

Full Length Research Paper

Phenotypic and molecular characterization of strains of *Ornithobacterium rhinotracheale* isolated from poultry in Turkey

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Accepted 11 May, 2012

Ornithobacterium rhinotracheale has been identified as a newly emerging respiratory bacterial pathogen that has been causing significant economic losses to the poultry industry. In this study, a total of 30 isolates of *O. rhinotracheale* isolated (2 from layer pullets, 5 from broilers, 21 from turkeys and 2 standart strains) from chickens and turkeys were characterized by Polymerase Chain Reaction (PCR), RAPD-PCR and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis. The isolates of *O. rhinotracheale* identified by biochemical tests were also confirmed by PCR. Amplification product of 784 bp was obtained, which is corresponding to the expected size. All of them were characterized as *O. rhinotracheale*. The random amplified polymorphic DNA (RAPD) profiles using OPG-11 primer and the protein profiles used in SDS-PAGE of the *O. rhinotracheale* strains show high variability in results of Neighbor Joining (NJ) and Unweighted Pair Group Method with Arithmetic Means (UPGMA). All field strains isolated from turkeys, broilers and layers show different RAPD and protein profiles.

Key words: *Ornithobacterium rhinotracheale*, broiler, layer, turkey, polymerase chain reaction (PCR), random amplified polymorphic DNA- polymerase chain reaction (RAPD-PCR), sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

Ornithobacterium rhinotracheale is an infectious agent which has an aetiologic role in the respiratory disease complex in poultry (van Beek, 1994; Vandamme et al., 1994; Hafez, 1996; Chin and Droual, 1997). *O. rhinotracheale*, a pleomorphic gram-negative, rod-shaped bacterium, is generally isolated from respiratory tracts of majority of affected birds (Sprenger et al., 2002; Szalay et al., 2002). The major economic losses due to *O. rhinotracheale* infection are ranging from rejection of carcasses as food consumption to growth retardation and even mortality (van Beek et al., 1994; van Veen et al., 2002). The infection of *O. rhinotracheale* can present

several different clinical signs such as tracheitis, airsacculitis, pericarditis, sinusitis, and exudative pneumonia (Hinz et al., 1994; Van Empel et al., 1996; Travers et al., 1996; Van Empel and Hafez, 1999).

Various pathogens (*Turkey rhinotracheitis* (TRT) virus, *Newcastle Disease virus*, *Escherichia coli*, *Bordetella avium*, etc.) have been identified as a cause to respiratory disease which are acting either as primary or secondary agent (Odor et al., 1997; van Empel and Hafez, 1999). *O. rhinotracheale* can be a primary or secondary cause which depends on strain virulence, adverse environmental factors (poor management, inadequate ventilation, high stocking density, poor litter condition, poor hygiene, high levels of respiratory ammonia), immunity state of flock, and presence of other infectious agents. The primary role of *O. rhinotracheale* in respiratory disease is open to debate (De Rosa et al., 1996;

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van Veen et al., 2000; Erganis et al., 2002; Hadimli et al., 2003; Canal et al., 2005).

The diagnosis of *O. rhinotracheale* infection is based on isolation and identification of strains, serological (Agglutination, ELISA and EIA etc.) and molecular (PCR) methods (Amonsin et al., 1997; van Empel, 1997; Hung and Alvarado, 2001; Turan and Ak, 2002; Chansiripornchai, 2004; Jansen et al., 2004; Canal et al., 2005; Türkyilmaz, 2005, Tachil et al., 2007). Because it grows slowly and needs specific culture conditions, culturing of *O. rhinotracheale* is really difficult. The attempts on strain isolation are often posing negative yields or plates are overgrown with other bacteria (van Beek et al., 1994; Erganis et al., 2002; Canal et al., 2005; van Veen et al., 2005).

O. rhinotracheale identified by Vandamma et al. (1994), after series of phenotypic and genotypic characterizations which includes protein profiles, DNA-DNA and DNA-rRNA hybridizations (Vandamme et al., 1994). Today, a total of 18 different serotypes which are marked from A to O have been reported (van Empel and Hafez, 1999). Of the chickens and turkeys, more than 95% of isolates are of serotype A (van Empel et al., 1997). The aims of the study was to biochemically and genotypically identify *O. rhinotracheale* strains isolated from layers, broilers and turkeys. In addition, genotypical differences was compared to among strains.

