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Original Article

Isolation, characterization, and genotyping of *Ornithobacterium rhinotracheale* isolated from broiler and broiler breeder flocks in Mazandaran province, Northern Iran

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Abstract

Background: *Ornithobacterium rhinotracheale* (ORT) is one of the most important pathogenic bacteria which cause significant economic losses in poultry breeder countries every year. **Aims:** The present study was conducted to isolate and investigate the ORT isolates' biochemical, antibiotic resistance, and genotypic characteristics of in industrial poultry flocks with respiratory signs in northern Iran. **Methods:** After sampling from 60 different flocks and cultivation of the samples on a selective medium, suspected colonies were subjected to biochemical and molecular identification of ORT. Then, confirmed isolates were aimed to antibiotic resistance assay, hemagglutination test, detection of *pOR1* plasmid, and DNA fingerprinting to survey the variability of the isolates. **Results:** A total of 13 isolates, including seven isolates from broiler flocks (19.44%) and six isolates from broiler breeder flocks (25%) were obtained. Almost all isolates showed similar results in terms of basically important biochemical tests. The most resistance rates among all ORT isolates were obtained for ampicillin, erythromycin, ceftriaxone, and penicillin (100%). The majority of ORT isolates were susceptible to furazolidone. The *pOR1* plasmid was detected in only two isolates, and analysis of the DNA fingerprinting phylogenetic tree showed four specific genotypic clusters. **Conclusion:** According to the results, the isolates showed different antibiotic resistance profiles, and most of the strains proved multiresistant. This can indicate the circulation of various multi-drug resistant strains among poultry farms in northern Iran. Isolates from broilers and broiler breeders were grouped into different clusters by genotyping.

Key words: Antibiotic resistance, ERIC-PCR, *Ornithobacterium rhinotracheale*, *pOR1* plasmid

Introduction

Respiratory infections cause severe economic losses in the poultry industry due to increased mortality, treatment costs, condemnation rates at slaughterhouses, reduced production and eggshell quality. Several pathogens are known as etiologies of respiratory disease. They injure either primarily or secondarily under the influence of non-infectious factors such as poor environmental conditions and management problems (Van Empel and Hafez, 1999). *Ornithobacterium rhinotracheale* (ORT) is a gram-negative, pleomorphic and non-motile bacterium that has been isolated from turkeys and chickens with respiratory symptoms, reduced growth, decreased egg production, increased mortality, and slaughterhouse condemnation (Chin *et al*., 2008). Early signs of the disease caused by this bacterium include depression, reduced consumption of food and water, weight loss, runny nose, cough, sneezing, and sinusitis, and in many cases, followed by severe respiratory disorders and eventually death (Barbosa *et al*., 2020). As definitive diagnosis based on clinical signs and necropsy findings seems difficult, diagnosis is primarily based on bacteria identification. Furthermore, serological methods for flock monitoring as well as molecular methods are valuable tools in supporting the diagnosis (Chin *et al*., 2013). To date, eighteen different serotypes have been identified (A-R) (Hafez and Sting, 1999). There are reports of ORT infections in different countries such as the United States, Germany, South Africa, Japan, Turkey, and Iran (Van Empel and Hafez, 1999; Sakai *et al*., 2000; Ozbey *et al*., 2004; Banani *et al*., 2017; Smith *et al*., 2020). The rapid spread of ORT-related diseases and the subsequent increase of epidemiological studies revealed intraspecies differences and specific characteristics of isolates based on the geographic region and source (Szabó *et al*., 2017; Barbosa *et al*., 2020; Alispahic *et al*., 2021). Especially that different treatment strategies in the use of different antibiotics can cause various resistant strains. In the present study, we surveyed phenotypic features including biochemical characteristics and antibiotic resistance

patterns of ORT strains isolated from broiler and broiler breeders in northern Iran. Furthermore, the genetic diversity of these strains was assessed using ERIC-PCR.

Materials and Methods

Sample collection and ORT strains

Between May 2020 and January 2021, tracheal swab samples from 42 broiler and 18 broiler breeder flocks with clinical signs of respiratory disease were collected in Mazandaran province Northern Iran. Ten swab samples were collected from each flock (a total of 600 swab samples). The collected samples were cultured onto 5% sheep blood agar (HiMedia, India) supplemented with gentamycin (5 µg/ml) and incubated at 37°C for 48 h in candle jars. After incubation, the growth features and colony morphology of the cultures were studied. The morphology of colonies obtained from isolates on culture media was examined in size and color. After 24 h incubation on selective media, colonies with 1-3 mm in diameter were considered as large colonies (LC) and colonies with <1 mm in diameter as small colonies (SC) (Vandamme *et al*., 1994; Van and Hafez, 1999; Tsai and Huang, 2006). Biochemical characterization of the isolates was done with catalase, oxidase, urease, nitrate reduction, growth on MacConkey agar, indole, arginine, lysine, ornithine, and carbohydrate fermentation test for lactose, maltose, galactose, and glucose. An ORT reference strain was obtained from the culture collection of the Department of Microbiology, School of Veterinary Medicine, University of Tehran, Iran.

