

## Characterization of *Ornithobacterium rhinotracheale* Isolates from Commercial Chickens

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### Summary

*Ornithobacterium rhinotracheale* (ORT) is a relatively recently discovered bacterium, which it is associated with respiratory diseases. Tracheal samples from carcasses of 100 poultry flocks submitted to Razi Institute were examined for ORT isolation. All these flocks were affected with respiratory disorders. Colonies of ORT were detected after 24h of incubation and identification of ORT isolates were carried out based on the biochemical and serological characteristics. Fifty-nine isolates from broilers, broiler breeders, and layer flocks were identified as ORT and analyzed for serotyping. The isolates were identified as serotype A, using standard antisera against ORT antigens in rapid slide agglutination, agar gel precipitation and immunodot tests. The identification of the species was confirmed by sodium dodecyl sulfate- polyacrylamide gel electrophoresis of whole cell proteins. High similarity of protein profiles of the isolates was observed. These preliminary results establish that ORT serotype A is associated with outbreaks of respiratory diseases in commercial poultry.

**Key words:** *Ornithobacterium rhinotracheale*, chickens, serotyping, characterization

### Introduction

*Ornithobacterium rhinotracheale* (ORT) is a relatively recently named bacterium that is associated with respiratory disease in avian species. ORT is a pleomorphic gram-negative rod shape bacterium that grows on blood agar. The first recorded isolation of

ORT was made from turkeys in Germany in 1981 (Hinz *et al* 1994). In 1991 van Beek *et al* reported respiratory disease of broiler chickens in South Africa that presented unusual clinical findings. The report described air sac lesions in broiler chickens that were different from those traditionally observed. At postmortem examination, the most striking feature was a foamy white, yoghurt-like exudate in the air sacs, although pneumonia was also found. Gram negative, pleomorphic, rod shaped organisms was isolated (van Beek *et al* 1994). Prior to being named in 1994 (Vandmme *et al* 1994), ORT had been identified as *Pasteurella*-like organism, *Kigella*-like bacterium, Taxone 28, and pleomorphic gram-negative rod bacterium (Chin & Charlton 1998). ORT has been isolated from turkey, chicken, rook, duck, partridge, guinea fowl, choker, pheasant, and pigeon (Lopes *et al* 2000, van Empel & Hafez 1999). This organism has been isolated in Germany, USA, Israel, South Africa, France, The Netherlands, Hungary, Austria, Slovenia, Belgium, Italy, England, Ireland, Canada, Peru, and Iran (Hung & Alvarado 2000, Banani *et al* 2000, Lopes *et al* 2000, Zorman-Rojs 2000, van Empel & Hafez 1999, Chin & Droual 1997). The first reported isolation of ORT in Iran was from a broiler and a pullet flock associated with respiratory disorders.

The world poultry industry has suffered significant financial losses because of the drop in egg production, growth suppression, mortality and condemnation of carcasses in flocks infected with this organism (Lopes *et al* 2000, van Empel & Hafez 1999). Gross lesions in infected birds include a fibrinopulurent, often unilateral pneumonia, airsacculitis, pericarditis, tracheitis, sinusitis, and arthritis (van Empel & Hafez 1999, Chin & Charlton 1998, Chin & Droual 1997). The role of ORT as a primary pathogen in respiratory diseases in turkeys (Sprenger *et al* 1998) and broilers (van Veen *et al* 2000) has been demonstrated. In most cases ORT is associated with other respiratory pathogens (van Empel & Hafez 1999). To date, 12 different ORT serotypes designated, as A to L has been reported (Lopes *et al* 2000). In chickens more than 95% of the isolates are of serotype A (van Empel & van den Bosch). The purpose of this study was isolation, identification, serotyping and determination of the protein

pattern of the isolates from commercial poultry flocks, submitted to Poultry Diseases Department of Razi Institute.

### **Material and Methods**

**Samples.** In the period lasted 6 months commencing in September 2000 and ending in March 2001 the carcasses from 100 broiler, broiler breeder, and layer flocks with respiratory disorders, which submitted to Razi Institute were examined for routine diagnostic procedures. ORT isolation was specifically targeted in these specimens. A complete necropsy was carried out on dead birds, and these birds were sampled via tracheal swabs for ORT isolation.

**ORT isolation and identification.** For selective isolation of ORT, 5µg gentamicin were added per ml of 5% sheep blood agar (SBA) media. Swab samples were inoculated on blood agar and MacConky agar plates. Plates were incubated under microaerophilic condition (7.5% CO<sub>2</sub>) at 37°C. Some phenotypic characteristics of the isolates were tested for differentiation from closely related agents (Chin & Charlton 1998, Banani *et al* 2000). Demonstrating the agglutination against polyvalent antiserum in rapid slide agglutination test (Abdul-Aziz 1997) did confirmation of ORT.