MATERIALS AND METHODS

Bacteriological examinations

The number of 165 visceras (lung and liver, n=75) and swapes from trachea (n=90) were taken from 21 turkey flocks which were affected by respiratory disease (nasal discharge, gasping, ruffled feathers, suffered from occasional head oedema, severe choked breathing and weakness). Tissue samples were streaked on blood agar containing 7% sheep eritocytes, supplemented with 5 mg/ml gentamycin and polymyxin B, and MacConkey Agar. The plates were incubated microaerobically in 37°C for 48-72 h. The biochemical identification was carried out as described previously (van Empel et al., 1997; Erganis et al., 2002).

Bacterial strain

A total 30 isolates of *O. rhinotracheale* were used in this study which consists of 2 from layer pullets, 5 from broiler (this isolates provided from Dr. TURKYILMAZ (Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, Aydin, Turkey) and 21 from turkeys. The strains of *O. rhinotracheale* were isolated from turkeys and chickens in different flocks which are located in 3 different geographical regions of the Turkey. The 2 strains of *O. rhinotracheale* were also used as a standart strains.

Biochemical reactions

Biochemical characterizations were as follows; oxidase, catalase, hemolysis, growth on MacConkey agar, triple sugar iron (TSI), indole, arginine, lysine, ornithine, phenylalanine, urea, and carbohydrate fermentation tests such as lactose, glucose,

mannose, sorbitol, sucrose, maltose, fructose, and dulcitol.

Molecular methods

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)

Whole cell proteins of total of 30 *O. rhinotracheale* isolates were separated by SDS-PAGE as described by Hung and Alvarado (2001). In short, whole cells were diluted (5:1, v/v) in SDS sample buffer (10% SDS, 0.5 M Tris-HCl, pH 6.8, 5% glycerol, 2.5% β -mercaptoethanol, 0.05% bromophenol blue), heated in boiling waterbath for 5 min, and then loaded into polyacryl-amide gel. Electrophoresis was performed with the constant current of 40 mAmp in vertical electrophoresis device (Owl Separation System, Porthmouths, USA) for 6 h at 110-220 volts. The separated proteins were stained overnight using 0.1% Coomassie blue R-250, made up in 40% methanol and 10% acetic acid, at 37°C. Then, the gel destained in 40% methanol and 10% acetic acid for 8 h. Finally, the gel was stored in a 10% methanol and 7% acetic acid solution.

SDS-PAGE analysis were used to determine polymorphisms among isolates of *O. rhinotracheale*. The polymorphisms and similarity coefficient were calculated using Unweighted Pair Group Method with Arithmetic Means (UPGMA) and Neighbor Joining (NJ).

DNA extraction

A total of 30 *O. rhinotracheale* isolates were grown on blood agar for 48 h and washed in PBS at pH 7.2. The mixture was microfuged (10,000xg, 5 min, 4°C), and the cell pellets were resuspended in 385 μ l of STE buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediamine tetraacetic acid [EDTA]). The extraction of DNA of ORT isolates was made by using a commercial kit (Promega, Wizard® Genomic DNA Purification Kit (Cat. #A1120, USA).

PCR

Primers used in our study were of those reported by Van Empel and Hafez (1999), OR16S-F1 (GAG AAT TAA TTT ACG GAT TAA G) and the 20-mer oligonucleotide OR16S-R1 (TTC GCT TGG TCT CCG AAG AT), which flanked a 784-bp DNA sequence within the 16S rDNA. The PCR mixture was consisting of 2 μ l template DNA, 10 μ l of PCR Buffer, 4 μ l 10 mM dNTPs (in equal volumes of each Deoxynucleotide triphosphat), 3 μ l of each primer (1.5 μ M), 0.25 μ l of Taq polimeraz (Promega) in a final volume of 50 μ l. The amplification was performed under the following conditions in thermal cycler (Eppendorf, England): a denaturation step of 94°C for 5 min followed by 35 cycles of 94°C for 30 sn, denaturation at 58°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 7 min. The PCR product was then analysed by agarose gel electrophoresis and visualised using ethidium bromide 0.5 μ g/ml under ultraviolet light. A 100 bp DNA ladder (Promega, Maddison, USA) was used in all runs (Canal et al., 2005).

