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Short Paper

Isolation, identification and antibiotic sensitivity determination of *Ornithobacterium rhinotracheale* in slaughtering broiler chicken flocks of Guilan province

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Summary

Ornithobacterium rhinotracheale (ORT), a species of bacteria, is known to be associated with respiratory disease, growth retardation, mortality and decreased egg production. The aim of the present study was to determine seroprevalence of the infection and to identify the microorganism by bacteriological and molecular methods. In this study, 460 serum samples and also 290 tracheal swabs were collected from 29 broiler chicken flocks in slaughter houses. Serological results showed that 24 out of 460 (5.12%) serum samples related to 5 flocks were positive for ORT by ELISA test. ORT was isolated from tracheal swabs of 3 flocks (1.03% out of 290 tracheal swabs) by culture and confirmed as ORT by specific primers in PCR method. Statistically, there was no significant difference between the rate of isolation and ORT serum titers ($P>0.05$). Antibiotic sensitivity test using standard disk diffusion technique was performed with 13 antibiotics. All the isolates were resistant (100%) to erythromycin, tetracycline, oxytetracyclin, enrofloxacin, ciprofloxacin, flumequin, lincospectin, furazolidon, and 100% of them were found to be susceptible to ceftriaxon and tiamulin, but 2 isolates (66.7%) were moderately sensitive to tylosin and amoxicillin and sensitive to florfenicol. This study is the first report of the prevalence of ORT, bacterial isolation and molecular method in broiler chickens after broiler breeder vaccination in Guilan province.

Key words: Isolation, Identification, *Ornithobacterium rhinotracheale*, Broiler chickens

Introduction

Respiratory infections can cause serious economic losses in the poultry industry, which may result in heavy costs, increased condemnation rates, a drop in egg production, a reduction in egg shell quality and decreased hatchability. Several pathogens are indicated as possible causes of respiratory disease, either alone, in synergy with other microorganisms, or influenced by non-infectious factors, such as climatic conditions and management-related problems.

Ornithobacterium rhinotracheale is a slow-growing, pleomorphic, Gram negative rod shaped bacterium and has not been classified into any of the known bacterial genera/families. The disease spreads horizontally by direct and indirect contact and vertical transmission is suspected. Clinical signs seen in birds include coughing, nasal discharge (Rahimi and Banani, 2007), arthritis, prostration, decreased egg production, growth suppression and mortality. Clinical signs and postmortem of ORT infections are similar to other bacterial and viral infections, and isolation, identification of the causative agent is essential for different diagnoses. Use of a reliable identification method is of great importance. Polymerase chain reaction (PCR) assay was recently shown to be useful for identification purposes (Van Empel and Bosch, 1998; Van Empel and Hafez, 1999). The aims of the present study were to determine seroprevalence, isolation, antibiotic resistance analysis and the

molecular characterization of ORT in broiler chicken flocks of Guilan province.

Materials and Methods

A total of 460 blood samples were collected from the individual birds by brachial venipuncture and 290 tracheal samples swabs were taken in BHI (Brain Heart Infusion) from 29 broiler chicken flocks in a slaughter house. Tracheal swabs were aseptically inoculated on blood agar with 5% sheep and 5 µg/ml gentamycin (to inhibit growth of other bacteria). The plates were incubated in a 5-7.5% CO_2 atmosphere at 37°C for at least 48 h. Identification of bacterial species was assessed by observation of the colonies morphology and gram staining results or biochemical methods, especially oxidase and catalase (Van Empel and Hafez, 1999).

Serology

The serum samples were tested for antibodies to *O. rhinotracheale* by the enzyme-linked immunosorbent assay (ELISA) with a commercial kit (IDEXX, Westbrook, ME, USA) test procedure and analysis of the results was performed as recommended by the manufacturer. Sample to positive (S/P) ratios of ≤0.4 were considered negative and samples with S/P values >0.4 were considered positive.

Antibiotic susceptibility test

Drug sensitivity test using standard disk diffusion technique was performed with 13 antibiotic disks obtained from Padtan Teb Ltd. Co. Iran: ceftriaxon CRO (30 µg), ciprofloxacin CP (5 µg), enrofloxacin NFX (5 µg), flumequin FM (30 µg), erythromycin E (15 µg), tylosin TY (30 µg), tiamulin TM (30 µg), tetracycline TE (30 µg), oxytetracyclin OTC (30 µg), amoxicillin AMX (25 µg), lincospectin LP (15/200 µg), florfenicol FF (30 µg), furazolidon FR (100 µg).

