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Isolation and Identification of *Ornithobacterium rhinotracheale* in Broiler Breeder Flocks of Guilan Province, North of Iran

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Abstract: The aims of the present study were to isolate and serotype, determine the Seroprevalence, Drug susceptibility and diagnosis of infection by Polymerase Chain Reaction (PCR). In this study 460 serum samples and 220 tracheal swabs, 90 ovaries and oviduct swabs, 90 misshapen egg shells swabs were collected from 22 broiler breeder flocks of 5 companies. Serological results showed that all of the 22 flocks (100%) were positive for ORT infection. *Ornithobacterium rhinotracheale* (ORT) antibodies were detected in 289 (62/83%) out of the 460 serum samples. ORT was detected from tracheal swabs of seven flocks (31/81% or 3/18% out of 220 tracheal swabs). There was significant correlation between flock different ages and ORT titers ($p<0.05$), but correlation of flock ages and ORT isolates was not significantly different ($p>0.05$). Seven flocks infected with ORT were detected positive in PCR but bacteria were Isolated from only five culture. No ovaries and oviducts, misshapen egg shell swabs yielded ORT. A 784 bp fragment of the 16S rRNA gene was amplified using ORT specific primers in the PCR. All the isolates were identified as serotype A by Rapid Agglutination Test. Drug sensitivity test using standard disk diffusion technique was performed with 27 antibiotics. Antibiotic susceptibility for Quinolons family was seen more than the others and Cephalosporins family except to Cephalexin. The isolates were 80-100% susceptible to Tetracycline family and the most antibiotic resistant were seen for Aminopenicillins, Polypeptides, Sulfanamides and 80-100% resistant to Aminoglycoside family. Eighty percent of the isolates were resistant to Licomycin and 60% were moderate sensitive to Lincomycin. This study is the first report of prevalence of ORT, bacterial isolation, biochemical characteristics, serotyping and molecular method (PCR) in broiler breeder flocks in Guilan province of Iran.

Key words: Isolation, identification, *Ornithobacterium rhinotracheale*, broiler breeder

INTRODUCTION

Respiratory diseases are continuing to cause heavy economic losses in the global Poultry industry. Various pathogens have been identified as causing respiratory diseases including *O. rhinotracheale* which is gram-negative, pleomorphic, rod-shaped bacterium named by Vandamme *et al.* (1994). The disease spreads horizontally by direct and indirect contact and Vertical transmission is suspected. Some researches have recently isolated ORT at very low incidence from reproductive organs and hatching eggs, infertile eggs and dead embryos (Van empel *et al.*, 1997; Hafez, 2002; Chin *et al.*, 2003). It is however not yet known if this vertical transmission is caused by ovarial or cloacal contamination (Hafez, 2002; Van veen *et al.*, 2004). ORT infections are common in broiler breeders at 24-52 weeks of age (Van veen *et al.*, 2001). There is a slight increase in mortality, a decrease in feed intake and some mild respiratory symptoms. Mortality is variable and relatively low in uncomplicated cases and influenced by environmental factor such as poor management, inadequate ventilation, high stocking density and poor hygiene (Hafez, 2002).

There can be a drop in egg quality. Fertility and hatchability are unaffected in many cases (Van empel and Hafez, 1999; Van empel, 2002; Chin *et al.*, 2003). Clinical signs and postmortem lesions of ORT infections are similar to other bacterial and viral infections, isolation and

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identification of causative agent are essential for differential diagnosis (Van empel and Hafez, 1999).

Use of a reliable identification method is of importance such as Polymerase Chain Reaction (PCR) assay was shown to be useful for identification purposes recently (Van empel and Bosch, 1998; Hafez and Beyer, 1997). The aims of the present study were to determine of Seroprevalence, Isolation, Serotyping, Analyse drug susceptibility and perform diagnosis of infection by PCR.

MATERIALS AND METHODS

Samples: A total of 460 blood samples from individual birds by brachial venipuncture, 220 tacheals (22 samples), 90 ovaries and oviducts (15 samples), 90 misshapen egg shells (15 samples) swabs samples were collected in BHI (Brain Heart Infusion) from 22 broiler breeder flocks of 5 companies. This study was conducted from May 2006 to June 2007.

Culture and biochemical properties: Tracheal, ovaries, egg shell swab samples were aseptically inoculated on blood agar with 5% Sheep erythrocytes and 5 μg mL^{-1} gentamycin (to inhibit growth of other bacteria).

The plates were incubated in a 5.0-7.5% CO_2 atmosphere at 37°C for at least 48 h. Each day, the agar plates were checked of suspected colonies. Primary identification of bacterial species was performed by observation of the colonial morphology and gram staining results or biochemical tests mainly Oxidase and Catalase.