RAPD-PCR

A random OPG-11 primer (5'-TGCCCGTCGT-3') was used as described by Leroy-Setrin et al. (1998). The RAPD reaction was performed in total of 25 μ l volume which consists of 2.5 μ l 10 \times PCR buffer (750 mM Tris-HCl, 200 mM (NH₄)₂SO₄, 0.1% Tween 20), 3.5 mM MgCl₂, 200 μ M deoxynucleoside triphosphates, 1.25 U of Taq DNA polymerase (Fermentas, Lithuania), 1 μ M of OPG-11 primer, 11 μ l of dsH₂O (sterile distilled water) and 2.5 μ l of template DNA. The amplification was performed in Touchdown Gradient

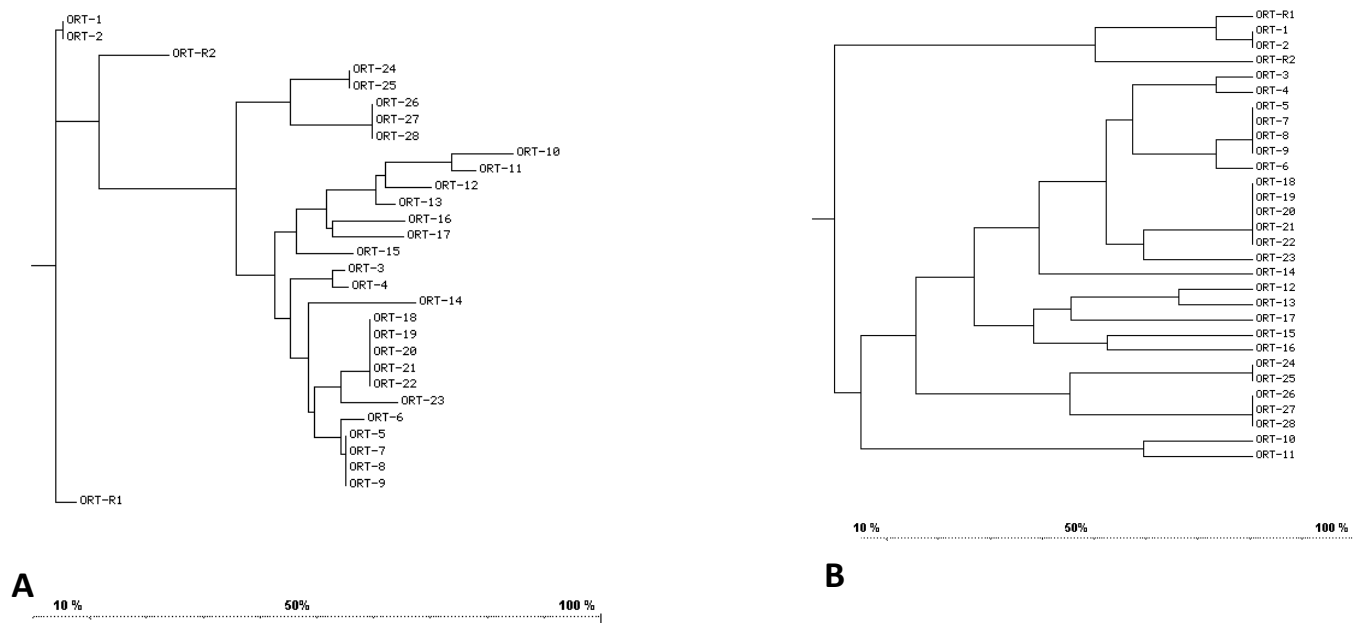


Figure 1. The dendrograms of SDS-PAGE analysis by NJ (A) and UPGMA (B) methods in strains of *O. rhinotracheale*.

Thermocycler (Eppendorf, England) using the following 40 cycles. These cycles were 94°C for 1 min, 35°C for 30 s and 72°C for 1 min 30 s for the first cycle then 92°C for 40 s, 35°C for 30 s and 72°C for 1 min 30 s. A final step of extension was applied in 72°C for 10 min. The amplified DNA products were resolved by electrophoresis on a 1.5% agarose gel using TBE buffer and stained using ethidium bromide, for 3 h. A 100 bp DNA ladder (Promega, Maddison, USA) was used as molecular weight marker on each gel.