DNA extraction and molecular identification

DNA extraction of *O. rhinotracheale* isolates was done using gram negative DNA extraction kit (Sinaclon, Tehran, Iran) according to the manufacturer instructions. The extracted DNA and the identified isolates were stored at -20°C. The isolates were identified by amplification and visualization of a 784 bp segment using primers according to the protocol of van Empel *et al*. (1999) with the forward sequence of 5´-GAG AAT TAA TTT ACG GAT TAA G-3´ and the reverse primer sequence of 5´-TTC GCT TGG TCT CCG AAG AT-3´. The polymerase chain reaction (PCR) products were evaluated using electrophoresis in 1.5% agarose gel with the assistance of a marker of 100 bp and safe stain (Sinaclon, Iran).

Hemagglutination assay

The hemagglutination test was done to determine hemagglutination activity (HA) of ORT isolates using 96-well plates. Fresh chicken erythrocytes were tested with the isolates. Bacteria were grown on sheep blood agar plates at 37°C for 24-48 h in microaerophilic condition, suspended, and serially diluted in 0.85% NaCl. To each well, 50 µL of a suspension of 0.75% erythrocytes was added and mixed. Wells containing only the suspension of erythrocytes served as a negative control. A small pellet of erythrocytes at the bottom was considered negative after 40 min of incubation at room temperature, and those containing an even layer of erythrocytes across the well were considered positive (Tsai and Huang, 2006).

Antibiotic susceptibility test

Antimicrobial susceptibility test of the ORT isolates was done by Kirby-Bauer disc diffusion method according to the standards set by Clinical and Laboratory Standards Institute (CLSI) for fastidious Gram-negative organisms and other previous studies (Bauer and Kirby, 1966; CLSI, 2017). The following antibiotic disks (PadtanTeb, Iran) were used with their particular concentrations: tetracycline (TE) (30 µg), doxycycline (D) (30 µg), erythromycin (ERY) (15 µg), gentamicin (GEN) (10 µg), neomycin (N) (10 µg), ciprofloxacin (CIP) (5 µg), cefalexin (CN) (30 µg), ceftriaxone (CRO) (30 µg), ceftizoxime (CT) (30 µg), nitrofurantoin (FM) (50 µg), chloramphenicol (C) (30 µg), penicillin (P) (10 IU), ampicillin (AM) (10 µg), and furazolidone (FR) (100 µg). The plates of Muller Hinton agar (HiMedia, India) with 5% sheep blood were incubated at 35 ± 2°C for 18 h, and diameter of growth inhibition zones was measured and compared with the standard tables. As a final point, the rate of multidrug-resistant (MDR) defined as being resistant to more than four antimicrobial classes.

Primer design and detection of pOR1 plasmid

The ORT isolates were examined for the presence of the *VapD* (virulence associated protein) and *CopA* (multicopper oxidase domain-containing protein) genes located on *pOR1* plasmid. At first, specific primers were designed using the Primer3 and BLAST softwares in NCBI (https://www.ncbi.nlm.nih.gov/) according to the sequence previously described by Jansen *et al*. (2004) with the accession number of NC_011414.1. Forward and reverse primers for *vapD* with the sequence of 5´-TGT ATG CGA TTG CGT TTG ATA TG-3´ and 5´-GAA TAT CGC GCA CGC TCA G-3´, respectively, with the expected product length of 222 bp. The forward and reverse primers for *copA* with the sequence of 5´-CCA GAT TCG CCT GGA ACT GT-3´ and 5´-ATC GTT CGG GTT GCC TTT CT-3´, respectively, with the expected product length of 427 bp were used in PCR reaction. PCR was performed in the final volume of 25 µL (including 12.5 µL of a PCR master mix (Sinaclon, Iran), 1 µL (0.5 µM) of both forward and reverse primers and 2 µL of DNA samples and reached to 25 µL using distilled deionized water). Then the PCR products were evaluated and confirmed using electrophoresis in 1.5% agarose gel with the assistance of a 100 bp DNA marker (Sinaclon, Iran). The presence of the genes was considered the presence of the *pOR1* plasmid.

DNA fingerprinting and phylogenetic tree

Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) was done for DNA fingerprinting comparison of the isolates. ERIC-PCR reactions were performed in a final volume of 25 µM including 1.5 µL of each primer in a final concentration of 2 pmol/µL, 12.5 µL of master mix (SinaClon, Iran), and 8.5 µL of

deionized distilled water. Primer ERIC-1 and primer ERIC-2 were used in ERIC reaction as previously described (Versalovic *et al*., 1991). The images of ERIC reactions were loaded in GelClust software for analysis. Genetic similarity was calculated using the Pearson correlation in which 2% of the optimization tolerance and 4% of the position tolerance shift were set. The dendrogram of the isolates was also created by the Dice correlation coefficient and the un-weighted pair group method with arithmetic averages (UPGMA).

