

Isolation and Serotyping of *Ornithobacterium rhinotracheale* from Poultry*

Süheyla TÜRKYILMAZ

Department of Microbiology, Faculty of Veterinary Medicine, Adnan Menderes University, Aydın - TURKEY
E-mail: suhturkyilmaz@yahoo.com

Received: 21.12.2004

Abstract: The aims of this study were the isolation of *Ornithobacterium rhinotracheale* from poultry and serotyping of the isolates and field sera by agar gel precipitation (AGP) test. For the isolation 257 chickens and 214 turkeys were used and for the AGP test 333 chickens and 250 turkeys were used. *O. rhinotracheale* was isolated from the sinus discharge of 3 broiler breeders at 37, 42 and 46 weeks of age. It was determined that 3 isolates were *O. rhinotracheale* serotype I having cross reactions with serotype L. Of 333 sera examined 86 were found seropositive in chickens by AGP test. It was determined that 30 sera were serotype A, 25 sera were serotype B, 9 sera were serotype I, and 6 sera were serotype L, while 13 sera were serotype A with cross reactions with serotype B, and 3 sera were serotype I with cross reactions with serotype L. Of the 250 turkey sera 64 were seropositive and of those 17 were serotype A, 29 were serotype B, 5 were serotype I, and 2 were serotype L, while 11 sera were serotype A with cross reactions with serotype B.

Key Words: *Ornithobacterium rhinotracheale*, poultry, isolation, agar gel precipitation

Kanatlı Hayvanlardan *Ornithobacterium rhinotracheale*'nin İzolasyonu ve Serotiplendirilmesi

Özet: Bu çalışmada, kanatlı hayvanlarda *Ornithobacterium rhinotracheale*'nin izolasyonu, izolatların ve saha serumlarının agar jel presipitasyon (AGP) testi ile serotiplendirilmeleri amaçlanmıştır. İzolasyon için 257 tavuk, 214 hindi; AGP testi için 333 tavuk 250 hindi kullanılmıştır. 37, 42 ve 46 haftalık üç broiler damızlığın sinus içeriğinden *O. rhinotracheale* izolasyonu yapılmıştır. Üç izolatın *O. rhinotracheale* serotip I olduğu, serotip L ile de kros reaksiyon verdiği tesbit edilmiştir. AGP testi ile incelenen 333 tavuk serumunun 86'sinin seropozitif olduğu saptanmıştır. Bunların 30'unun serotip A, 25'inin serotip B, 9'unun serotip I, 6'sinin serotip L olduğu, 13'ünün serotip A olup B ile, 3'ünün serotip I olup L ile kros reaksiyon verdiği belirlenmiştir. İncelenen 250 hindi serumunun 64'ünün seropozitif olduğu, bunların 17'sinin serotip A, 29'unun serotip B, 5'inin serotip I, 2'sinin serotip L ve 11'inin serotip A olup B ile kros reaksiyon verdiği belirlenmiştir.

Anahtar Sözcükler: *Ornithobacterium rhinotracheale*, kanatlı hayvanlar, izolasyon, agar jel presipitasyon

Introduction

Respiratory diseases are still a major problem in poultry. *Ornithobacterium rhinotracheale*, which is a respiratory pathogen, has been described by Vandamme et al. (1). It was initially regarded as a Pasteurella-like organism. *O. rhinotracheale* can cause severe respiratory clinical signs in turkeys and chickens (2-4). *O. rhinotracheale* was proven to be a primary pathogen in broilers (5) and potentially pathogenic for fowl (4,6). Although *O. rhinotracheale* infections are frequently associated with other respiratory diseases, *O. rhinotracheale* has sometimes been isolated from asymptomatic flocks (7). Blood-stained mucus in the

mouth is an occasional finding. The organism has been isolated from the partridge, pheasant, pigeon, rook, quail duck, ostrich, goose, guinea fowl, chicken and turkey (1,6,7). The disease has been described in many countries (7-10). In Turkey, 2 *O. rhinotracheale* isolations were first obtained from 2 commercial pullets aged 12 and 15 weeks. One of these isolates was determined as serotype B by agar gel precipitation (AGP) test but the other was not described (8). In serological investigations it is better to take a blood sample during the acute stage of the infection to detect antibodies to *O. rhinotracheale*, because antibody titres decrease rapidly, especially in field infections. This makes serological detection very difficult.

* This manuscript is prepared from the author's PhD thesis. Institute of Health Science, Ankara University, Ankara, 2001.