RESULTS

Isolation of *O. rhinotracheale*

The 21 *O. rhinotracheale* strains were isolated from 21 turkey flocks that were suffering respiratory diseases. While 19 isolates were isolated from lungs and trachea of turkeys in 2 different companies in same providence, 2 strains were recovered from another providence. Also, 7 isolates (previously 5 isolated from broilers and 2 layers) were added from our culture collection. In addition, 2 strains were used as references strains.

Biochemical reactions

All isolates reacted positively in the p-nitrophenyl-b-D-galactopyranoside (PNPG), urease and oxidase tests, but, were negative to catalase, nitrate reduction, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, indole, TSI, and growth on MacConkey agar. Acid produced from glucose, mannose, lactose, maltose, and fructose were variable. For all isolates, no acid was produced from sucrose, sorbitol, and dulcitol. Not all

strains were having hemolysis.

SDS-PAGE analysis

The dendrograms of protein profiles by SDS-PAGE of all ORT strains are shown in Figure 1. The protein profiles of *O. rhinotracheale* strains were shown high variability in results of NJ and UPGMA methods. All of field strains isolated from turkeys, broilers and layers were shown different protein profiles. However, it has shown that the isolates of broilers, layers or turkeys among themselves were given similar bands. In addition, although isolates of turkey, 2 strains (10 and 11) isolated from different region and flocks were formed to be different bands than others.

PCR

The isolates of ORT identified by biochemical tests were also confirmed by PCR. Amplification product of 784 bp was obtained which was corresponding to the expected size (Figure 2). All of them were determined as *O. rhinotracheale*. The dendrograms of RAPD-PCR profiles of all ORT strains are shown in Figure 4. Using OPG-11 primer, the RAPD profiles of the ORT strains were shown high variability in results of NJ and UPGMA methods. All of field strains isolated from turkeys, broilers and layers were shown different RAPD profiles (Figure 3). The isolates of *O. rhinotracheale* isolated from layers and broilers were characterized as to be different than isolates of turkeys.

Also, the isolates of turkey were observed as to be similar

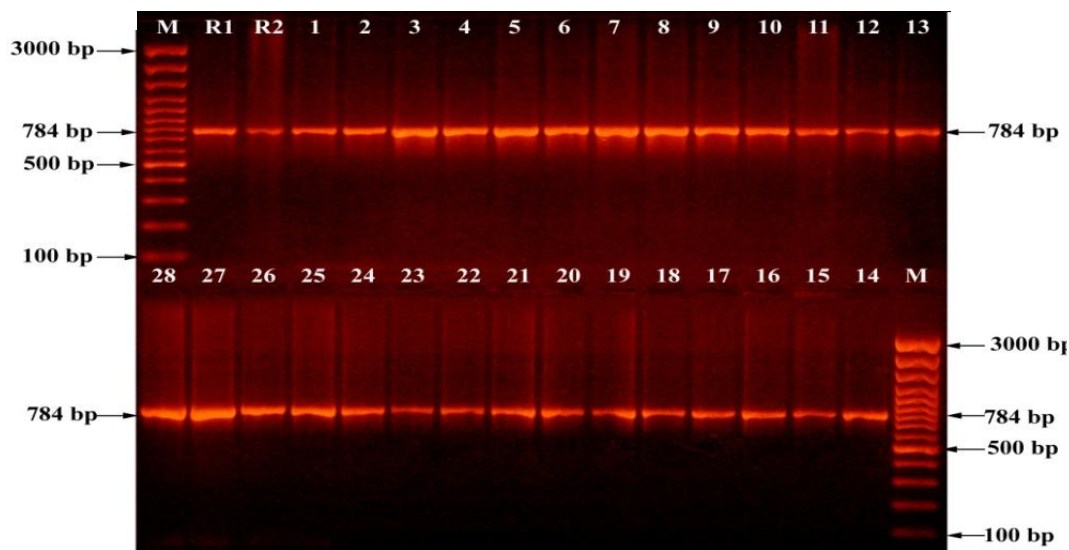


Figure 2. PCR analysis of ORT isolates (M: 100 bp DNA Marker, 80 and 85 of number: references strains, 78 (serotype B) and 79 of number: layer chickens, 157, 154, 152, 150 and 149 of number: broiler chickens, 1-21 of number: turkeys).