DNA extraction

An amount of a 0.5 ml tube containing BHI of tracheal swabs or a few colonies from suspicious ORT cultures were transferred into new micro tubes. They were vortexed and an equal volume of Lysis buffer (10 Mm Tris-Hcl, pH = 8 10 mM EDTA + 1 mM SDS 1% + Proteinase K 200 µg/ml) was added to the suspension and incubated at 56°C for 4 h in a water bath. An equal volume of phenol was then added to the suspension which was shaken vigorously by hand and then centrifuged at 13000 rpm for 15 min. The upper phase was transferred into a new micro tube and an equal volume of phenol-chloroform was added to it, then centrifuged at 13000 rpm for 15 min. The upper phase was transferred into a new micro tube and an equal volume of chloroform was added and vortexed, then micro tube was centrifuged at 13000 rpm for 5 min. The upper phase was transferred into a new micro tube and twofold of the sample volume of 0.1% sodium acetate 3 mM was added, as well as an equal volume of 90% ethanol and then incubated at -20°C for 15-20 min. The samples were then centrifuged at 13000 rpm for 15 min and all of materials in the micro tube were discarded. 200 µl Ethanol 70% was added to the DNA, precipitated and after turning upside down, was then centrifuged at 13000 rpm for 5 min the micro tube was discarded from the suspension with the tip vigorously and incubated *in vitro* convert to dry. 50 µl sterile distilled water was added and used as a target DNA in PCR.

Primers

Primers used in this study were designed by Van Empel and Hafez (1999). The sequence of primers pairs were as follows: OR 16S-F_1 (5´- GAG AAT TAA TTT ACG GAT TAA G-3´) and OR 16S-R_1 (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 thermocycler (Eppendorf, mastercycler gradient, Germany) in a total reaction volume of 25 µl containing: 2.5 µl of 10 X PCR buffer, 1.5 µl of 25 mM $mgCl_2$, 0.5 µl of each dNTP, 2 µl of *taq* DNA polymerase (Fermentas Co., Cinnagen Co., Iran), and 1+1 *p*mol primers and 4 µl of DNA template sample. Amplification was obtained with an initial

denaturation step at 94°C for 7 min followed by 40 cycles at 94°C for 30 s, and 53°C for 1 min, 72°C for 2 min. The final extension cycle was at 72°C for 7 min. 10 µl of PCR products were separated on a 1% agarose gel with 0.5 µg/ml ethidium bromide. DNA fragments were visualized by UV illumination and photographed with Polaroid film. The molecular size of the PCR products were compared with a 100 bp DNA ladder. In this study, we used *Pasteurella multicida*, *Haemophillus paragallinarum*, *Mycoplasma gallisepticum*, *Salmonella enteriditis* and distilled water (Hung and Alvarado, 2001) as negative controls. The positive control was provided from Razi Vaccine and Serum Research Institute.

Statistical analysis

Statistical analysis between ORT serum titers and the rate of isolation were performed by SPSS Software and K^2 test.

Results

The result showed that 5 out of 29 broiler flocks were positive for ORT infection by ELISA (17.2%). *Ornitho-bacterium rhinotracheale* antibodies were detected in 24 (5.12%) out of the 460 serum samples. There was no significant difference between the rate of isolation and flock ORT titers (P>0.05). After 24 h of incubation on blood agar, pin point Grey to grey/white colonies were observed. The colonies were larger after 48 h of incubation. Non hemolytic colonies were subcultured on blood agar plates. A unique characteristic of the colonies was their poor adherence to agar. Pure cultures of the isolates had a 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 and catalase negative.

Ornithobacterium rhinotracheale was isolated from 3 flocks of tracheal swabs (10.3% out of 29 flocks) or (1.03% out of 290 tracheal swabs) by culture. All ORT isolates were positive in PCR and produced the predicted 784 bp amplification product (Table 1, Fig. 1).

Table 1: ORT antibodies titer of slaughtered broiler chickens in Guilan province by ELISA test and the results of its culture and PCR assay

Flock	Serum	Serum positive	%	Titer	PCR/ Culture
F1	15	4	26.6	1551	-/-
F2	16	-	-	169	+/+
F3	15	6	40	1120	+/+
F4	15	4	26.6	1543	-/-
F5	17	5	29.4	2370	-/-
F6	15	5	33.33	1823	-/-
F7	16	-	-	190	+/+

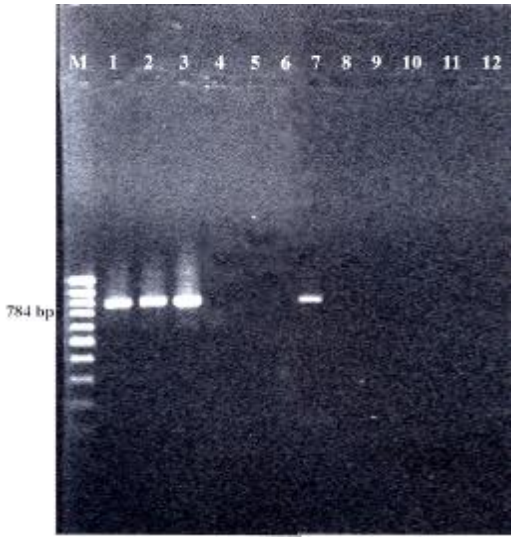

Fig. 1: Electrophoresis of PCR products on 1% agarose gel stained with ethidium bromide (Lane M) 100 bp molecular weight marker, (Lane 1-3) ORT strain, (Lane 4-6) negative sample, (Lane 7) positive control, (Lane 8) *Haemophillus Paragallinarum*, (Lane 9) *Pasteurella multicida*, (Lane 10) *Salmonella Enteriditis*, (Lane 11) *Mycoplasma gallisepticum*, and (Lane 12) distilled water