**Serotyping.** For serotyping of ORT isolates, rapid slide agglutination (RSA), agar gel precipitation (AGP) and immunodot tests were done using standard antisera (Joubert *et al* 1999) against ORT antigens. These standard antisera had been obtained from Dr. Amer Silim (Faculty of Veterinary Medicine, University of Montreal, Canada).

**Preparation of ORT proteins.** 5% SBA used to propagate and purify of ORT isolates. For purification of the bacteria, each isolate cloned three times on SBA. After 2 days of incubation, ORT cells were harvested from plates by overlaying with phosphate-buffered saline, pH 7.2, and washed three times with the same buffer by centrifugation (5000g×10 min). 5µl of each ORT isolated cells suspension were lysed in 20µl of sample (solubilizing) buffer (60mM Tris-HCL, pH 6.8, 2.5% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, and 0.02% bromophenol

blue), heated for 10min in a boiling water bath. These samples were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting tests.

**SDS-PAGE.** SDS-PAGE was performed in a 12.5% acrylamide separating (running or resolving) gel with 5% stacking gel according to the discontinuous buffer system of Laemmli 1970. The dimension of the separating gel was 140×100×1mm, and sample-loading slots (wells) were 6 mm wide. Prepared samples of ORT isolates as described above, and prestained protein molecular-weight markers (Gibco BRL, Life Technologies, Germany) as standard, which was prepared with the same procedure, were loaded into wells. Samples were electrophoresed under constant current in mini gel apparatus (Paya Pajooresh Co., Mashhad, Iran) until the dye front reached the end of the separating gel. The gel was run at 200 volts at room temperature for about 4h. Electrophoresis buffer consisted of 25mM Tris-HCL (pH8.3), 0.2M glycine, and 0.1% SDS. Proteins were stained with 0.25%-0.5% (w/v) Coomassie Brilliant Blue R-250 and destained in 15% methanol and 5% glacial acetic acid with two to three changes. Photographs were taken with a UVP camera (UVP International, Ultra-Violet Products LTD, England).

**Western blot.** Proteins of the ORT isolates separated by SDS-PAGE were transferred to nitrocellulose (NC) sheets by electrophoretic blotting (Uniform Electro-Transfer, Paya Pajooresh Co., Mashhad, Iran) at 100 volts for 2h in 20mM Tris, 150mM glycine, and 20% methanol (pH8.6). After transfer, a blocking solution (5%skim milk in PBS) was added to NC sheets, and incubated overnight at 4°C. Washed NC sheet (three times with PBS, each one 5min) was incubated 90min at room temperature, with primary serum (antiserum against ORT serotype A, obtained from Dr. Amer Silim) diluted 1:250 in PBS. Next, the appropriate peroxidase-labeled secondary antibody (goat anti-chicken IgG [H+L] peroxidase-labeled, Kirkegaard & Perry Laboratories, INC, USA) diluted 1:1000 in PBS was added to washed NC sheet, and incubated 90min at room temperature. Followed by washing as above, freshly prepared developing solution (3mg 4-chloro-1-naphthol [Sigma] in 1ml

methanol, 5ml PBS, 0.015% H<sub>2</sub>O<sub>2</sub>) was then added. Color development was terminated by several washes in distilled water, and the NC sheets were then dried.

## **Results**

*Isolation and identification of ORT.* After 24h of incubation at 37°C in a 7.5% CO<sub>2</sub> atmosphere, pinpoint grayish opaque colonies were detected only on blood agar plates. The colonies were larger after 48h of incubation. Nonhemolytic colonies were subcultured on blood agar plates. A unique characteristic of the colonies was their poor adherence to agar. A wire loop used to displace or slide a colony along the agar without deforming the colony. Incomplete hemolysis was noted only after 96h of incubation. Pure cultures of the isolates had distinct smell similar to butyric acid. The gram examination revealed the presence of gram-negative, pleomorphic, rod-shaped microorganisms, and, according to biochemical tests, isolated organisms were oxidase positive, catalase and indole negative, and grew poorly in triple sugar iron slants with no change in the butt or slant portions of the tube. Other biochemical characteristics of ORT isolates that have been described previously (Banani *et al* 2000) were also observed. All the presumptive diagnosis was confirmed by RSA test. Fifty-nine out of 100 flocks affected by respiratory disorders were positive for ORT.