Statistical analysis

We tried to find correlations among different phenotypic and genotypic variables including the source of the sample, antibiotic susceptibility results, ERIC-PCR genotypes, and the presence of the plasmid genes, so; the results of the study were analyzed using SPSS version 22 software (IBM Armonk, North Castle, NY, USA).

Results

Of 60 chicken flocks, 13 (21.6%) ORT isolates were obtained. Seven isolates originated from broilers, and six were from broiler breeders. All isolates proved Gram-negative (Fig. 1) and gave the following biochemical features: all 13 isolates were negative for catalase and indole tests and positive for oxidase and urease tests. The size of bacterial colonies ranges from 1-2 mm, presenting the grey-white color. Ten (76.9%) isolates, including all broiler breeder isolates, showed a positive hemagglutination test. The number of isolates with large colonies among broiler isolates was higher than broiler breeder isolates, but this difference was not statistically significant (P>0.05). All broiler breeder isolates showed positive HA test, but this difference was not statistically significant (P>0.05). Detailed results of biochemical and enzymatic features of the ORT isolates obtained from chicken farms are listed in Table 1. The antibiotic resistance patterns of all ORT isolates were determined, and all 13 isolates were considered MDR. The antibiotic resistance patterns of the 13 isolates to 14 antibiotics are presented in Table 2. In the present study, high resistance rate of the isolates to tetracycline, doxycycline, ciprofloxacin and ceftizoxime was observed. Twelve isolates were susceptible to furazolidone. ORT specific PCR products of 784 bp length were obtained from all 13 strains (Fig. 2). All 13 ORT isolates were successfully genotyped using ERIC-PCR technique (Fig. 3 and Fig. 4). Based on DNA fingerprinting analysis of the isolates, four different clusters named C1 to C4 were identified. Five (71.5%) isolates recovered from broilers belonged to cluster C1, and one isolate each to cluster C2 and C3, respectively. Broiler breeder isolates could be attributed to the following clusters: three isolates to cluster C2, one to cluster C3, and two to cluster C4. Distribution of the ERIC-PCR patterns is shown in Table 3. The *pOR1* plasmid related genes, *vapD*, and *copA*, were only present in two broiler isolates (ORT3 and ORT5) (Fig. 5 and Table 4). Differences of isolates in the incidence of antibiotic resistance were also not statistically significant. According to the statistical analysis, cluster C1 was significantly associated with broiler isolates (P<0.05), however, the presence of other clusters among the isolates did not show a significant difference (P>0.05).

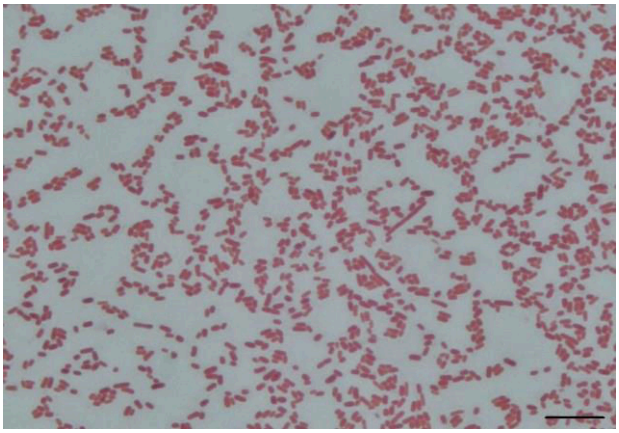

Fig. 1: Gram-stained ORT1 isolate showing characteristic pleomorphism (scale bar: 10 µm)

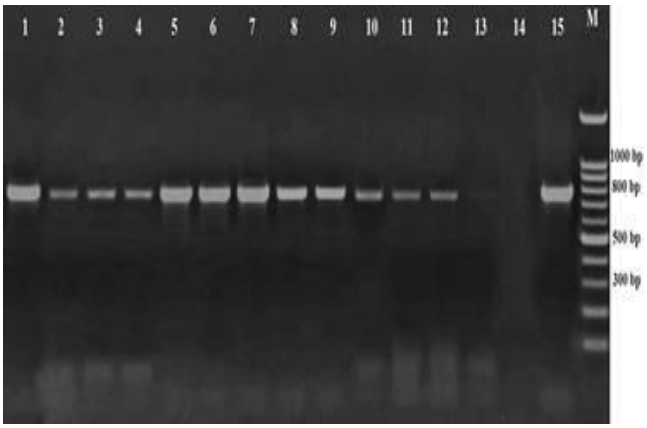

Fig. 2: Electrophoresis of PCR products of ORT specific band with 784 bp length. Lane M: 100 bp DNA marker, Lanes 1-13: The positive isolates, Lane 14: Negative control, and Lane 15: Positive control

