

Investigations on *Ornithobacterium rhinotracheale* in broiler flocks in Elazig province located in the East of Turkey

G. OZBEY¹, H. ONGOR¹, D. T. BALIK², V. CELIK², A. KILIC³, A. MUZ¹

¹Department of Microbiology, Faculty of Veterinary Science, ²Department of Biology, Faculty of Art and Science, University of Firat, Elazig, Turkey

³Veterinary Control and Research Institute, Elazig, Turkey

ABSTRACT: In the present study, lung, trachea and serum samples from broiler flocks slaughtered at an abattoir in Elazig province located in the East of Turkey were examined for the presence of *Ornithobacterium rhinotracheale* using culture and enzyme-linked immunosorbent assay (ELISA). The identity was latter proved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, and polymerase chain reaction (PCR) assays. A total of 324 serum and 250 lung and trachea samples were collected from 10 commercially reared chicken flocks showing respiratory manifestations. The samples were obtained from different flocks. The causative agent (ORT) was isolated from trachea (1.5%) of five chickens and from both lung and trachea (0.4%) of only one chicken in the bacteriological examination of tissues. The presence of antibodies against ORT was detected in 33 (10.2%) of the 324 sera by ELISA. A 784 bp fragment of the 16S rRNA gene was amplified using specific primers in the PCR. All ORT isolates that were positive by culture were also detected to be positive by the PCR. SDS-PAGE protein profiles of whole cell extracts showed a high similarity for all the isolates with a major band of the molecular weight of 33 kDa (kiloDalton). Results of Western blot analysis indicate four antigenic fractions predominantly with molecular weights of 33, 42, 52 and 66 kDa.

Keywords: *Ornithobacterium rhinotracheale*; ELISA; SDS-PAGE; western blot analysis; PCR; chicken

Ornithobacterium rhinotracheale (ORT) that was originally identified by Charlton et al. (1993 as well as Hafez et al. (1993) and subsequently named by Vandamme et al. (1994) has been reported in chickens and turkeys in many countries worldwide and incriminated as a causative agent in the respiratory disease complex in poultry (Hinz et al., 1994; van Beek et al., 1994; van Empel, 1994; Bock et al., 1995; Tanyi et al., 1995; Leorat and Mogenet, 1996; Travers, 1996; Travers et al., 1996; Ryll et al., 1997; Salem et al., 1997; Hafez and Friedrich, 1998; Abdul-Aziz and Weber, 1999; Joubert et al., 1999; Erganis et al., 2002a).

As clinical signs and postmortem lesions of ORT infections are similar to other bacterial and virus in-

fections, isolation and identification of the causative agent are essential for differential diagnosis (van Empel and Hafez, 1999). Accurate diagnosis must be substantiated by isolation and identification of the causative bacteria and/or detection of antibodies using serological examination (Hafez, 1998). The advantage of the serological tests over bacteriological examination is that antibodies persist for several weeks after infection and the bacterial shedding is short (Hafez, 2002).

Serological examination for detection of antibodies could be carried out using the slide agglutination test prepared from different serotypes (Bock et al., 1995; Back et al., 1996) enzyme-linked immunosorbent assay (ELISA) tests (van Empel, 1994; Hafez and

Sting, 1996) or DOT immunobinding assay (Erganis et al., 2002b).

Because ORT is difficult to identify, use of a reliable identification method is of importance. PCR assays were shown to be useful for identification purposes recently (Hafez and Beyer, 1997; van Empel, 1998).

The aim of this study was to detect ORT antibodies by ELISA from serum samples collected from commercially reared chicken flocks in Elazig region showing respiratory disease symptoms and to isolate and identify the causative bacteria from lung and trachea samples by both culture and PCR. In addition protein profiles and immunogenic structures of ORT isolates were determined by SDS-PAGE and Western Blotting, respectively.

MATERIAL AND METHODS

Samples. In this study a total of 250 lung and trachea samples and 324 serum samples were collected from 10 commercially reared chicken flocks showing respiratory disease symptoms slaughtered at an abattoir in Elazig province located in the East of Turkey. Each sample was obtained from a different chicken flock. A total of 25 lung and trachea samples were collected per flock and 18 and 34 serum samples were collected from only one flocks in 10 flocks and the remaining flocks, respectively. Lung and trachea samples were aseptically removed from chicken and stored at -20°C until required.