*Serotyping, proteins profile and immunoblotting.* All of the ORT isolations examined in this study reacted with chicken antiserum serotype A in RSA, AGP (Fig. 1), immunodot (Fig. 2), and western blot tests, indicating that they corresponded to serotype A.

Protein profiles of the ORT isolates showed that about 16 proteins with sizes ranging from 12-100kDa, some of them were more concentrated, and few variations were observed among isolates (Fig. 3). Western blot analysis identified at least 10 immunoreactive proteins, with that of 42kDa having the strongest reaction.

## **Discussion**

Respiratory signs in ornithobacteriosis are not pathognomonic, and signs of this disease are also associated with other respiratory diseases. Therefore, the diagnosis of

Figure 1. All ORT isolates tested developed a single well-defined precipitin line against antiserum serotype A ORT in AGP test. 1-5-antigen prepared from different ORT isolates, 6-negative control S-avian standard antiserum against serotype A ORT

Figure 2. Immunodot of the ORT isolates using standard antiserum against serotype A ORT. A1-3, B1-3, C1-2-antigen prepared from different ORT isolates, C3 negative control

Figure 3. Lane A-H- proteins of the ORT isolates separated by SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue and destained in methanol and glacial acetic acid. At the left side relative molecular masses are indicated in kDa

ORT infection should be based on the isolation and identification of the causative agent (Abdul-Aziz 1997). In this study, ORT infection was observed in various types of commercial chicken including broilers, layers, and broiler breeders. The

importance of ORT as a serious infection has been increased throughout the world (Abdul-Aziz 1997, Chin & Droual 1997, van Empel & Hafez 1999, van Veen *et al* 2000), so ORT can be recognized as an important cause of losses in poultry industry of our country. In this study all of the ORT isolations examined by AGP test were of serotype A. The AGP test was found to be serotype-specific, but could also be used to distinguish ORT from other avian pathogens, such as, *P.multocida*, *R.anatipestifer*, and *H.paragalinarum* (van Empel & Hafez 1999). In one study, 1091 chicken and turkey isolates of ORT from all over the world were investigated serologically in the AGP test, serotype A was found to have the highest prevalence. In chickens, more than 95% of the isolates are of serotype A. In turkeys, the predominant serotype is also type A (van Empel & Hafez 1999, van Empel & van den Bosch 1998).

In addition to biochemical and serological tests, SDS-PAGE was also used in this study for confirmation of the isolates as ORT. Other workers confirmed the identity of ORT by SDS-PAGE of whole-cell proteins (vandamme *et al* 1994; Joubert *et al* 1999). In this study, few variations were observed among the protein profiles of the ORT isolates. Similarly, van Empel (1998), vandamme *et al* (1994), and Hung and Alvarado (2000) reported a high similarity of outer-membrane proteins and/or total proteins of ORT, among strains isolated in France, Germany, Israel, USA, The Netherlands, United kingdom, and Peru. The high similarity of profiles among the tested ORT strains, without any correlation with serotyping results, indicates that the isolates originating from all over the world and from several bird species are represented by a small group of closely-related clones. Some genetic investigations using by amplified fragment length polymorphism and random amplified polymorphic DNA methods revealed that more species or subspecies probably exist within the genus *Ornithobacterium* (van Empel and Hafez 1999). These apparently controversial results suggested that ORT should be investigated more thoroughly, for example, by sequencing the total 16S rRNA gene of relevant strains.

Therapeutic treatment of ornithobacteriosis can be difficult. The sensitivity of ORT to antibiotics is very inconsistent and appears to depend on the source of the strain (van Empel & Hafez 1999). Because acquired antibiotic resistance is very common

with ORT (Abdul-Aziz 1997, van Empel & Hafez 1999), it is recommended to do sensitivity tests before beginning treatment of an infected flock. The earlier treatment is commenced, the better the results (Abdul-Aziz 1997). Because of acquired antibiotic resistance and various results of antibiotic therapy, it must be stress to prevent the disease. Obviously, the best way to prevent the disease is by vaccinating birds against ORT (van Empel & van den Bosch 1998, Abdul-Aziz 1997). Vaccinations with autogenous inactivated oil adjuvant vaccines were successful in reducing outbreaks of ORT (van Empel & Hafez 1999). Any commercially available vaccines require attention to the specific serotype induced (Abdul-Aziz 1997), so upon the results of this study, it is recommended to use autogenous or serotype A vaccine against ORT in infected areas of poultry industry.

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