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Full Article

Isolation and characterization of *Ornithobacterium rhinotracheale* in the commercial turkey, quail flocks and domestic pigeons by bacteriological and molecular methods

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ABSTRACT

Ornithobacterium rhinotracheale (ORT) is a respiratory pathogen which has been isolated throughout the world from numerous bird species. The present study was designed to isolate and characterize the ORT from domestic turkeys, quails and pigeons. For this purpose, 250 samples from each bird species (turkey, quail and pigeon) with or without respiratory signs were tested by taking of tracheal swabs. In addition, respiratory tissue samples (tracheal and lung), from 250 slaughtered turkeys, 50 slaughtered quails and 100 dead pigeons were also subjected to culture for ORT as tracheal swabs. Respiratory tissues were also tested for bacterial DNA by using polymerase chain reaction (PCR). In general, 30 isolates including 4 isolates from turkeys, 3 isolates from quails and 23 isolates from pigeons were identified as ORT by bacteriological method and then confirmed by PCR. Bacterial DNA was detected in 20%, 50% and 35% of respiratory tissues in turkeys, quails and pigeons respectively. Five ORT isolates from pigeon and all four isolates from turkey showed smaller colony size, while other isolates had larger colonies when cultured in blood agar. Fifty percent of the isolates with larger colony but none of the isolates with small colony size could agglutinate red blood cells (RBCs). All of the isolates were sensitive to danofloxacin and chloramphenicol while more than 90% of pigeon isolates were resistant to ampicillin. All of turkey and quail and 30% of pigeon isolates were resistant to tetracycline. Our ORT isolates showed high identity (98%- 100%) in sequence of 16S rRNA gene to related data in GeneBank.

Keywords: *Ornithobacterium rhinotracheale*, turkeys, quails, pigeon, polymerase chain reaction

INTRODUCTION*

Ornithobacterium Rhinotracheale (ORT) is a gram negative, pleomorphic, rod shaped bacterium which can infect numerous bird species including chicken, turkey, partridge, pheasant, quail, duck, goose, guinea fowl, gull, ostrich and pigeon (Hafez & Sting 1999, Banani *et al* 2001a & 2003, Soriano *et al* 2002, Tsai & Huang 2006, Hassanzadeh *et al* 2010). ORT was first identified as *Pasteurella*- like bacterium and Taxon 28 until the present name was suggested by Vandamme in 1994 (Chin *et al* 2008). Since then, it has been reported

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from various wild and domesticated birds in diverse countries throughout the world such as Taiwan, Mexico, Brazil, Canada, Germany and South Africa (Travers 1996, Hafez & Sting 1999, Soriano *et al* 2002, Canal *et al* 2005, Tsai & Huang 2006). ORT can be a primary or secondary avian respiratory pathogen. Manifestation of clinical signs or the severity of infection are highly influenced by environmental factors such as poor management, high stocking density, poor hygiene, strain virulence of the pathogen and host factors such as immune statues and concurrent diseases. Other infectious agents of the respiratory tract such as *Escherichia coli*, *Bordetella avium* and Newcastle disease virus have also a triggering effect on the incidence of ORT infections (Schuijffel *et al* 2005). Determination of the occurrence of this relatively new pathogen in a population of host species is essential for selecting and implementing appropriate control measures. In Iran there have been several reports on ORT infection in commercial chicken and sporadic turkey cases (Banani *et al* 2003 & 2004, Asadpour *et al* 2008, Hassanzadeh *et al* 2010) but no investigation has been done in the case of other susceptible bird species like quails and pigeons. So, the aim of this study was to investigate the existence of ORT in commercial turkeys, quails and pigeons and to characterize this organism.