Bacterial strain and antisera. ORT strain B3263/91 of serotype A and antisera against serotype A (obtained from Paul van Empel, Intervet-International, Boxmeer, The Netherlands) used as an antigen for coating the ELISA plates. These antisera were used as a positive control. Sera from specific-pathogen-free (SPF) chickens were used as negative control.

Culture. Swabs from the lung and trachea samples were aseptically inoculated on blood agar supplemented with 7% sheep blood and on blood agar supplemented with 7% sheep blood and 10 $\mu\text{g}/\text{ml}$ gentamicin (to inhibit growth of other bacteria). The plates were incubated in a 5–10% CO_2 atmosphere at 37°C for at least 48 hours. Each day the agar plates were checked for suspected colonies. Storage of isolates was achieved by inoculating 10 ml of nutrient broth with 15% glycerol and freezing at -20°C . Identification or confirmation of bacterial species was assessed by observation of the colonial morphology and Gram staining results or confir-

mation of bacterial species was assessed by observation of the colonial morphology, Gram staining results and biochemical methods. These biochemical methods were as follows; catalase, nitrate reduction, H_2S production in triple sugar iron (TSI), ornithine decarboxylase, growth on MacConkey, β -D-galaktosidase, Indole, Urease, Metil Red, Voges Proskauer, Jelatinase, oxidase reaction, motility, nitrate, carbohydrate fermentation tests such as glucose, trehalose, xylose, fructose, galactose, maltose, lactose, dulcitol, inositol, sorbitol, raffinose (Chin et al., 2003).

Antigen extraction. The extraction of the antigen with sodium dodecyl sulphate (SDS-antigen) for ELISA test was performed according to the method described by Hafez and Sting (1999). The ORT strain (B 3263/91) of serotype A was incubated on blood agar supplemented with 7% sheep blood under microaerophilic conditions at 37°C for 48 h and thereafter was homogenized with 6 ml phosphate-buffered saline (PBS). The bacterial suspensions were then centrifuged at $13\,000 \times g$ for 30 min and the pellet were resuspended in 1 M Tris-HCl buffer, pH 6.8, supplemented with 2% SDS and 0.05 M dithiotreitol (Sigma). The suspensions were boiled for 15 min and then followed by centrifugation at $13\,000 \times g$ for 30 minutes. The obtained supernatants were used as antigen for ELISA test.

Antigen titrations were performed according to checkerboard titration method. The protein content of the antigens was measured by the method of Lowry et al. (1951). Ten $\mu\text{g}/\text{ml}$ of protein per well was used.

ELISA. The indirect ELISA was carried out using a modification of the method described previously by Hafez and Sting (1999). The antigen was diluted 1 : 100 with 0.06 M carbonate-bicarbonate buffer (pH 9.6) and 100 μl was added to each well of plate. The plates were covered and held at 4°C for 18 hours. The plates were washed three times with PBST (Phosphate buffered saline 0.01 M, 0.15 M NaCl, 0.05% Tween 20, pH 7.2). For neutralization of polystyren non-adsorption of antigen (Blocking step), 100 microliters of PBS supplemented 1% Bovine Serum Albumin (BSA) was added to each well (Crowther and Smith, 1995). The plates were placed on a low speed, continuous shaker and incubated at 37°C for 1 hour. Then, the plate was washed again three times as above. All serum samples (diluted 1 : 100) were tested in duplicate and the plates were incubated at 37°C for 1 hour. After the plates were washed again 100 μl

of a 1 : 5 000 dilution (in sample dilution buffer) of goat anti-chicken immunoglobulin G peroxidase-labelled antibody as conjugate (Nordic Laboratories, Tilburg, The Netherlands) was added to each well and incubated at 37°C for 1 h and washed again. Then 100 µl of chromogen substrate (ABTS, 2,2'-azino-di-ethylbenzothiazolinesulfonic acid) (Sigma) was added to each well. The plate was incubated at room temperature for 20 minutes. The reaction was stopped by adding 50 µl of stop solution and the absorbance values were evaluated photometrically ELISA reader (Medispec ESR 200) at a wavelength of 405 nm.

The ELISA cut off value for positive reaction was calculated as the average optical density (OD) of five negative serum samples plus three times the standard deviation (SD).