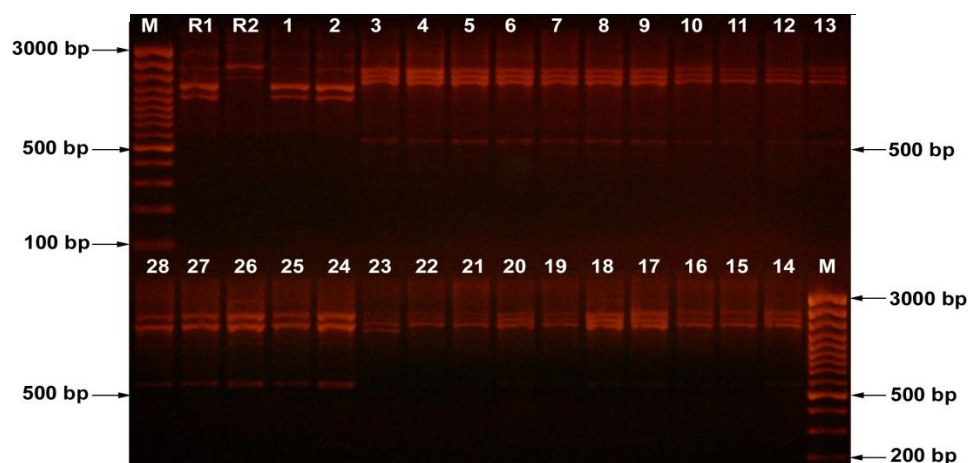


Figure 3. The RAPD-PCR profiles of ORT isolates (M: 100 bp DNA Marker, 80 and 85 of number: references strains, 78 (serotype B) and 79 of number: layer chickens, 157, 154, 152, 150 and 149 of number: broiler chickens, 1-21 of number: turkeys).

to each other.

DISCUSSION

The diagnosis of naturally occurred *O. rhinotracheale* infection may commonly be difficult, according to findings of clinical and histopathological examinations (van Beek et al., 1994; Hafez, 1996). Because, similar clinical symptoms in respiratory diseases caused by many microorganisms were often formed (Travers et al., 1996; Szalay et al., 2002). Therefore, the diagnosis of *O.*

rhinotracheale infection based on isolation and identification of bacteria (van Empel et al., 1997; Banani et al., 2001). The levels of antibodies after infection can be determined in early 5 days, otherwise barely determined in later stages. For this reason, *O. rhinotracheale* infections in infected poultry houses and/or the respiratory system can be under-diagnosed or misdiagnosed (van Leen et al., 2005). Furthermore, the slow-growing *O. rhinotracheale* strains can be overgrown by other more rapidly reproducing bacteria and may also be overseen due to their highly variable biochemical properties or their bypassing of clinical practitioners.