To detect *O. rhinotracheale* infections serologically, sera should be taken during the first 4 to 6 weeks of the infection when the titre is very high. Additionally, more serum samples at several times from suspected animals would increase the chance of diagnosis (6). This condition is a disadvantage in the detection of pathogenic agents such as *O. rhinotracheale* that do not have any specific symptoms at the beginning of the infection.

This study aimed to isolate of *O. rhinotracheale* from poultry, and to serotype the isolates and field sera by AGP test.

Materials and Methods

Materials: For the isolation 257 chickens and 214 turkeys were used and for the AGP test 333 chickens and 250 turkeys with the symptoms of respiratory disease (nasal discharge, gasping, ruffled feathers, occasionally head oedema, severe laboured breathing and weakness) were used. Blood serum samples of poultry used for isolation were also used in the AGP test. Lung, trachea, tracheal swabs, sinus discharges (if present), and blood samples were collected from the animals for bacteriological and serological tests. The numbers of chickens and turkey breeders investigated in this study and their distribution according to location are given in Table 1.

Bacteriological examinations: Lungs, trachea and tracheal swabs were streaked on Blood Agar with 7% sheep blood and MacConkey Agar. The plates were incubated at 37 °C under aerobic conditions as well as at 37 °C under microaerobic conditions for 2-3 days. The

biochemical identification was carried out as described previously (3,7,11,12).

Antimicrobial sensitivity of *O. rhinotracheale* isolates: Antimicrobial sensitivity tests were applied to the isolated strains by the Kirby-Bauer disk diffusion method described by Bauer et al. (13). Interpretation of the in vitro susceptibility findings should be done with caution since standards have not been established for this organism. Since a standard protocol for antibiotic sensitivity tests for *O. rhinotracheale* does not exist the method described by the Clinical and Laboratory Standards Institute (CLSI) for fastidious Gram-negative organisms was followed (8,14,15).

Standard bacterial strains: *O. rhinotracheale* strains (serotype A (B3263/91), serotype B (GGD 1261), serotype C (K 91-201), serotype D (ORV 94108 nr. 2), serotype E (O-95029 nr. 12229), serotype F (ORV 94084 k858ort), serotype G (O-95029 nr. 16279), serotype H (E-94063 4.2), serotype I (BAC 960334 minn18), serotype J (O-97091 HEN 81-2), serotype K (BAC 970321 101 sm), serotype L (O-97071 BUT 2237), serotype M (TOP 98036 98.4500), serotype N (TPO 99023 LMG 13114) and serotype O (TOP 99023 LMG 11553) were obtained from Dr. Paul van Empel (Intervet International, Boxmeer, the Netherlands).

Preparation of antiserum: Preparation of antiserum was carried out on specific-pathogen-free (SPF) chickens in accordance with a method described by Hafez and Sting (16). Antiserum against each strain (serotypes A to O) was prepared in SPF chickens at 4 weeks of age. The *O. rhinotracheale* strains were grown in Brain Heart Infusion Broth (BHIB) for 48 h at 37 °C under

Table 1. The number of chicken and turkey breeders and their distribution by location.

Location	Layer Chickens	Broiler Breeders	Total Chickens	Total Turkey Breeders
Ankara	45	105	150	100
Bolu	10	40	50	50
Afyon	20	10	30	-
Konya	-	10	10	-
İzmir	15	22	37	100
Aydın	29	17	46	-
Cyprus	10	-	10	-
Total	129	204	333	250

microaerobic conditions. The total viable bacterial count was determined on Brain Heart Infusion Agar (BHIA) containing 7% sheep blood. Live bacterial cells were inoculated by intranasal, intraocular and intramuscular route into the animals at a dose level of 10^7 CFU/bird. After 4 weeks, this procedure was repeated at the same dose and the birds were bled 8-10 days after the second inoculation.

Bacterial suspensions for antigen extractions:

Preparation of bacterial suspensions for antigen extractions was carried out in accordance with a method described by Hafez and Sting (16). The *O. rhinotracheale* strains belonging to serotypes A to O were grown on BHIA containing 7% sheep blood under microaerophilic conditions at 37 °C for 48 h. Thereafter, each plate was flooded with 6 ml of PBS. The bacterial suspensions were centrifuged at 3000 g for 30 min and cell sediments were washed 3 times with PBS. Following the final centrifugation, bacterial pellets were resuspended with 3 ml of PBS (adjusted to a 0.5-0.7 optical density by a wavelength of 600 nm).