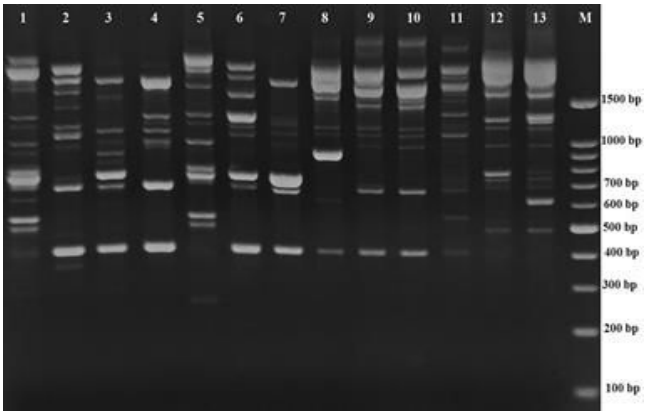

Fig. 3: Electrophoresis of the ERIC-PCR products of *O. rhinotracheale* isolates, demonstrating different genotypic patterns. Lane M: 100 bp DNA marker. Lanes 1-13: ORT isolates

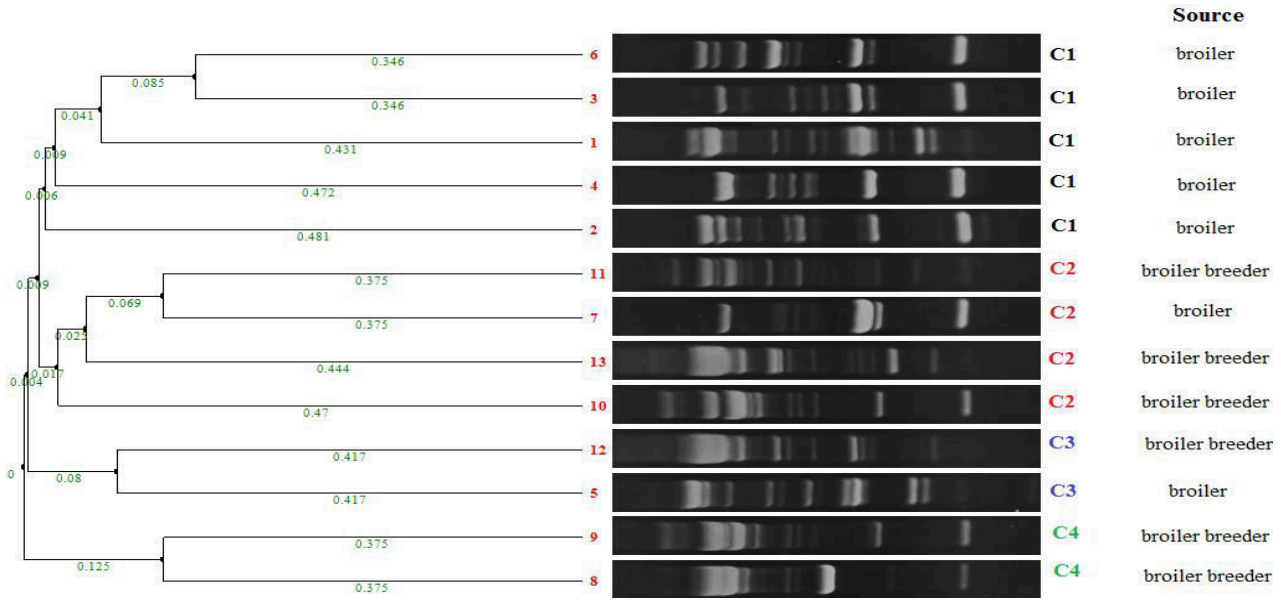

Fig. 4: Dendrogram grouping the *O. rhinotracheale* isolates based on the ERIC-PCR results using the unweighted pair group method