SDS-PAGE. Whole cell proteins of six ORT isolates were separated by SDS-PAGE as described by Laemmli (1970) for protein profile analysis. The SDS-PAGE was performed with 12% (w/v) separating gel and 4% stacking gel. Whole cells for SDS-PAGE were diluted in SDS sample buffer (10% SDS, 0.5 M Tris-HCl, pH 6.8, 5% glycerol, 2.5% 2-β-mercaptoethanol, 0.05% bromophenol blue), boiled for 5 min prior to loading onto the gel. Electrophoresis was performed in the Protean II electrophoresis cell (BioRad) at 20 mA until the bromophenol dye front had reached the bottom of the gel. The gels were stained in Coomassie Brilliant Blue solution (0.1% Coomassie blue R250, 40% methanol, 10% glacial acetic acid, 40% H₂O) for 30 min at 67°C and destained in destaining solution (5% methanol, 7% acetic acid, 88% H₂O) for 3–4 h at 67°C to visualise the proteins.

Western blot analysis. Electrophoretic transfer of protein from unstained SDS-PAGE was performed onto nitrocellulose membrane (Immobilon-P transfer membrane, Millipore) at 100 V in a Mini Trans-Blot Transfer Cell (BioRad) with transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 1 hour. After the transfer, the membrane was washed three times for 5 min each with PBST (0.15 M NaCl, 0.1 M Na₂HPO₄, 0.02% Tween 20) and then block in 5% skimmed milk in PBST for 2 hours. The membrane was then washed and incubated with ORT positive serum samples. Serum samples were diluted in PBST containing 5% skimmed milk and incubated at 4°C for 1 h or overnight. The blot was washed three times in PBST to remove non-bound antibody and then incubated for 1 h at 4°C with peroxidase-conjugated goat anti-chicken immunoglobulin G diluted 1 : 500 in PBST contain-

ing 5% skimmed milk. Following three washes in PBST, the membrane was developed with di-aminebenzidine (Sigma). Color development was stopped by washing the membrane in distilled water and then dried.

DNA extraction. A few colonies from suspicious ORT cultures were transferred into an eppendorf tube containing 300 µl distilled water. The tubes were vortexed and incubated at 56°C for 30 minutes. The suspension was then added in to 300 µl of TNES buffer (20 mM Tris pH 8.0 + 150 mM NaCl + 10 mM EDTA + 0.2% SDS) and 200 µg/ml Proteinase K. Following 30 min boiling, an equal volume of phenol was added to the suspension which was shaken vigorously by hand for 5 min and then centrifuged at 11 600 g for 10 minutes. The upper phase was transferred into a new eppendorf tube. Genomic DNA was precipitated with absolute ethanol and 0.3 M sodium acetate at –20°C for one hour or overnight. The mixture was then centrifuged at 11 600 g for 10 min and the upper phase discarded. The pellet was washed twice with 300 µl of 90% and 70% ethanol, respectively, each step followed by 5 min centrifugation. The pellet was dried and resuspended in 50 µl sterile distilled water and used as a target DNA in PCR.

Primers. Primers used in this study were designed by van Empel and Hafez (1999). The sequences of primer pairs were as follows: OR16S-F1 (5'-GAG AAT TAA TTT ACG GAT TAA G-3') and OR16S-R1 (5'-TTC GCT TGG TCT CCG AAG AT-3'). These primers amplify a 784 bp fragment on the 16S rRNA gene of ORT.

PCR. PCR was performed in a Touchdown Thermocycler (Hyaid, Middlesex, England) in a total reaction volume of 50 µl containing 5 µl of 10 × PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton[®] X-100), 5 µl of 25 mM MgCl₂, 250 µM of each deoxynucleoside triphosphate, 2 U of Taq DNA Polymerase (Fermentas, Lithuania) and 40 pmol of primers and 5 µl of template sample DNA. Amplification was obtained with an initial denaturation step at 94°C for 5 min followed by 45 cycles at 94°C for 30 s, and 52°C for 1 min and 72°C for 1 min 30 s. The final cycle was at 72°C for 7 min (van Empel et al., 1999). A 10 µl of PCR products were separated on a 1.5% agarose gel with 0.5 µg/ml ethidium bromide. The DNA fragments were visualized by UV illumination and photographed with Polaroid film. The molecular sizes of the PCR products were compared with a 100 bp DNA ladder.

RESULTS

Culture. After 48 h of incubation on blood agar at 37°C for 48 h, small grey to grey/white colonies, were observed. Biochemical reactions of isolates were typical of ORT; negative for indole, ornithine decarboxylase, glucose, sucrose, maltose and mannitol and positive for urease, arginine dehydrolase, and β -galaktosidase. All isolates were positive for oxidase and negative for catalase. A total of six (2.4%) isolates consisting of only one (0.4%) isolate from both lung and trachea samples and five (1.5%) isolates from trachea were biochemically identified as ORT.