Serology: The serum samples were tested for antibodies to *O. rhinotracheale* by the Enzyme-Linked Immunosorbent Assay (ELISA). Test was performed using a commercial kit (IDEXX, Westbrook. ME. USA) which was able to determine antibodies of 12 serotypes (A-M) of *O. rhinotracheale*. The test procedure and analysis of results were performed as recommended by manufacturer. Samples were diluted one of 500 and the Optical Density (OD) was measured on ELISA reader at 650 nm. Result were determined by calculating the sample to positive (S/P) ratio. Samples with S/P ratios of ≤0.4 were considered negative and samples with S/P-values >0.4 were considered positive.

Serotyping: For serotyping of ORT isolates, Rapide Slide Agglutination Test (RSA) were done using standard antisera (Joubert *et al*., 1999) against ORT antigen. These standard antisera were kindly provided by Dr. Amer Silim (Faculty of Veterinary Medicine, University of Montreal Canada).

Drug susceptibility test: The test was performed as described by Bauer *et al*. (1996). The following 27 antibiotic disks were obtained from Padtan teb LTD. CO. Iran.

DNA extraction: Tracheal, ovaries and oviducts, egg shell swabs in 0.5 mL BHI or a few colonies from suspicious ORT culture were transferred into new micro tube. Then were vortexed and an equal volume of Lysis buffer (10 mM Tris-HCl, pH = 8+1 mM EDTA + 1% SDS + 200 μg mL^{-1} Proteinase K) was added to the suspension and incubated at 56°C for 4 h in water bath. Then equal volume of it, phenol was added to the suspension which was shaken vigorously by hand the centrifuged at 13000 rpm for 15 min. The upper phase was transferred into a new micro tube and equal volume of phenol-chloroform was added to it, then centrifuged at 13000 rpm for 15 min, upper phase Transferred into a new micro tube and equal volume of it, chloroform added and vortexed, then micro tube centrifuged at 13000 rpm for 5 min. Upper phase transferred into a new micro tube and was added twofold of sample volume of 0.1% Sodium acetate 3 mM, equal volume to the sample, 90% Ethanol and then incubated in -20°C for 15-20 min then samples centrifuged 13000 rpm for 15 min and all of materials micro tube discarded. 200 μL Ethanol 70% added to the DNA was precipitated after up side down, centrifuged 13000 rpm for 5 min the micro tube quite discarded from suspension with tip vigorously and incubated *in vitro* convert to dry. Fifty microliter 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 (Mastercycler Gradient) in a total reaction volume of 25 μL containing: 2.5 μL of 10X PCR buffer, 1.5 μL of 25 mM $MgCl_2$, 0.5 μL of each deoxynucloside triphosphate, 2 μL of *taq* DNA polymerase (Fermentas Company, Cinagen Iran) and 1+1 *p*mol primers and 4 μL of template sample DNA.

Amplification was obtained with an initial denaturation step at 94°C for 7 min followed by 40 cycles at 94°C for 30 sec and 53°C for 1 min, 72°C for 2 min the final cycle was at 72°C for 7 min. A 10 μL of PCR products were separated on a 1% agarose gel with 0.5 μg mL^{-1} ethidium bromide. The DNA fragment were visualized by UV illumination and photograph with Polaroid film. The molecular size of PCR products were compared with a 100 bp DNA ladder.

RESULTS

The result showed that all of the 22 broiler breeder flocks were positive to ORT infection by ELISA (100%). *O. rhinotracheale* antibodies were detected in 289 (62/83%) out of the 460 serum samples. After 24 h of incubation on blood agar at 37°C in a 5-7.5% CO_2 atmosphere, pin point grey to grey/white colonies, were observed. the colonies were larger after 48 h of incubation (Fig. 1).

Non hemolytic colonies were subcultured on blood agar plates. A unique characteristics of the colonies was their poor adherence to agar, A wire loop used to displace or slide a colony along the agar without deforming the colony Pure cultures of the isolates had distinct smell similar to butyric acid, the gram staining revealed the presence of gram-negative, pleomorphic, rod-shaped microorganisms (Fig. 2) and according to Biochemical tests, isolated organisms were oxidase positive and catalase negative.