MATERIALS AND METHODS

Sample collection. A total of seven hundreds and fifty tracheal swab samples from different flocks of turkey, pigeon and quail (250 samples per specie), with or without respiratory signs, were randomly collected in different part of country at the period of June 2009 to July 2010. In addition, lung and tracheal tissue samples were taken from different flocks of turkey (250 samples) and quail (50 samples), in the slaughter house and 100 same tissue samples from dead pigeons. Lung and tracheal tissues as well as tracheal swabs were cultured immediately for ORT isolation, so the remained tissue samples were kept frozen for later assessment by PCR test.

Tissue DNA extraction. To determine the presence of ORT genomic DNA in tissues, five lung and tracheal tissue samples from each bird species which were negative in bacterial culture, were pooled. DNA extraction was done by using AccuPrep® genomic DNA extraction kit (Bioneer, Republic of Korea).

Isolation of ORT. Tracheal swabs were transported in test tubes with a broth transport medium consisted of peptone and yeast extract to reserve the bacteria and prevent swabs from drying out after sampling. They were kept cool until they were incubated at 37°C in atmosphere containing 7.5% CO_2. After 24 hrs, swabs were streaked on 5% sheep blood agar containing 10 µg per ml gentamicin. Trachea and lung tissues were separately cultured for ORT isolation as above. The plates were incubated for 24 to 48 hrs at the described conditions. Colonies which were circular, non-hemolytic, small and opaque to grayish in color were selected. Suspected colonies were stained by Gram's Method, then identified biochemically to confirm the main characteristics and phenotypic traits and also genetically identified by PCR.

Biochemical identification. The biochemical characterization was carried out with phenotypic tests such as oxidase, catalase, growth on MacConkey agar, lysine, urea, indole, nitrate, and triple sugar iron (TSI). Some carbohydrate fermentation tests such as glucose, lactose, sorbitol, maltose and mannitol were also implemented (Van Empel *et al* 1997).

Hemagglutination assay. Hemagglutinating (HA) activity of ORT isolates was determined by a hemagglutination test by using 96 well round bottom plates. Isolates from three different host species were tested with each host specific erythrocytes in addition to fresh chicken erythrocytes. Colonies from pure cultures were suspended and serially diluted in normal salin solution. To each well, 50 µl of a suspension of 0.75% erythrocytes was added and mixed (Tsai & Huang 2006). Wells with only the suspension of erythrocytes served as a negative control and another

wells containing Newcastle Disease virus (NDV) antigen that could agglutinate birds red blood cells (RBC) was used as a positive control. After 40 min of incubation at room temperature, the results were evaluated (Tsai & Huang 2006).

Antimicrobial susceptibility. Bacterial isolates were taken from 48 hrs blood culture plates. The inoculums were prepared by making a direct suspension of isolated colonies from agar plates in Brain- Heart Infusion (BHI) broth and then applied with a sterile cotton swabs on surface of 5% sheep blood agar. Discs of 7 antimicrobial agents were used: ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), danofloxacin (30 µg), erythromycin (15 µg), norfloxacin (5 µg) and tetracycline (30 µg). After 20 hrs of incubation at 37°C, zones of inhibition (halo) were measured and the results were interpreted with comparison to standards.

Polymerase chain reaction. Polymerase chain reaction (PCR) was performed in a 25 µl final volume with 5 µl of boiled extract DNA, 2 U of *Taq* polymerase, 12.5 µM KCl, 5 µM Tris- HCl, 0.1 µM of each dNTP, 0.5 µM $MgCl_2$ and 0.2 µM of each specific primer to the 16S ribosomal RNA gene of ORT (Van Empel & Hafez 1999) (Table 1). Initial denaturation was at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30s, annealing at 58 °C for 60s and extension at 72 °C for 90s with a final extension at 72 °C for 7 min. The inactivated vaccine and distilled water were used as the positive and negative controls (Hassanzadeh *et al* 2010).

DNA sequencing. PCR products of four ORT isolates including two from pigeons and one from each turkey and quail isolates were sequenced in both directions by an automatic sequencer provided by a commercial sequencing facility (Gen Fanavaran Co.). The obtained sequences were compared with 15 reference sequences obtained from GeneBank (accession numbers DQ195248, DQ195250, DQ195249, DQ195247, DQ195245, DQ195242, DQ195252, DQ195258, DQ195240, DQ860700, DQ860701, HM246652, EU730706, U87100 and AY162321) by using the BLAST program.