All the isolates were resistant (100%) to erythromycin, tetracycline, oxytetracyclin, enrofloxacin, ciprofloxacin, flumequin, lincospectin, and furazolidon, 100% of the isolates were susceptible to ceftriaxon and tiamulin, but 2 isolates (66.7%) were moderately sensitive to tylosin and amoxicillin and sensitive to florfenicol (Table 2).

Discussion

Ornithobacterium rhinotracheale is a new problem in the poultry industry and it is also a possible additional causative agent in the respiratory disease complex (Van Empel and Hafez, 1999). Since ORT is a difficult

Table 2: Antibiotic susceptibility of 3 ORT isolates in slaughtering broiler chickens of Guilan province

Antibotic	CRO	CP	NFX	FM	E	TY	TM	TE	OTC	AMX	LP	FF	FR
No. & % (S)	3(100)						3(100)					2(66.7)	
No. & % (Ms)						2(66.7)				2(66.7)		1(33.3)	
No. & % (R)		3(100)	3(100)	3(100)	3(100)	1(33.3)		3(100)	3(100)		3(100)		3(100)

S = Sensitive, Ms = Moderate sensitive, and R = Resistant

bacterium to culture, the serology test is useful for flock monitoring or as an aid in the diagnosis of ORT (Van Empel and Hafez, 1999; Van Empel, 2002).

This is the first report of seroprevalence, isolation, identification and the antibiotic sensitivity of *O. rhinotracheale* from the broiler chickens after vaccination of the broiler breeder flocks schedule in Guilan province.

Allymehr (2006) examined 463 serum samples from 50 broiler flocks in west Azarbaijan. The result showed that 41 broiler flocks (82%) were positive for ORT. Ganbarpour and Salehi (2009) reported the seroprevalence of ORT in the south east of Iran. 134 (31.9%) out of 420 serum samples or 17 (81%) out of 21 broiler flocks were positive. Keleidari *et al*. (2008) took 211 blood samples from 13 broiler flocks in Mashhad city, the result showed that 206 out of 211 serums were positive for ORT infection. Haghighi Khoshkhoo *et al*. (2008) examined 864 serum from 48 broiler flocks in Tehran province. The result showed that 24 flocks (50%), or 384 sera out of 864 (44.5%) in each individual flock of the birds were positive.

Canal *et al*. (2003) collected 1550 serum related to 50 broiler flocks during the slaughter time in southern Brazil. The prevalence of ORT antibodies was 63.83%, but in each individual flock only 6.52% of the birds were positive. Chansiripornchai *et al*. (2007) randomly examined 17 broiler farms (19 flocks) in Thailand. The seropositive flocks were 63% and the sera analysis showed that the individual 280 broiler sera antibody responses were 19.6% positive.

Seroprevalence of *O. rhinotracheale* in broiler and broiler breeder flocks of Guilan province were also investigated during 2006-2007. During this period no ORT vaccine was applied and the data showed that seropositive to ORT infection in broiler breeder and broiler flocks were 100 and 30.4%, respectively (Asadpour and Mosavi, 2010). The present research has been performed after broiler breeder vaccination in Guilan province. It demonstrates that vaccination of broiler breeder flocks against ORT might be responsible for the protection of progeny chicks against ORT infection, as was reported by (Van Empel and Bosch, 1998). Indeed, it was found that the mean percentage of broiler flocks infected by ORT at slaughter time was lower in the progeny from vaccinated breeder flocks in comparison with the non vaccinated broiler breeder flocks.

Antibiotic sensitivity of 5 ORT isolates in broiler breeders of Guilan province were performed with 29 antibiotics. Antibiotic susceptibility for quinolons family was seen more than the others and cephalosporins family with the exception of cephalexin, respectively. The isolates were 80-100% susceptible to tetracyclin family and the most antibiotic resistance was seen for minopenicillins, polypeptids, sulfanamids, 80-100% was resistant to aminoglycoside family, 80% of the isolates were resistant to licomycin and 60% were moderately sensitive to lincomycin (Asadpour *et al*., 2008). In comparison with the above study, more antibiotic resistance was observed, perhaps from the inappropriate use of antibiotics for treatment of secondary infections due to the prevalence of respiratory diseases complex in broiler chicken farms in the study areas.

There were no clinical signs of disease at the time of sampling. Therefore, the role of this bacterium in respiratory disease is similar to other bacteria involved in secondary infection. It is the first report related to *O. rhinotracheale* characterization in progeny of ORT vaccinated breeder flocks. This indicates the protective effect of such vaccination on broiler chicken farms contamination.

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