirect contact. Vertical transmission is suspected since some recent research has isolated ORT at very low incidence from reproductive organs, hatchery eggs, and dead embryos (2).



Figure 2. Electrophoresis of PCR products on a 1% agarose gel stained with ethidium bromide. 100 bp molecular weight marker (lanes 1 and 15), positive control (lane 2), negative control (lane 3), tracheal swab isolate (lane 4), lung isolates (lanes 5 to 7), DNA extracted from tracheal (lane 8), and DNA extracted from lung tissues (lanes 9 to 14).

On the basis of clinical features and pathological lesions, the diagnosis of ORT is often difficult, because they may be confused with other infectious conditions. The proof of infection therefore must be confirmed by isolation and identification of the causative agents (11).

In the present study, only 4 ORT bacteria were isolated from the respiratory tract of broiler chickens and one of the main reasons for low isolation could be the overgrowth by rapid-growing bacteria masking the ORT colonies, as reported earlier by many authors (1,12,13). Regarding the etiology of the organism, the majority of ORT isolates are resistant to gentamycin (12) and this antibiotic has to retard the growth of other bacteria in media cultures. However, in our study sometimes gentamicin did not prevent the growth of all other bacteria species, which might be due to overuse of antibiotics such as gentamicin in commercial broiler flocks as suggested earlier (2,13).

The biochemical characteristics observed in our ORT isolates were similar to those in earlier reports (1,13). Under optimal conditions, all 4 isolates were positive for oxidase and urease tests; however, they were negative for catalase, triple sugar iron agar, MacConkey agar, indole, and other properties (Table), in accordance with the results of Canal et al. (13). Three out of 4 isolates, taken from glucose, galactose, and lactose cultures, were positive for acid production; however, no acid was produced from these 3 sugars by the fourth ORT isolate. Vandamme et al. (4) observed no acid production from glucose, in agreement with the results of Canal et al. (13). One out of 4 isolates, taken from maltose, was positive for acid production. Nevertheless, no acid production was observed from sucrose, which was not in accordance with the results of Canal et al. (13). The variability of biochemical results obtained here was compatible with the literature reports and probably reflected the great genetic variability found in different regions (1,12,13), even in one country.

Four ORT isolates together with 7 DNA extractions from tissue samples generated amplification products of 784 bp after the PCR with specific primers. The findings of our investigation have an important implication for the diagnosis of ORT. Regarding our results, the number of detected-ORT by PCR assay was almost double (7/150) in



tissue samples when compared with the number of ORT identified by germ isolates (4/150). This indicates that the ORT contamination of broiler flocks was much higher than those of flocks that could isolate only bacteria. Additionally, the isolation of ORT from 3 out of 150 (3/150) tissue samples when compared with the identification of 7 ORT in PCR assay in the same tissues again confirmed the phenomenon of such slowly growing organism and the sensitivity of PCR assay for diagnosis. Since the dead cells of ORT could be detectable by PCR, it was possible that broiler flocks had already been infected with this germ and samples had a greater number of contaminated cells but exited a lower number of viable ORT (13).

Based on the sequence analysis of 16S rRNA, ORT isolates in our study was close to the isolates from GenBank with identity ranging from 98% to 100%. DNA sequences obtained here showed the identity to be according to the fall of sequences in cluster ORT isolated from sequences of U87100 and AY162321. Sequences were obtained from strains that were isolated from a turkey flock in Minnesota (14), broiler flocks in Brazil (13), and were obtained from broiler chickens and pigeons in Taiwan with the sequence of

DQ195252 (10). This was in agreement with many reports that indicated that ORT strains from all over the world were closely related clones (1,14).

The high similarity of biochemical reactions and great homology between our isolates with the isolates (all without any correlation with serotyping results) of other regions all over the world from several bird species support the suggestion of Canal et al. (13), in which ORT has only recently been introduced from a common source. Therefore, most disease episodes in domesticated poultry could have resulted from infection with a single clone complex even from wild bird populations (14). Such homology between the isolates demonstrates that vaccination could be a successful strategy for the control of this disease in poultry flocks.

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