RESULTS

Isolation of ORT. ORT was isolated from 1.6% (4 of 250) in turkey, 1.2% (3 of 250) in quail and 4.8% (12 of 250) in pigeons, by culture of tracheal swabs. ORT was also isolated from 11% (11 of 100) of pigeons by culturing of lung and trachea tissue samples. So, the overall isolation rate of ORT for pigeons was 6.6 % (23 of 350). No isolate was found by culture of turkey or quail respiratory tissue samples (Table 2).

Table 1. PCR primers used in this study

Primer	Sequence	Target gene
OR16S-F_1	5- GAG AAT TAA TTT ACG GAT TAA G – 3	16S RNA
OR16S-$_1$	5- TTCGCTTGGTCTCCGAAGAT- 3	16S RNA

Table 2. Origin, colony morphology and hemagglutination ability (HA) of 30 *Ornithobacterium rhinotracheal*e isolates

Origin of ORT isolate	Number of isolates/ Tracheal swabs	Colony morphology / Lung tissue	Number of isolates Small colony (SL)	Number of samples with HA Large colony (LC)	
Turkey	4/250	0/250	4	0	0
Quail	3/250	0/50	0	3	1
Pigeon	12/250	11/100	5	18	9
Total	30				10

Morphological characteristics. There was a paramount difference in colony size within ORT isolates. Five isolates of pigeon origin and all the four isolates of turkeys had small colonies, while quail isolates and other isolates of pigeon (18 samples), were of larger colonies after 24 hrs of incubation. This difference in colony morphology could be yet seen after 48 hrs of incubation. From one of pigeon lung samples, ORT isolates with both small and large colony size were grown. The ORT isolates displayed pleomorphism with rod shaped and filamentous form in Gram stain especially when grown in liquid media (Figure 1).

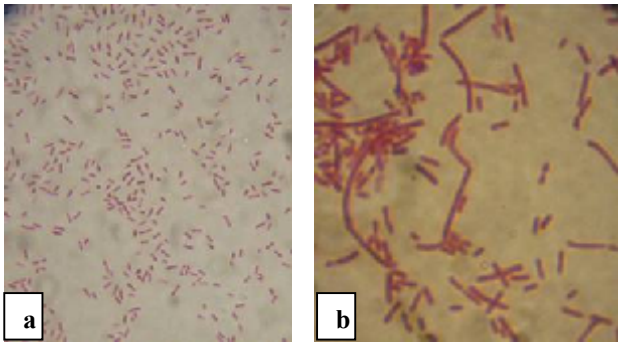

Figure 1. Gram stain of *Ornithobacterium rhinotracheal*e, a: ORT from 48 hrs culture on blood agar, showing short and plump rods; b: bacteria from fluid medium, showing very long rod.

Biochemical identification. Thirty ORT isolates were identified biochemically by means of some phenotypic tests. The results for catalase test, TSI, growth on MacConkey agar and production of indole were negative as predicted. It was interesting that some differences were observed in phenotypic characteristics of our pigeon isolates compared with preponderant literature reports. All of the pigeon isolates but none of turkey or quail isolates were oxidase and urease negative. Acid production from glucose, maltose, sucrose, lactose and mannitol were variable.

Hemagglutination assay. Nine of 23 pigeon isolates (39.1%) showed hemagglutination activity with chicken or pigeon red blood cells. Among these, five isolates could agglutinate both chicken and pigeon RBCs while three and one isolates were only positive for pigeon and chicken RBCs respectively. None of turkey isolates could react with either turkey or chicken RBCs, while one of three quail isolates showed hemagglutination activity with both quail and chicken RBCs. All hemagglutinating strains were of large colony size and none of ORT isolates with small colony could agglutinate RBCs.