Preparation of antigens: Boiled extract antigens (BEAs) were prepared as described by Heddleston et al. (17) by washing well-grown cultures from sheep blood agar with 0.02 M PBS-8.5% NaCl-0.3% formaldehyde (pH 7.2). The same buffer was used to adjust the suspensions to an optical density at 660 nm of 0.5 to 0.6 when the suspensions were diluted 1:20. Subsequently, the suspensions were boiled for 60 min at 100 °C. After centrifugation, the supernatants were sterilised by filtration through a 0.22 µm pore size filter and were then used as antigen in AGP tests.

AGP test: The test was carried out in petri dishes with 1.5% Noble agar-8.5% NaCl-0.1% thimerosal in accordance with a method described by Van Empel et al.

(18). A hexagonal pattern was cut into the agar layer that consisted of a central well surrounded by 6 peripheral equidistant wells approximately 5 mm from the central well. The diameter of each well was 3 mm. The central well was filled with antigen extract and the peripheral ones with the antisera against different serotypes. The petri dishes were incubated for at least 72 h in a moist chamber at 37 °C and then were observed for precipitation lines under UV light.

Results

Bacteriological identification: Two hundred and fifty-seven chickens were necropsied. While there was no bacteriological growth in 46 chickens, *Staphylococcus aureus*, *Klebsiella* spp., *Escherichia coli*, *Mannheimia haemolytica*, *P. multocida*, *Proteus* spp., *Pseudomonas* spp., *Bacillus* spp., *Corynebacterium* spp., *Acinetobacter* spp., *Citrobacter* spp., *Streptococcus* spp. and *O. rhinotracheale* were isolated from 257 broilers. *O. rhinotracheale* was isolated from 2 different broiler breeder flocks in Ankara province. The broiler breeders were 37, 42 and 46 weeks of age. The biochemical test results of the isolated strains are shown in Table 2.

Two hundred and fourteen turkeys were necropsied. While there was no bacteriological growth in 16 turkeys *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* spp., *Mannheimia haemolytica*, *P. multocida*, *Pseudomonas* spp., *Proteus* spp., *Corynebacterium* spp. and *Citrobacter* spp. were isolated. *O. rhinotracheale* could not be isolated from any turkeys. All micro-organisms isolated and the isolation rates of the chickens and turkey breeders are given in Table 3.

Antimicrobial sensitivity of the isolates: The results of the antibiotic susceptibility test are shown in Table 4.

Table 2. Biochemical characterisation of isolated strains.

Reaction	Result	Reaction	Result
Oxidase	+	Indole	-
Catalase	-	Growth on MacConkey Agar	-
Nitrate reduction	-	Lysine decarboxylase	-
Urease	+	Ornithine decarboxylase	-
β-Galactosidase	+	Fermentation of oxidation of:	
ADH	-	Fructose, Lactose, Maltose, Galactose	+

Table 3. All micro-organisms isolated and isolation rates of the chickens and turkey breeders investigated.

Isolated micro-organisms	The number of chickens (Total: 257)	%	The number of turkey breeders (Total: 214)	%
<i>S. aureus</i>	134	52.1	91	42.5
Klebsiella spp.	121	47.1	88	41.1
<i>E. coli</i>	98	38.1	127	59.3
<i>P. haemolytica</i>	43	16.7	33	15.7
<i>P. multocida</i>	22	8.6	20	9.3
Proteus spp.	22	8.6	17	7.9
Pseudomonas spp.	20	7.8	19	8.9
Bacillus spp.	17	6.6	-	-
Corynebacterium spp.	11	4.3	12	5.6
Acinetobacter spp.	8	3.1	12	5.6
Citrobacter spp.	6	2.3	4	1.9
Streptococcus spp.	3	1.2	-	-
<i>O. rhinotracheale</i>	3	1.2	-	-

Table 4. Antibiotic sensitivity test results of *O. rhinotracheale* isolates in chickens.

Antibiotic disc.	Sensitivity of isolated strains	Antibiotic disc	Sensitivity of isolated strains
Gentamicin	-	Amoxicillin	++
Polymyxin B	-	Amoxicillin/clavulanic acid	++
Nalidixic acid	-	Oxytetracycline	++
Flumequine	-	Neomycin	++
Enrofloxacin	-	Cefoperazone sulbactam	++
Ciprofloxacin	-	Ampicillin sulbactam	++
Erythromycin	-	Tetracycline	++
Novobiocin	-	Danofloxacin	++
		Lincomycin	++

- : Resistant, ++: Sensitive

Three isolates were determined as sensitive to danofloxacin, lincomycin, amoxicillin, amoxicillin/clavulanic acid, oxytetracycline, neomycin, cefoperazone sulbactam, ampicillin sulbactam and tetracycline and as resistant to gentamicin, polymyxin B, nalidixic acid, flumequine, enrofloxacin, ciprofloxacin, erythromycin and novobiocin.