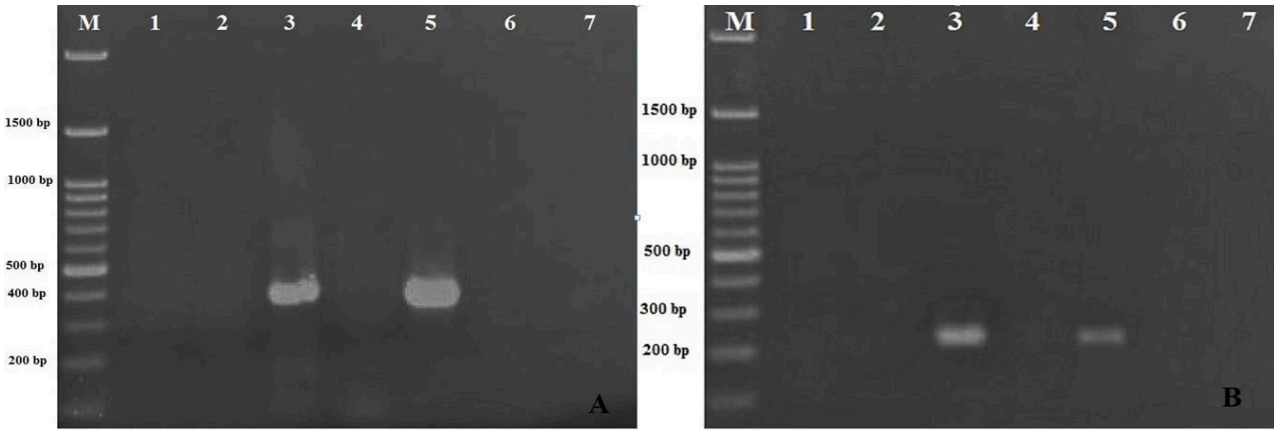

Fig. 5: Electrophoresis of the PCR products of *vapD* gene with the 222 bp length and *copA* gene with the 427 bp length. **A**: Lanes 3 and 5: Positive samples (ORT3 and ORT5), Lane M: 100 bp DNA marker, and **B**: Lanes 3 and 5: Positive samples (ORT3 and ORT5), and Lane M: 100 bp DNA marker

Table 1: Results of the biochemical and hemagglutination assays of the ORT isolates

Isolates	Farm type	Type of the assay														
		Hemagglutination	Colony type	Catalase	Oxidase	Urease	Nitrate reduction	Growth on MacConkey	Indole	Arginine	Lysine	Ornithine	Lactose	Maltose	Galactose	Glucose
ORT-1	Broiler	+	LC	-	+	+	-	-	-	-	-	-	+	-	+	+
ORT-2	Broiler	+	LC	-	+	+	-	-	-	-	-	-	+	-	-	+
ORT-3	Broiler	-	LC	-	+	+	-	-	-	+	-	-	+	+	-	+
ORT-4	Broiler	-	LC	-	+	+	-	-	-	+	-	-	+	-	-	-
ORT-5	Broiler	+	SC	-	+	+	-	-	-	-	-	-	-	+	-	+
ORT-6	Broiler	-	LC	-	+	+	-	-	-	-	-	-	+	+	+	+
ORT-7	Broiler	+	LC	-	+	+	-	-	-	+	-	-	+	+	+	+
ORT-8	Broiler breeder	+	SC	-	+	+	-	-	-	-	-	-	-	-	-	+
ORT-9	Broiler breeder	+	LC	-	+	+	-	-	-	-	-	-	-	+	-	+
ORT-10	Broiler breeder	+	SC	-	+	+	-	-	-	-	-	-	+	+	+	-
ORT-11	Broiler breeder	+	LC	-	+	+	-	-	-	+	-	-	+	-	+	-
ORT-12	Broiler breeder	+	LC	-	+	+	-	-	-	-	-	-	+	+	-	+
ORT-13	Broiler breeder	+	SC	-	+	+	-	-	-	+	-	-	+	+	-	+
Total positive	13	10 (76.9)	-	0 (0)	13 (100)	13 (100)	0 (0)	0 (0)	0 (0)	5 (38.4)	0 (0)	0 (0)	10 (76.9)	8 (61.5)	5 (38.4)	10 (76.9)

LC: Large colony, and SC: Small colony

Table 2: Resistance of the ORT isolates to the different antibiotics

Isolates	Farm type	Isolates resistant to the antibiotic													
		TET	D	ERY	GEN	N	CIP	CN	CRO	CT	FM	C	P	AM	FR
ORT-1	Broiler	R	R	R	R	S	R	S	R	R	I	R	R	R	S
ORT-2	Broiler	R	R	R	S	R	R	S	R	R	R	R	R	R	S
ORT-3	Broiler	R	R	R	R	S	R	R	R	R	I	R	R	R	S
ORT-4	Broiler	R	R	R	R	R	R	R	R	R	R	S	R	R	S
ORT-5	Broiler	R	R	R	R	R	R	S	R	I	R	S	R	R	S
ORT-6	Broiler	R	R	R	R	R	S	S	R	S	R	R	R	R	S
ORT-7	Broiler	R	R	R	R	R	S	S	R	R	R	R	R	R	S
ORT-8	Broiler breeder	R	R	R	S	I	R	S	R	S	I	R	R	R	S
ORT-9	Broiler breeder	R	R	R	R	R	R	R	R	R	R	S	R	R	S
ORT-10	Broiler breeder	R	R	R	R	R	R	R	R	R	R	R	R	R	S
ORT-11	Broiler breeder	R	R	R	S	S	R	R	R	S	I	S	R	R	S
ORT-12	Broiler breeder	S	S	R	R	R	R	R	R	R	R	R	R	R	R
ORT-13	Broiler breeder	R	R	R	R	R	R	S	R	I	R	R	R	R	S
Total		12	12	13	10	9	11	6	13	8	9	9	13	13	1
		(92.3)	(92.3)	(100)	(76.9)	(69.2)	(84.6)	(46.1)	(100)	(61.5)	(69.2)	(69.2)	(100)	(100)	(7.6)