Antimicrobial susceptibility. The results of the antimicrobial susceptibility test are shown in Table 3. All of the 30 isolates were sensitive to chloramphenicol and danofloxacin. The majority of pigeon isolates were resistant to ampicillin, erythromycin and norfloxacin. Both turkey and quail isolates were sensitive to ampicillin, erythromycin and norfloxacin but showed resistance against tetracycline. Quail isolates in contrast to turkey isolates were resistant against ciprofloxacin.

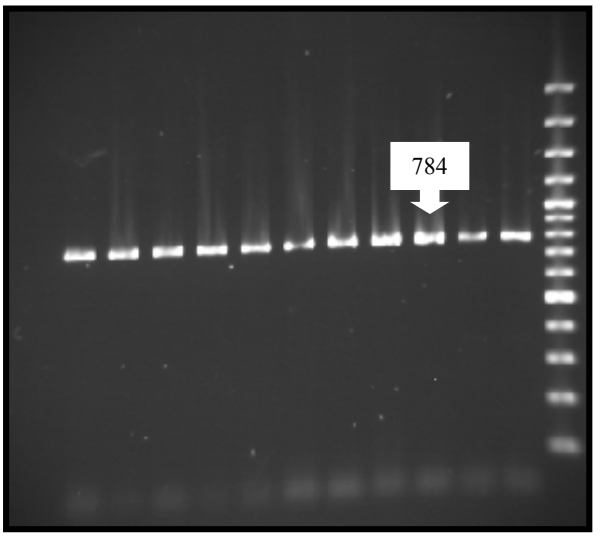

Figure 2. Electrophoresis of PCR products on 1.5% agarose gel stained with ethidium bromide: 100 bp molecular weight marker (lane M), positive control (lane a), amplification products from a number of ORT isolates (lanes b- k) and negative control (lane l).

Polymerase chain reaction. All isolates reacted positively in the PCR assay and produced the predicted 784 bp amplification product. In the case of extracted tissue samples, 10 out of 50 (20%), 5 out of 10 (50%) and 7 out of 20 (35%) of pooled samples were positive in turkeys, quails and pigeons respectively (Figure 2).

DNA sequencing. The nucleotide sequences had been submitted to GeneBank and had been assigned accession numbers HQ696787 for turkey, HQ696786 for quail and HM234094 and HM246651 for pigeon isolates. The sequences of the 16S rRNA fragment showed 98% to 100% identity with other sequences from GeneBank without regard to the origin of isolates. In this study, two pigeon ORT isolates showed more than 99% identity with related data from Taiwanese pigeon isolates obtained from GeneBank.

DISCUSSION

Ornithobacterium rhinotracheale has recently been recognized in many countries as possible additional bacterial respiratory pathogen of birds especially turkeys and chickens. In a study, investigation of the ORT

Table 3. Antibiotic susceptibility of *Ornithobacterium rhinotracheal*e isolated from turkeys, quails and pigeons.

Antibiotic	Concentration (µg)	Number of resistant isolates/ % antibiotic resistance		
		Turkey isolates n= 4	Quail isolates n= 3	Pigeons isolates n= 23
Ampicillin	10	0/0	0/0	21/91
Chloramphenicol	30	0/0	0/0	0/0
Ciprofloxacin	5	0/0	2/100	0/0
Danofloxacin	30	0/0	0/0	0/0
Erythromycin	15	0/0	0/0	14/61
Norfloxacin	5	0/0	0/0	12/52
Tetracycline	30	3/100	2/100	7/30

infection in commercial turkeys by tracheal swab culture revealed that 43% of flocks were positive for ORT (Roepke *et al* 1998).