Fig. 1: Subculture of ORT colonies after 48 h of incubation

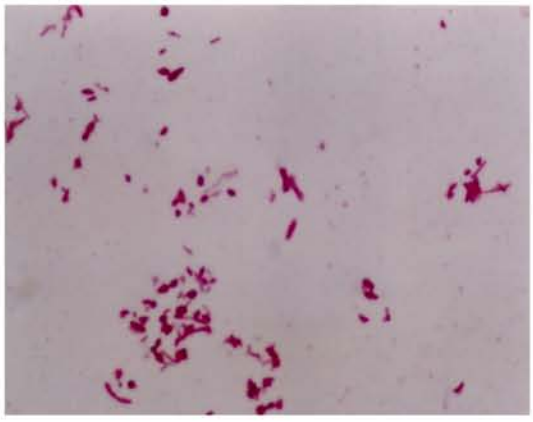

Fig. 2: Pleomorphic and gram negative bacteria from a 4 h culture (x100)

Infection agent (ORT) was detected only from seven flocks of tracheal swabs (31/81%) or (3/18% out of 220 tracheal swabs) (Table 1). 784 bp fragment of the 16s rRNA gene was amplified using ORT specific primers in PCR but out of seven, only five bacteria were isolated in culture (Fig. 3). ORT was not isolated from ovaries and oviducts, misshapen egg shell swabs in this study. All the isolates were susceptible (100%) to: ceftriaxon, ceftizoxim,

Table 1: *O. rhinotracheale* antibodies in broiler breeder flocks of Guilan by ELISA (Flockchek® IDEXX kit) and Culture and PCR results

Flock	Age (week)	Serum	Serum positive	(%)	Mean titer	PCR/Culture result
F_1	32	23	22	95.65	10584	
F_2	32	23	21	91.30	10498	
F_3	30	22	19	86.36	12554	
F_4	41	21	18	85.71	4323	
F_5	47	22	16	72.73	5317	
F_6	51	17	6	35.29	1759	
F_7	53	21	9	42.85	2042	
F_8	38	22	16	72.73	9175	
F_9	42	21	15	71.43	6241	
F_{10}	47	19	7	36.84	1806	+/+
F_{11}	41	19	10	52.63	4595	
F_{12}	52	21	11	52.38	2955	+/-
F_{13}	55	18	6	33.33	2087	+/+
F_{14}	68	17	10	58.82	2220	
F_{15}	55	22	15	68.18	4218	+/-
F_{16}	68	22	10	45.45	3528	
F_{17}	48	20	11	55.00	2838	+/+
F_{18}	51	22	11	50.00	5220	
F_{19}	42	21	15	71.43	3641	+/+
F_{20}	74	22	5	22.73	3523	
F_{21}	52	22	19	86.36	3860	
F_{22}	55	23	17	73.91	2545	+/+

+: Positive, -: Negative

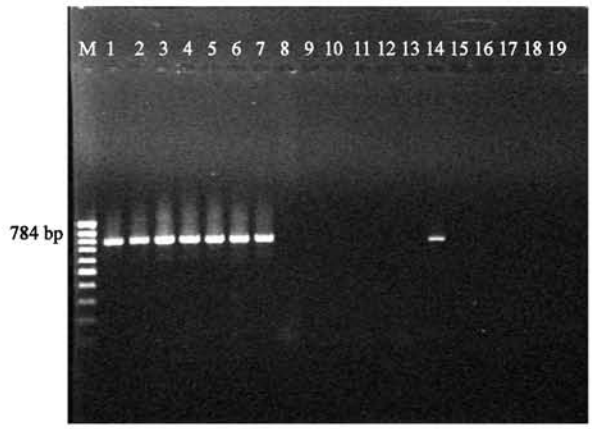

Fig. 3: Electrophoresis of PCR products on a 1% agarose gel stained with ethidium bromide (lane M) 100 bp molecular weight marker, (lane 1-7) ORT strains, (lane 8-13) negative Samples, (lane 14) positive control, (lane 15) *Haemophillus Paragallinarum*, (lane 16) *Pasteurella Multocida* (lane 17) *Salmonella Entrididis* (lane 18) *Mycoplasma Gallisepticum* (lane 19) Distilled water

Table 2: Drug susceptibility of 5 ORT isolates in broiler breeder flocks

Antibiotic	NO and (S)*	NO and (%) (MS)**	NO and (%) (R)***
Ceftriaxon	5(100)		
Ceftizoxim	5(100)		
Ceftiofur	5(100)		
Cephalexin			5(100)
Ciprofloxacin	5(100)		
Enrofloxacin	5(100)		
Difloxacin	5(100)		
Flumequin	5(100)		
Erythromycin			5(100)
Tylosin	5(100)		
Tiamulin	5(100)		
Kanamycin	1(20)		4(80)
Amikacin	1(20)		4(80)
Gentamycin			5(100)
Neomycin			5(100)
Tetracycline	5(100)		
Doxycycline	4(80)		1(20)
Penicillin			5(100)
Ampicillin			5(100)
Colistin			5(100)
Lincomycin	1(20)		4(80)
Lincospectin	1(20)	3(60)	1(20)
Chloramphenicol	5(100)		
Sulfametoxazol-trimetoprime			5(100)
Nitrofnrantoin	5(100)		
Fnrazolidone	2(40)	1(20)	2(40)
Fnraltadone	5(100)		

S: Sensitive*, MS: Moderate Sensitive**, R: Resistant***

ciprofloxacin, enrofloxacin, difloxacin, flumequin, tylosin, tiamulin, tetracycline, chloramphenicol, Nitrofurantoin, Furaltadon and 100% isolates were resistant to: cephalexin, erythromycin, gentamycin, neomycin, penicillin, ampicillin, colistin, sulfametoxazol-trimetoprim. 80% of the isolates were susceptible to doxycycline and 40% to furazolidone, 80% of the isolates were resistant to licomycin and 60% were moderate sensitive to Lincomycin (Table 2).