TE: Tetracycline, D: Doxycycline, EYR: Erythromycin, GEN: Gentamicin, N: Neomycin, CIP: Ciprofloxacin, CN: Cephalexin, CRO: Ceftriaxone, CT: Ceftizoxime, FM: Nitrofurantoin, C: Chloramphenicol, P: Penicillin, AM: Ampicillin, FR: Furazolidone, R: Resistant, S: Susceptible, and I: Intermediate

Table 3: Resistance patterns of the ORT isolates to the different antibiotics

Isolates	Source	Pattern of antibiotic resistance
ORT-1	Broiler	TET, D, ERY, GEN, CIP, CRO, CT, C, P, AM
ORT-2	Broiler	TET, D, ERY, N, CIP, CRO, CT, FM, C, P, AM
ORT-3	Broiler	TET, D, ERY, GEN, CIP, CN, CRO, CT, C, P, AM
ORT-4	Broiler	TET, D, ERY, GEN, N, CIP, CN, CRO, CT, FM, P, AM
ORT-5	Broiler	TET, D, ERY, GEN, N, CIP, CRO, FM, P, AM
ORT-6	Broiler	TET, D, ERY, GEN, N, CRO, FM, C, P, AM
ORT-7	Broiler	TET, D, ERY, GEN, N, CRO, CT, FM, C, P, AM
ORT-8	Broiler breeder	TET, D, ERY, CIP, CRO, C, P, AM
ORT-9	Broiler breeder	TET, D, ERY, GEN, N, CIP, CN, CRO, CT, FM, P, AM
ORT-10	Broiler breeder	TET, D, ERY, GEN, N, CIP, CN, CRO, CT, FM, C, P, AM
ORT-11	Broiler breeder	TET, D, ERY, CIP, CN, CRO, P, AM
ORT-12	Broiler breeder	ERY, GEN, N, CIP, CRO, CN, CT, FM, C, P, AM, FR
ORT-13	Broiler breeder	TET, D, ERY, GEN, N, CIP, CRO, FM, C, P, AM

TE: Tetracycline, D: Doxycycline, EYR: Erythromycin, GEN: Gentamicin, N: Neomycin, CIP: Ciprofloxacin, CN: Cephalexin, CRO: Ceftriaxone, CT: Ceftizoxime, FM: Nitrofurantoin, C: Chloramphenicol, P: Penicillin, AM: Ampicillin, and FR: Furazolidone

Table 4: Distribution of the ERIC-PCR fingerprinting patterns and *pOR1* genes among ORT isolates

Number of isolates	Source	Frequency of ERIC analysis patterns (%)				Presence of *pOR1* related genes	
		C1	C2	C3	C4	*VapD*	*CopA*
ORT-1	Broiler	+	-	-	-	-	-
ORT-2	Broiler	+	-	-	-	-	-
ORT-3	Broiler	+	-	-	-	+	+
ORT-4	Broiler	+	-	-	-	-	-
ORT-5	Broiler	-	-	+	-	+	+
ORT-6	Broiler	+	-	-	-	-	-
ORT-7	Broiler	-	+	-	-	-	-
ORT-8	Broiler breeder	-	-	-	+	-	-
ORT-9	Broiler breeder	-	-	-	+	-	-
ORT-10	Broiler breeder	-	+	-	-	-	-
ORT-11	Broiler breeder	-	+	-	-	-	-
ORT-12	Broiler breeder	-	-	+	-	-	-
ORT-13	Broiler breeder	-	+	-	-	-	-
Total	13	5 (38.4)	4 (30.7)	2 (15.3)	2 (15.3)	2 (15.3)	2 (15.3)

Discussion

ORT is one of the leading causes of respiratory infections in poultry flocks worldwide. The prevalence and spread of *O. rhinotracheale* among poultry and other susceptible birds varies greatly in different studies around the world (Numee *et al*., 2012; Barbosa *et al*., 2020). Proper isolation and identification of these bacteria are necessary for disease control and epidemiological investigations. Some molecular-based methods in identification and detection of ORT will show more accurate and reliable results (Van Empel and Hafez, 1999; Bordoloi *et al*., 2020). In the present study, 16.6% of broiler farms and 25% of broiler breeder farms

were positive for ORT using culture methods that are in accordance with other studies reporting a higher prevalence of this bacterium among broiler breeder and laying hen flocks (Murthy *et al*., 2008; Numee *et al*., 2012). This issue can be due to the longer period of breeding and maintenance in broiler breeder flocks as well as different nutritional and medicinal conditions. In terms of biochemical tests and enzymatic properties, no significant difference was observed between broiler isolates. The broiler breeder isolates and there was also no significant relationship between genotypes and biotypes. The biochemical characteristics of ORT isolates recovered in the present study are similar to further isolates of other regions (Mohd-Zain *et al*., 2008; Szabó *et al*., 2017). In addition, two types of colonies have been found among the isolates according to the colony diameter. Although most isolates (69.2%; 9/13) presented large colonies, 30.7% (4/13) presented small colonies. This is of certain interest, as it was previously reported that small colony variants might harbor increased virulence features (Zahra *et al*., 2013).