AGP test: It was determined that 3 isolates were *O. rhinotracheale* serotype I having cross reactions with serotype L. Of 333 sera examined 86 were found

seropositive in chickens by AGP test. It was determined that 30 sera were serotype A, 25 sera were serotype B, 9 sera were serotype I and 6 sera were serotype L, while 13 sera were serotype A having cross reactions with serotype B, and 3 sera were serotype I having cross reactions with serotype L. Of the 250 turkey sera 64 sera were seropositive and of those 17 sera were serotype A, 29 sera were serotype B, 5 sera were serotype I, 2 sera were serotype L, 11 sera were serotype A having cross reactions with serotype B.

AGP test results of the field sera are shown in Table 5.

Furthermore, at the end of the serologic investigation, it was determined that only 3 sera taken from layers were seropositive. Serologically positive blood sera were collected from broiler breeders, layers and turkeys at 22-46, 25-37 and 17-30 weeks of age, respectively.

Discussion

In the present study, while *O. rhinotracheale* infections were investigated in poultry, serotyping of the isolates and the field sera were performed.

O. rhinotracheale serotype A was determined as a predominant serotype among the isolates of *O. rhinotracheale* from chickens (6). Serotype A was also a predominant serotype in this study. The bacteriological isolation rate for *O. rhinotracheale* was determined as low. It is very resistant to routinely used antibiotics (5,19). *O. rhinotracheale* is often overgrown by *E. coli*, *Proteus* spp. and *Pseudomonas* spp., which have a rapid growth rate, especially in materials taken from hens. TRT/ART and *O. rhinotracheale* infections are concomitant in field conditions (19). Two flocks used for the isolation of *O. rhinotracheale* also had a positive reaction for ART infection by ELISA.

O. rhinotracheale strains were sensitive for danofloxacin, lincomycin, amoxicillin, amoxicillin/clavulanic acid, oxytetracycline, neomycin, cefoperazone sulbactam, ampicillin sulbactam and tetracycline. Isolates investigated by Erganiş et al. (8) in Turkey were

sensitive for ofloxacin, erythromycin, lincomycin, amoxicillin, amoxicillin/clavulanic acid. The antibiotic sensitivity of *O. rhinotracheale* strains could differ according to region, possibly caused by inherent genetic differences between breeds and the antibiotic resistance of agents (3,15).

O. rhinotracheale infections were generally reported in turkey and broiler breeders having respiratory symptoms in many countries (2,5,6). Bacteriological isolation was achieved from broiler breeders in this study. Age groups described as seropositive revealed that the *O. rhinotracheale* infection was commonly seen in older birds, parallel to a previous study (5). The findings revealed that 25% of serum samples were positive for *O. rhinotracheale* infection. Hafez (19) reported that 1096 sera from chickens and turkeys showed respiratory disease symptoms. The results showed that 77.3% of broilers, 22.2% of layers and 96.2% of turkeys were *O. rhinotracheale* positive. In a field survey performed by Odor et al. (3) in the USA, 20% seropositiveness was detected in chickens. All these studies revealed that *O. rhinotracheale* infections can be detected serologically at high rates.

The presence of different *O. rhinotracheale* serotypes in Turkey was determined bacteriologically and serologically in this study. *O. rhinotracheale* strains were resistant to gentamicin, polymyxin B, nalidixic acid, flumequine, enrofloxacin, ciprofloxacin, erythromycin and novobiocin. These findings on antibiotic resistance should eventually be helpful in planning strategies for the control of *O. rhinotracheale*

Table 5. The AGP test results of field sera.

Serotype	(+) Chicken Sera (Total: 333 sera)	%	(+) Turkey Sera (Total: 250 sera)	%
A	30	34.9	17	26.6
B	25	29.0	29	45.3
A and B	13	15.1	11	17.2
I	9	10.5	5	7.4
L	6	7.0	2	3.5
I and L	3	3.5	-	-
Total positive sera	86	100.0	64	100.0

infections in poultry. However, it is very important to design more detailed studies for the investigation of *O. rhinotracheale* infections, which cause more antibiotic usage and decrease productivity.