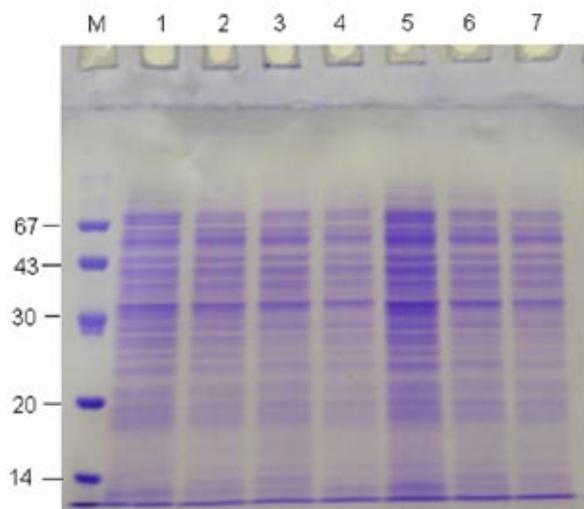


Figure 1. SDS-PAGE of ORT isolates. M: molecular size standards, lane 1: positive control, lanes 2–7: ORT isolates. Molecular size standards are shown in kilodaltons on the left

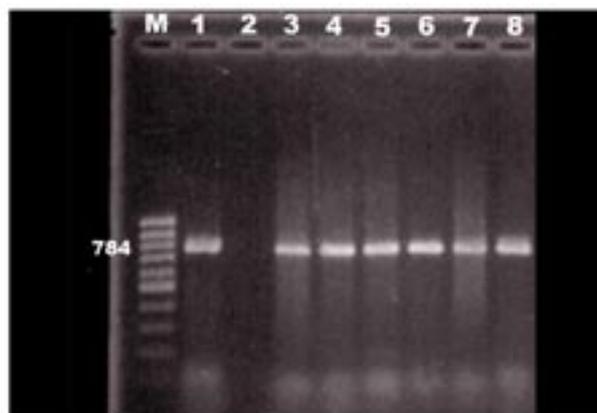


Figure 3. An agarose gel stained with ethidium bromide, with PCR products of ORT isolates (M: 100 bp DNA ladder, lane 1: positive control, lane 2: negative control, lanes 3–8: ORT isolates)

These six isolates belonged to two of the 10 flocks. Four isolates were obtained from one flock and the remaining two isolates were from another flock.

ELISA. Of the 324 serum samples collected from chickens, 33 (10.2%) samples were positive for the presence of antibodies to ORT by ELISA. Thirty three sera belonging to two chicken flocks were found to be positive with respect to antibodies against ORT.

SDS-PAGE of the six isolates is shown in Figure 1. SDS-PAGE revealed that the isolates possessed similar protein profiles. The 42 kD protein band appears weakly stained while the band with a molecular weight of 33 kD was detected as the major band.

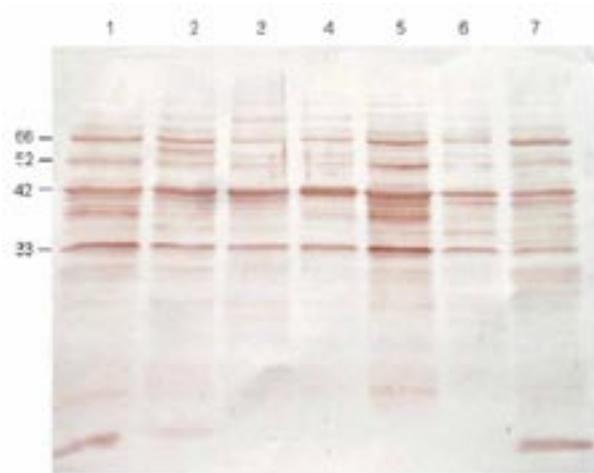


Figure 2. Western Blot analysis of whole-cell antigens of ORT isolates reacted with the ORT positive serum samples. M: molecular size standards, lane 1: positive control, lanes 2–7: ORT isolates. Molecular size standards are shown in kilodaltons on the left

Western blot analysis. Immunoblot analysis showed predominantly antigenic fractions with molecular weights of 33, 42, 52 and 66 kD (Figure 2).

PCR. The biochemical test results were confirmed by PCR. All ORT suspicious isolates were positive in PCR. A 784 bp amplification product was obtained, corresponding to the expected size (Figure 3). No amplified products were obtained from the negative control.