One of the most important aspects that should be constantly monitored is antibiotic resistance patterns and the occurrence of MDR strains. Although the use of autogenous vaccines is very common, the application of effective antibiotics is essential, in case of disease outbreaks. In the present study, all isolates proved multidrug resistant, showing mainly resistance to ampicillin, penicillin, ceftriaxone, erythromycin, tetracycline, and doxycycline. Studies of other regions also previously showed hundred percent resistances to amikacin, cloxacillin, gentamicin, metronidazole, cephalexin, erythromycin, neomycin, clindamycin, penicillin, ampicillin, and sulfamethoxazole-trimethoprim. These amounts of antibiotic resistance are not limited to these antibiotics alone, and high resistance is also observed for other antimicrobial agents such as fosfomycin and sultrim (Tsai and Huang, 2006; Asadpour *et al*., 2008; Murthy *et al*., 2008; Mayahi *et al*., 2016; Peña-Vargas *et al*., 2016; Umali *et al*., 2018). In recent years, antibiotic resistances appear to have developed rapidly and widely among ORT isolates, and treatment of *O. rhinotracheale* infections with antibiotics is very difficult due to the variability of susceptibility of the isolates. Therefore, antibiotic resistance status based on the antibiotics used in the field should be continuously monitored in each area.

Although the significance of the hemagglutination ability of ORT isolates is not clear, some bacteria with the hemagglutination ability are more virulent and more likely to cause disease (Tsai and Huang, 2006; Zahra *et al*., 2013). All broiler breeder isolates were positive in the hemagglutination test, but four broiler isolates showed positive results; however, this was not a significant difference. Tsai and Huang (2006) showed that 22 of 28 chicken isolates and none of the pigeon isolates, could agglutinate chicken and pigeon red blood cells. Nevertheless, the role of the hemagglutination ability of ORT strains in regard to virulence needs further investigation.

Determining genetic linkages of different isolates can help track the origin of infections as well as the distribution of certain clusters in geographic areas. This knowledge can contribute to setting more effective disease control measures. ERIC-PCR is known for its discriminatory capacity in regard to ORT isolates. For example, Tachil *et al*. (2007) obtained six different fingerprints from 58 ORT isolates using this method. Furthermore, they reported the detection of distinct fingerprint patterns within each ORT serotype. In a study by Szabo *et al*. (2017), thirteen distinct ERIC patterns were identified among 37 isolates obtained from commercial and backyard birds (Szabó *et al*., 2017). Although the number of isolates was limited in the present investigation, ERIC-PCR presented four different genotypic patterns.

Interestingly, cluster C1 comprised only broiler and cluster C4 only broiler breeder isolates. Clusters C2 and C3 showed isolates derived from broilers and broiler breeders. Previously, the presence of *pORI* plasmid was reported in ORT carrying possible virulence factors, heavy metal resistance genes, and other sequences related to putative proteins. It seems that this plasmid is present in 36% of the isolates, but is related to the origin and genotypic clades of the isolates (Jansen *et al*., 2004; Smith *et al*., 2020). In the present study, only two broiler isolates carried the plasmid and showed the presence of two genes of *pOR1* plasmid.

In conclusion, the present study showed apparent differences in ORT isolates in terms of colony type, biochemical properties, antibiotic resistance profiles, and genotypes. A comprehensive study on isolates from different parts of the country might reveal more aspects of virulence and aid disease prevention strategies.

Acknowledgements

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Conflict of interest

Authors have no conflict of interest.

References

Alispahic, M; Endler, L; Hess, M and Hess, C (2021). *Ornithobacterium rhinotracheale*: MALDI-TOF MS and whole genome sequencing confirm that serotypes K, L and M deviate from well-known reference strains and numerous field isolates. Microorganisms. 9: 1006.

Asadpour, Y; Bozorgmehrifard, MH; Pourbakhsh, SA; Banani, M and Charkhkar, S (2008). Isolation and identification of *Ornithobacterium rhinotracheale* in broiler breeder flocks of Guilan province, north of Iran. Pak. J. Biol. Sci., 11: 1487-1491.

Banani, M (2017). Infection of *Ornithobacterium rhinotracheale* (ORT) and its signs, importance and

pathogenicity in the poultry industry of Iran: a review article. Vet. Res. Biolog. Product., 4: 2-16.

Barbosa, EV; Cardoso, CV; Silva, RDCF; Cerqueira, ADMF; Liberal, MHT and Castro, HC (2020). *Ornithobacterium rhinotracheale*: An update review about an emerging poultry pathogen. Vet. Sci., 7: 3.

Bauer, AW; Kirby, WMM; Sehris, JC and Turck, M (1966): Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol., 36: 493-496.

Bordoloi, S; Nayak, A; Jogi, J; Shakya, P; Rai, A and Sharma, S (2020). *Ornithobacterium rhinotrachalae*: An emerging poultry pathogen. J. Entomol. Zool., 8: 92-97.