References

1. Vandamme, P., Segers, P., Vancanneyt, M., Van Hove, K., Mutters, R., Hommez, J., Dewhirst, F., Paster, B., Kersters, K., Falsen, E., Devriese, L.A., Bisgaard, M., Hinz, K. H., Mannheim, W.: *Ornithobacterium rhinotracheale* gen. nov., sp. nov., isolated from the avian respiratory tract. *Int. J. Syst. Bacteriol.*, 1994; 44: 24-37.
2. Hinz, K.H., Blome, C., Ryll, M.: Acute exudative pneumonia and airsacculitis associated with *O. rhinotracheale* in turkeys. *Vet. Rec.*, 1994; 135: 233-234.
3. Odor, E.M., Salem, M., Pop, C.R., Sample, B., Prim, M., Vance, K., Murphy, M.: Isolation and identification of *Ornithobacterium rhinotracheale* from commercial broiler flocks on the Delmarva Peninsula. *Avian Dis.*, 1997; 41: 257-260.
4. Sprenger, S.J., Back, A., Shaw, D.P., Nagaraja, K.V., Roepke, D.C., Halvorson, D.A.: *Ornithobacterium rhinotracheale* infection in turkeys: experimental reproduction of the disease. *Avian Dis.*, 1998; 42: 154-161.
5. Van, Veen, L. Van Empel, P., Fabria, T.: ORT, a primary pathogen in broilers. *Avian Dis.*, 2000; 44: 896-900.
6. Van Empel, P., Hafez, H.M.: *Ornithobacterium rhinotracheale*: a review. *Avian Pathol.*, 1999; 28: 217-227.
7. Charlton, B.R., Channing-Santiago, S.E., Bickford, A.A., Cardona, C.J., Chin, R.P., Cooper, G.L., Droual, R., Jeffrey, J.S., Meteyer, U.C., Shivaprasad, H.L., Walker, L.R.: Preliminary characterization of a pleomorphic gram-negative rod associated with avian respiratory disease. *J. Vet. Diagn. Invest.*, 1993; 5: 47-51.
8. Erganiş, O., Ateş, M., Hadimli, H.H., Çorlu, M.: Tavukçuluk işletmelerinde *O. rhinotracheale*'nin araştırılması. S.Ü. Araştırma Fonu Proje No: 97/061, 1999.
9. Turan, N., Ak, S.: Investigation of the presence of *Ornithobacterium rhinotracheale* in chickens in turkey and determination of the seroprevalance of the infection using the ELISA. *Avian Dis.*, 2002; 46: 442-446.
10. Erganiş, O., Hadimli, H.H., Kav, K., Çorlu, M., Öztürk, D.: A comparative study on detection of *Ornithobacterium rhinotracheale* antibodies in meat-type turkeys by dot immunobinding assay, rapid agglutination test and serum agglutination test. *Avin Pathol.*, 2002; 31: 201-204.
11. Erganiş, O.: Enfeksiyöz koriza benzeri enfeksiyonlarda mikrobiyolojik durum. III. Ulusal Veteriner Mikrobiyoloji Kongresi. 1998, 23-25 Eylül, Bursa, Türkiye.
12. Hadimli, H.H., Erganiş, O., Kav, K.: Hindilerde *Ornithobacterium rhinotracheale* enfeksiyonu. *Vet. Bil. Derg.*, 2003; 19: 105-108.
13. Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M.: Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.*, 1966; 45: 493-496.
14. National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Disk Dilution Susceptibility Tests for Bacteria Isolated from Animals. 2002, 2nd ed., NCCLS Approved Standard, Wayne, PA. pp. M31-A2.
15. Malik, Y.S., Olsen, K., Kumar, K., Goyal, S.M.: In vitro antibiotic resistance profiles of *Ornithobacterium rhinotracheale* strains isolated from Minnesota turkeys during 1996-2002. *Avian Dis.*, 2003; 47: 588-593.
16. Hafez, H.M., Sting, R.: Investigations on different *Ornithobacterium rhinotracheale* "ORT" isolates. *Avian Dis.*, 1999; 43: 1-7.
17. Heddleston, K.L., Gallagher, J.E., Rebers, P.A.: Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. *Avian Dis.*, 1972; 16: 925-936.
18. Van Empel, P., van den Bosch, H., Loeffen, P., Storm, P.: Identification and serotyping of *Ornithobacterium rhinotracheale*. *J. Clin. Microbiol.*, 1997; 35: 418-421.
19. Hafez, H.M.: Respiratory diseases in turkey: serological surveillance for antibodies against *Ornithobacterium rhinotracheale* and turkey rhinotracheitis (TRT). 1st International Symposium on Turkey Diseases, 1998, 19-21 February, Berlin, Germany.

Acknowledgements

The author would like to thank Prof. Dr. Ömer M. Esendal, Prof. Dr. H. M. Hafez and Dr. P. van Empel for their help and support.