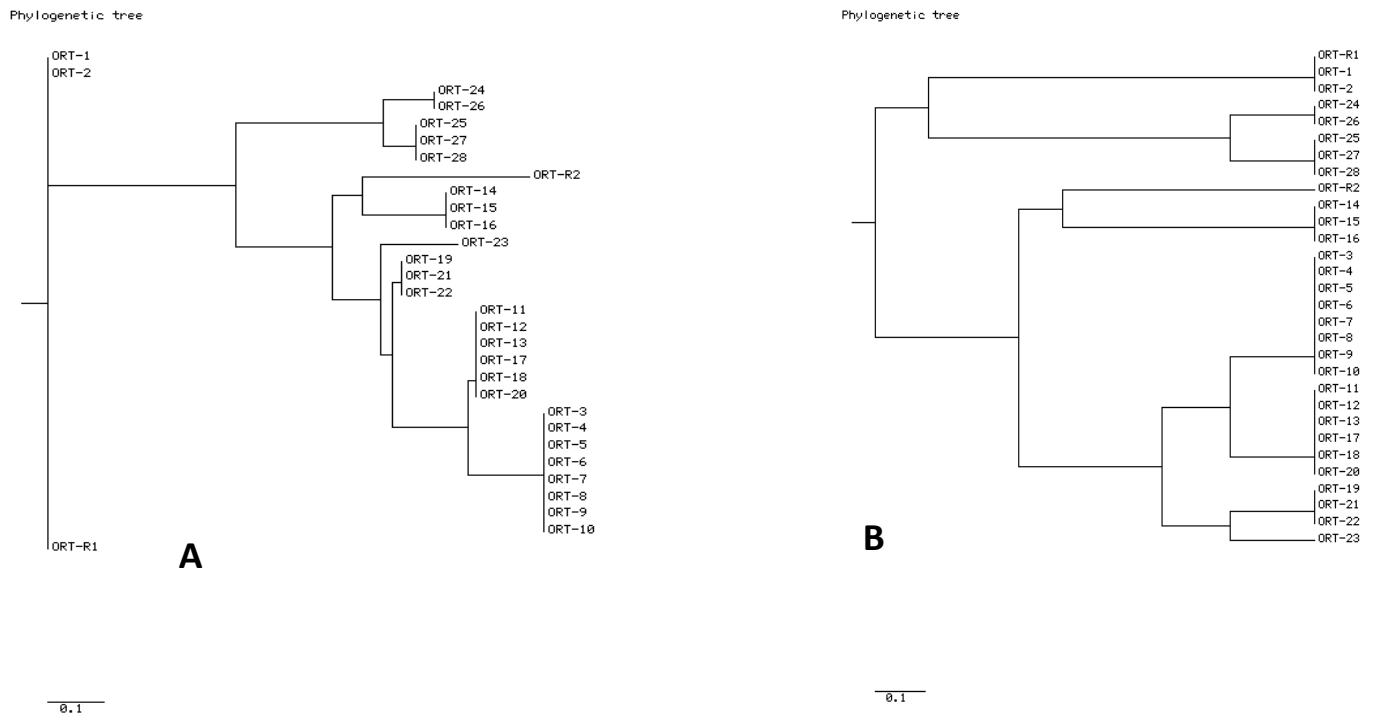


Figure 4. The dendrograms of RAPD-PCR profiles by NJ (A) and UPGMA (B) methods in strains of *O. rhinotrachele*.

Therefore, in the event of *O. rhinotracheale* infections, there is a need for the identification and confirmation of the causative agent (Tsai and Huang, 2006; Hassanzadeh et al., 2010). In this study, all the *O. rhinotracheale* isolates have been isolated from chickens and turkeys suffering respiratory diseases. However, a relationship between the number of samples from flocks with respiratory diseases and the rate of isolation was not intended.

PCR is fast, sensitive, and specific method to identify and characterize bacterial strains and is an alternative method to determine the etiologic agents of respiratory diseases in poultry (Amonsin et al., 1997; Hung and Alvarado, 2001; Özbey et al., 2004; Hassanzadeh et al., 2010; Tachil et al., 2010). However, the cost and availability of equipments, as well as incorrect manipulation of the reagents with the amplification of contaminants, may lead to the misinterpretation of the results (Koga and Zavaleta, 2005). Many researchers (Amonsin et al., 1997; Hung and Alvarado, 2001; Özbey et al., 2004; Hassanzadeh et al., 2010; Tachil et al., 2010) have used the PCR method with success for confirmation of isolated and identified strains and the detection of *O. rhinotracheale* DNAs in tissues. Kiliç et al. (2009), reported that *O. rhinotracheale* strain inoculated alone (4.8×10^8 cfu) into 14 day old broiler chickens using the aerosol route. After inoculation, microbiological (culture) and molecular (PCR) analyses were performed on different parts of respiratory system (sinuses, larynx,

trachea, lungs and air sacs) and on visceral organs. Finally, *O. rhinotracheale* strain was successfully re-isolated in culture and PCR from samples of trachea and lungs. All isolates positive by *O. rhinotracheale* culture were also positive by PCR and culture negative samples were also PCR negative. In a study conducted by Hassanzadeh et al. (2010), in slaughtered broiler chickens, it was reported that the number of ORT-positive tracheal swapes and tissue samples increased from 4 to 7 with the use of the PCR. Due to the overgrowth of the causing agent by other bacteria, it is indicated that the isolation yields of this particular bacterium is rather low (Hassanzadeh et al., 2010). In an another study, *O. rhinotracheale* DNA was detected in 75 samples (19.93%) of broiler turkeys in Iran (Doosti et al., 2011). In the present study, all the *O. rhinotracheale* isolates obtained from layer hens, broiler chickens and turkeys shown the same bands (784 bp) using PCR. This result suggests that the PCR can be used for confirmation of isolates as part of common diagnosis in field (Hassanzadeh et al., 2010; Doosti et al., 2011).