DISCUSSION

The first documented isolation and identification of *O. rhinotracheale* in Iran was made from 4 week old broiler in 2000 (Banani *et al*., 2001). To the best our knowledge, this is the first report of isolation and identification of *O. rhinotracheale* in broiler breeder flocks of Guilan province in north of Iran.

ORT is a difficult bacterium to culture, it grows slowly and needs special growth Conditions and so attempts at isolation are often negative and plates are overgrown by other bacteria such as *Escherichia*, *Proteus* sp. or *Pseudomonas* sp. (Van veen *et al*., 2001). In this study, colonies easily could be replaced in the blood agar culture. Bacteria pleomorphic features and biochemical characteristics and its serotype had been similar to that of reported previously by other research. Abdul-Aziz (1997) declared the isolation of ORT appears to be more successful during the first 10 days after infection. Non isolated bacteria from ovaries, oviducts and egg shells swabs can not reject vertical transmission. Due to biosequrity conditions, sampling implemented at specific times. Therefore chance for isolation was impossible and so more research is needed. Bacterial isolation in this research could be described as, long breeding and production period, surviving of the bacteria as well in cold environmental condition and environmental factors of the houses. Up to now 18 serotype were detected and serotype A is predominant in the most of the countries. Fifty percent of the France isolates, 100% of Germany, Italy, South Africa and 94% USA isolates, 100% Peru isolates and in this study belong to serotype (Van empel and Hafez, 1999). Serology is useful for flock monitoring or as an aid in the diagnosis of *O. rhinotracheale*. ELISA have been developed using different serotypes and extracted antigens of *O. rhinotracheale*. Two commercial ELSA kits (BIOCHEKand IDEXX) were used for detection of specific ORT antibodies. These were able to determine antibodies of 12 serotype (A-M) (Chin *et al*., 2003; Van empel and Hafez, 1999).

Canal *et al*. (2003) reported that the prevalence ORT antibodies in broiler breeder flocks of Southern Brazil was 100% and in each individual flock 94/62% of the birds were positive. Refaei *et al*. (2005) used the IDEXX ELISA kit for testing 338 serum samples from 6 broiler breeder flocks of Egypt, showed a 84/6% rate of positive reaction.

Allymehr (2006) were examined 472 samples of 42 broiler breeder flocks of West Azarbaijan of Iran for the presence of antibodies against ORT. The result showed 39 flocks (92/8%) were positive. In this study all of the 22 flocks were positive to ORT infection (100%) and similar to the others research.

In broiler breeder, the disease affects the birds in the laying period, primarily at the peak of production or soon before entering production. In this study, we detected ORT antibodies in higher ages (68 and 74 weeks). Egg production stress is one of the important environmental factors in the molting flocks which that infection is influenced.

The treatment of *O. rhinotracheale* infections with antibiotics is very different, because of the variable susceptibility of strains and sensitivity pattern depends on the source of the strains. *O. rhinotracheale* can acquire resistant against current antibiotics.

We observed high sensitivity to the antibiotics and this subject was probably due to severe biosequrity and antibiotics low consumption in broiler breeder flocks.

The primers combination OR16S-F_1 and OR16S-R_1 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 and Bosch, 1998; Hung and Alvarado, 2001).

In this study, we used the *Pasteurella multocida*, *Haemophillus paragallinarum*, *Mycoplasma gallisepticum*, *Salmonella entritidis* and distilled water as negative control which did not show any DNA bands in PCR method (Fig. 3).

In the present study, out of the 22 flocks, in 7 flocks were detected ORT infections by PCR, but only in 5 flocks were isolated bacteria by culture and two flocks were negative in culture, perhaps it is related to tracheal swab samples of the birds necropsy and probably in during of sample transporting, the bacteria became reliable but detected positive by PCR.

In spite of, seropositivity of the all flocks, culture and PCR results, there were no clinical signs of disease at the time of sampling and therefore the role of the *O. rhinotracheale* in respiratory disease in poultry is similar to other bacteria involved in secondary infection.

For the first rime, this study reported the presence of the ORT in broiler breeder flocks of Guilan province of Iran. However, further studies are needed to understand epidemiological importance of this diseases in poultry population of the region.

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