DISCUSSION

The purpose of this study was to examine ORT from chickens by culture, ELISA, SDS-PAGE, Western Blotting and PCR tests.

To date, several methods were used for the extraction of the ELISA-antigen; boiled extraction, extraction with SDS; and extraction of proteinase K-stable antigen. Among them, SDS-extraction appears to give the most suitable results (Hafez and Sting, 1999). An ELISA using an antigen extraction with SDS was proven to be less serotype-specific than the antigen with boiled extraction (Hafez and Sting, 1997) and also less sensitive (P.C.M. van Empel, personal communication). Hafez et al. (2000) showed that the self-made ELISA was more sensitive as observed by the end titration of antisera against different ORT serotypes in both self-made ELISA and the commercial ELISA kit. For these reasons, we used a self-made ELISA based on SDS-antigen extraction of ORT strain B3263/91 of serotype A.

Hafez and Sting (1996) reported that antibodies to ORT were detected in 79% of broiler breeder flocks, and in 26% broiler flocks by an indirect self-made ELISA test. Ryll et al. (1997) and Heeder et al. (2001) detected specific antibodies in 9.4% and 52% of broiler and layer flocks, respectively. Sakai et al. (2000) found that 13.5%, 13.9% and 12.7% of the tested sera of broilers, broiler breeders and layers, respectively were positive. Another study showed antibodies in 64.4% of the tested sera of broiler and layer flocks (Turan and Ak, 2002).

In this study, serum samples were positive in 10.2% of samples by ELISA. These findings are lower than those results of Heeder et al. (2001) and Turan and Ak (2002), but is in agreement with the findings of Sakai et al. (2000) and Ryll et al. (1997). The differences in the results of reports might be due to that most of our samples were not taken from young chickens and in the early stage of infection in this study. Antibody titres peak between 1 and 4 weeks after field infection but decline rapidly thereafter, indicating that serum samples for flock screening should be taken at different ages (van Empel and Hafez, 1999).

The SDS-PAGE showed a high similarity of protein profiles among ORT isolates. This finding is in agreement with the results reported by Vandamme et al. (1994), Amonsin et al. (1997), van Empel et al. (1999), Lopes et al. (2000) and Hung and Alvarado (2001) who showed a high similarity levels between the total protein profiles and the outer membrane protein (OMP) profiles of ORT isolates despite differences in their origin and/or serotype. The high similarity among total protein and OMP profiles indicates that the isolates originating from all the over the world and from several bird species are

represented by a small group of closely-related clones (van Empel et al., 1999).

Results of western blot analysis used in this study showed that the protein of 33 kD is the major band and the 42 kD protein band appears weakly stained as reported by Hung and Alvarado (2001). Proteins of 33 kD and 42 kD showed greater immunogenicity, whereas proteins of 52 kD and 66 kD had less immunogenicity.

The primer combination OR16S-F1 and OR16S-R1 was very specific in amplifying a 784 bp fragment of the 16S rRNA gene of ORT but not of other closely related bacteria with which ORT could be confused (van Empel, 1998; Hung and Alvarado, 2001). In this study, we detected ORT DNA from all the isolates by PCR.

Although *post mortem* lesions of ORT do show specificity in affecting the abdominal air sacs before the lungs and trachea, it was not possible to take air sacs during slaughter because samples are taken from chickens slaughtered an abattoir in this study.

In this study, a proportion of 2.4% of ORT was obtained from chickens. This proportion is lower than the results (8.8%, 11.46%) recorded in some other parts of the world and in Turkey (El-Sukhon et al., 2002, Turan and Ak, 2002) but is in agreement with the results (0.4% and 1.2%) reported by Erganis et al. (2002a,b) and Turkyilmaz (2001). The cause might be correlated to the difficulty of the isolation of ORT, especially since ORT is often overgrown by other bacteria fast growing bacteria such as *E. coli*, *Proteus* spp. or *Pseudomonas* spp., ORT colonies may be overgrown and therefore cannot be detected in routine investigation (Hafez, 1998).

This study reported the presence of ORT in Elazığ for the first time. However, further studies are needed to understand epidemiological importance of this disease in poultry population of the region.

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Corresponding Author

Gokben Ozbey, Department of Microbiology, Faculty of Veterinary Science, University of Firat, 23119 Elazig, Turkey
 Tel.: +90 424 2370000/6451, fax +90 424 2388173, e-mail: gokbenosbey@yahoo.com