SDS-PAGE can be used for confirmation of *O. rhinotracheale* isolates and demonstration of similarities or differences between isolates. Several researchers (van Empel, 1998; van Damme et al., 1994; Amonsin et al., 1997; Lopes et al., 2000; Hung and Alvarado, 2000) have been reported a high rate of similarity for the outer membrane and/or proteins of the outer membrane between *O. rhinotracheale* strains of different origin

and/or different serotypes. Banani et al. (2001), reported that 59 *O. rhinotracheale* isolates they isolated from broiler chickens and commercial layer hens with clinical signs of respiratory disease belonged to serotype A. Based on SDS-PAGE results; they also pointed out to the presence of close similarities between the protein profiles of these isolates. These researchers (van Empel, 1998; van Damme et al., 1994; Amonsin et al., 1997; Lopes et al., 2000; Hung and Alvarado, 2000) reported minor differences between isolates and predominating similarities among some strains. Furthermore, Özbey et al. (2004), reported that the 6 *O. rhinotracheale* strains isolated from the trachea (n=5) and lungs and trachea (n=1) of slaughtered broiler chickens yielded similar protein profiles when SDS-PAGE was performed.

In the present study, although the isolates obtained from broiler chickens, layer hens and turkeys displayed close similarities, they yielded rather different bands. It was noted that strains of *O. rhinotracheale* isolated from layers, broilers and turkeys formed distinct clusters. While the layer and broiler isolates has existed in same cluster, turkey isolates took part in different group. Tsai and Huang (2006), reported that 40 *O. rhinotracheale* strains were isolated from 28 chickens and 12 in Taiwan. All isolates were identified using culturing and confirmed using PCR. The 16S ribosomal RNA (rRNA) sequences of 23 Taiwanese *O. rhinotracheale* isolates showed high identity (98-100%) to sequences.

It is reported that RAPD-PCR method was relatively simple and easy to use for the characterizations of *O. rhinotracheale* and gave a level of discrimination. Most genotypes could be identified by this method using one or two primers only: OPG 11 (D:0.786) or OPG 11 (D:0.850) (Leroy-Setrin et al., 1998). Both Leroy-Setrin et al. (1998) and Van Empel (1998), showed that no relationship between serotypes and either ribotypes or RAPD types could be established Özbey et al. (2005), reported that RAPD assay showed almost similar DNA profiles in 6 ORT isolates of serotypes A, B, D and E. In this study, eight random-amplified-polymorphic DNA (RAPD) types by UPGMA method were found among the *O. rhinotracheale* strains. In addition, *O. rhinotracheale* isolates of layers, broiler and turkey by SDS-PAGE analysis were demonstrated 19 different clusters. Tachil et al. (2007), reported that fingerprinting methods may be more discerning tool for characterization of *O. rhinotracheale* isolates than the serological tests using the agar gel precipitation test. Also, fingerprinting method can potentially be a valuable tool in identifying an isolate from a clinical outbreak of *O. rhinotracheale* infection for development of autogenous vaccine.

Conclusion

As the study reveal, *O. rhinotracheale* strains isolated from different geographical regions, sources and animal species (turkeys, broiler chickens and layer hens) were

confirmed using PCR. The protein profiles of these strains were determined using SDS-PAGE and their similarities were evaluated using RAPD-PCR. It is considered that further studies are required to confirm the *O. rhinotracheale* strains via different molecular methods such as using pulse-field gel electrophoresis, the random-amplification-fragment-length-polymorphism (AFLP) method, and entire length of the 16S rRNA gene or the sequence divergence.

ACKNOWLEDGEMENT

This project was granted by The Scientific and Technological Research Council of Turkey (Project No 104O456).

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