Chin, RP; Van Empel, PCM and Hafez, HM (2008). *Ornithobacterium rhinotracheale* infection. In: Saif, YM (Ed.), *Diseases of poultry*. (12th Edn.), UK, Blackwell Publishing. PP: 765-774.

Chin, RP; Van Empel, PCM and Hafez, HM (2013). *Ornithobacterium rhinotracheale* infection. In: Swayne, DE (Ed.), *Diseases of poultry*. (13th Edn.), Hoboken, NJ, USA, Wiley-Blackwell. PP: 807-858.

Hafez, HM and Sting, R (1999). Investigations on different *Ornithobacterium rhinotracheale* "ORT" isolates. Avian. Dis., 43: 1-7.

Jansen, R; Chansiripornchai, N; Gaastra, W and Van Putten, JP (2004). Characterization of plasmid pOR1 from *Ornithobacterium rhinotracheale* and construction of a shuttle plasmid. Appl. Environ. Microbiol., 70: 5853-5858.

Mayahi, M; Gharibi, D; Ghadimipour, R and Talazadeh, F (2016). Isolation, identification and antimicrobial sensitivity of *Ornithobacterium rhinotracheale* in broilers chicken flocks of Khuzestan, Iran. Vet. Res. Forum., 7: 341-346.

Mohd-Zain, ZAINI; Jee, TL and Jusoff, K (2008). Phenotypic characteristics, antibiotic susceptibility and pathogenicity of *Ornithobacterium rhinotracheale*. WSEAS Transact. Biol. Biomed., 7: 133-142.

Murthy, TRGK; Dorairajan, N; Balasubramanium, GA; Dinakaran, AM and Saravanabava, K (2008). *In vitro* antibiotic sensitivity of *Ornithobacterium rhinotracheale* strains isolated from broiler breeder hens in India. Vet. Arh., 78: 49-56.

Numee, S; Hauck, R and Hafez, HM (2012). Detection and typing of *Ornithobacterium rhinotracheale* from German poultry flocks. Avian. Dis., 56: 654-658.

Ozbey, G; Ongor, H; Balik, DT; Celik, V; Kilic, A and Muz, A (2004). Investigations on *Ornithobacterium rhinotracheale* in broiler flocks in Elazig province located in the East of Turkey. Vet. Med., 49: 305-311.

Peña-Vargas, ER; Vega-Sánchez, V; Morales-Erasto, V; Trujillo-Ruíz, HH; Talavera-Rojas, M and Soriano-Vargas, E (2016). Serotyping, genotyping, and antimicrobial susceptibility of *Ornithobacterium rhinotracheale* isolates from Mexico. Avian Dis., 60: 669-672.

Sakai, E; Tokuyama, Y; Nonaka, F; Ohishi, S; Ishikawa, Y; Tanaka, M and Taneno, A (2000). *Ornithobacterium rhinotracheale* infection in Japan: preliminary investigations. Vet. Rec., 146: 502-503.

Smith, EA; Miller, EA; Weber, BP; Munoz Aguayo, J; Flores Figueroa, C; Huisinga, J; Nezworski, J; Kromm, M; Wileman, B; Johnson, TJ and Johnson, TJ (2020). Genomic landscape of *Ornithobacterium rhinotracheale* in commercial turkey production in the United States. Appl. Environ. Microbiol., 86: e02874-19.

Szabó, R; Wehmann, E; Makrai, L; Nemes, C; Gyuris, É; Thuma, Á and Magyar, T (2017). Characterization of *Ornithobacterium rhinotracheale* field isolates from Hungary. Avian Pathol., 46: 506-514.

Tsai, HJ and Huang, CW (2006). Phenotypic and molecular characterization of isolates of *Ornithobacterium rhinotracheale* from chickens and pigeons in Taiwan. Avian. Dis., 50: 502-507.

Umali, DV; Shirota, K; Sasai, K and Katoh, H (2018). Characterization of *Ornithobacterium rhinotracheale* from commercial layer chickens in eastern Japan. Poult. Sci., 97: 24-29.

Van Empel, PCM and Hafez, HM (1999). *Ornithobacterium rhinotracheale*: a review. Avian Pathol., 28: 217-227.

Van Empel, P; Vrijenhoek, M; Goovaerts, D and van den Bosch, H (1999). Immunohistochemical and serological investigation of experimental *Ornithobacterium rhinotracheale* infection in chickens. Avian Pathol., 28: 187-193.

Wikler, MA (2017). Performance standards for antimicrobial susceptibility testing: 27th Information Supplement. Wayne, USA: Clinical and Laboratory Standards Institute; M100-S26. PP: 1-249.

Zahra, M; Ferreri, M; Alkasir, R; Yin, J; Han, B and Su, J (2013). Isolation and characterization of small-colony variants of *Ornithobacterium rhinotracheale*. J. Clin. Microbiol., 51: 3228-3236.