Unlike in chickens, there was no documented report regarding the extent of ORT infection in turkey, quail and pigeon flocks in our area. The present study was the first report confirming the presence of *Ornithobacterium rhinotracheale* in pigeons of Iran. In our study the total isolation rate of ORT from pigeons was lower than Taiwanese pigeons, (6.6% in compare with 9%) (Tsai & Huang 2006), but ORT genomic DNA could also be detected in 35% of pigeon carcasses. Lower isolation rate for ORT but more positive results in bacterial DNA detection in respiratory tissues, may indicate limited value of culture method in comparison with PCR test to show infection with this organism. ORT can be present in apparently healthy or sick birds and participate to avian respiratory complexes in many susceptible bird species, but due to its slower growth rate and smaller colony size, it may be overgrown by fast growing bacteria such as *E. coli* and therefore is hard to be detected. It has been proven that most ORT isolates are resistant to gentamycin, so in the present study, this antibiotic was added at the rate of 10 µg per ml of blood agar medium to increase the chance of isolation (Chin *et al* 2008).

Inconsistency in conventional biochemical tests are usually observed among ORT strains (Chin *et al* 2008). In this study urease and oxidase tests for pigeon isolates showed negative reactions in contrast to the previous results which were reported by Canal *et al*. (2005). Such results in biochemical tests were in agreement with the reports of Tsai and Huang (2006) which indicated lower positive rates and variable results in biochemical tests of pigeon isolates compare with chicken isolates and it may be related to host origin.It has been shown that ORT strains have very variable susceptibility to antibiotics (Chin *et al* 2008, Tsai & Huang 2006). Acquired resistance was seen with quinolones, tetracyclines and macrolides among ORT isolates (Ak & Turan 2001). The sensitivity pattern of the different strains of ORT depends on the antibiotics which are used more commonly from the source it is isolated. In this study danofloxacin of quinolone group as well as chloramphenicol showed antimicrobial activity against all ORT strains. It has been reported that most of the chicken isolates in Iran were sensitive to chloramphenicol but showed varying degrees of resistance to erythromycin and tetracycline (Banani *et al* 2001a & 2004). Contrary to Taiwanese pigeon ORT isolates, which were sensitive to ampicillin, more than 90% of our isolated pigeon strains were resistant to this antibiotic. In this study it has also been determined that resistance to erythromycin among pigeon isolates was at a higher rate with comparison to Taiwanese counterparts (61% and 25% respectively). The overall antimicrobial resistance rate in both turkey and quail isolates were low except for tetracycline which some pigeon isolates also showed resistance against that antimicrobial.

Fitzgerald *et al* (1998) reported that certain chicken isolates of ORT could agglutinate red blood cells. It has been reported that there is a correlation between the ability of certain isolates of ORT to agglutinate RBCs and their colony size (Tsai & Huang 2006). All of our hemagglutinating isolates had large colony size in agreement with Tsai and Huang (2006). In the present study we demonstrated the different hemagglutination ability of pigeon ORT isolates with both chicken and pigeon red blood cells. Some of the hemagglutinating isolates however could react only with RBCs from one source. The relationship between colony morphology and hemagglutintion abilities of ORT and their virulence is yet to be determined.

High identity which was found between sequencing results of our isolates and other isolates for example isolates from broiler chicken in Iran (Hassanzadeh *et al* 2010), Brazil (Canal *et al* 2005) and Taiwan (Tsai & Huang 2006), turkeys in United State (Amonsin *et al* 1997) and Taiwan (Chang *et al* 2006), partridge in Iran (Mirzaie *et al* 2010) and pigeons in Taiwan (Tsai & Huang 2006), denoted that ORT isolates originating from different bird species all over the world constitute a small group of closely related clones (Van Empel & Hafez 1999). Other workers by employing SDS-PAGE also confirmed high identities within ORT strains (Banani *et al* 2001b, Vandamme *et al* 1994, Joubert *et al* 1999). However some genetic investigations including AFLP and rep-PCR revealed variations within ORT isolates which may be used to describe subspecies or species for the genus *Ornithobacterium* (Van Empel & Hafez 1999, Amonsin *et al* 1997). Further genetic studies are needed to confirm divergence among ORT strains.

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