Molecular characterization of Ornithobacterium rhinotracheale *strains isolated from chickens in the Northwestern Iran*

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RAPD assay.

Summary
Ornithobacterium rhinotracheale is the etiological agent of chickens and turkeys' respiratory diseases, reduction of eggs, growth retardation, and death. Present research aimed to conduct the isolation, recognition, and molecular investigations of the bacterium in commercial broiler chicken flocks in East Azerbaijan province, Northwest of Iran, by the partial sequencing of 16S rRNA gene, ERIC-PCR, and RAPD-PCR with the OPG11 and M13 primers. We obtained 330 specimens from tracheal swabs of 33 slaughtered broiler flocks, of which we found 14 isolates (4.24%) of five flocks (15.15%) to be *O. rhinotracheale*. Typing by RAPD assay with the OPG11 primer, and ERIC-PCR, classified the isolates in two types of 1 and 2 molecular patterns, most of which belonged to type 1. However, the M13 primer-based RAPD technique was inappropriate for distinguishing and categorizing the isolates and generating all of them in the same pattern. In a phylogenetic analysis of *O. rhinotracheale* based on 16S rRNA sequences, the strains generated three clusters (I-III), in which all of the studied isolates fell in one cluster (cluster I). Based on the results obtained from the RAPD and ERIC-PCR assays, the genetic patterns of broiler-chicken-isolated *O. rhinotracheale* strains in Northwestern Iran had no significant differences.

Introduction

Ornithobacterium rhinotracheale is a gram-negative, rod-shaped, pleomorphic bacterium that causes respiratory distress, growth retardation, death, and drops in egg production among chickens and turkeys (Markey *et al.* 2013). *O. rhinotracheale* has been recently isolated in the USA, France, Mexico, the Netherlands, Germany, Belgium, Spain, Brazil, South Africa, Taiwan, Hungary, Korea, United Kingdom, Turkey, and other countries (Van Empel *et al.* 1997, Sakai *et al.* 2000, Canal *et al.* 2005, Tsai and Huang 2006). In Iran, Banani and colleagues (Banani *et al.* 2000) first reported *O. rhinotracheale* infection. The infection was found in several avian hosts, such as turkey, chicken, duck, goose, ostrich, gull, quail, partridge, pigeon, pheasant, guinea fowl, and rook (Hafez 2002).

Clinical symptoms and mortality rate of *O. rhinotracheale* infections can be affected by the virulence and the pathogenicity of the causative isolate as well as by several environmental factors, including improper control, inappropriate ventilation, high amounts of ammonia, elevated stocking densities, high temperatures and humidity, and co-infections with other respiratory pathogens, like *Bordetella avium*, Newcastle disease virus, infectious bronchitis virus, *Escherichia coli, Chlamydophila psittaci*, or *Mycoplasma synoviae* (Van Empel and Hafez 1999, Schuijffel *et al.* 2005).

Several epidemiologists considering the global spread of *O. rhinotracheale* (Van Empel and Hafez 1999), have tried various typing techniques to characterize *O. rhinotracheale* strains. The serological typing of *O. rhinotracheale* strains via agar gel precipitation (AGP) method has been widely used for its simplicity and performability. (Van Empel *et al.* 1997). Eighteen different *O. rhinotracheale* serotypes (A-R) have been identified. Ninetyfive percent of chicken isolates and more than 50% of turkey isolates belong to serotype A (Van Empel *et al.* 1997, Numee *et al.* 2012). However, AGP is more subjective and inefficient regarding the subgrouping *O. rhinotracheale* field isolates due to the unavailability of the antisera and cross-reactions

among serotypes of *O. rhinotracheale* (Van Empel *et al.* 1997).

Phenotypic and genotypic markers are necessary for epidemiological typing investigations, and several methodological techniques have been developed (Chaslus-Dancla *et al.* 1996). The 16S rRNA gene sequencing is applied to differentiate the bacteria at the genus level over the main phylum. A high similarity rate of 98-100% is reported in *O. rhinotracheale* strains for the 16S rRNA gene (Amonsin *et al.* 1997, Tsai and Huang 2006). Multifarious fragment-based typing techniques, such as enterobacterial repetitive intergenic consensus (ERIC) sequences, and random amplified polymorphic DNA (RAPD) assays are used to differentiate *O. rhinotracheale* strains at subspecies or strain levels (Leroy-Setrin *et al.* 1998, Olive and Bean 1999). Several studies on veterinary strains successfully employed these techniques (Leroy-Setrin *et al.* 1995, Chaslus-Dancla *et al.* 1996).

The current research aimed at isolation, identification, and molecular epidemiologic characterization of the organism in commercial broiler chicken flocks in East Azerbaijan province, Northwest of Iran, by RAPD assay, the partial 16S rRNA gene sequencing, and ERIC-PCR.

Materials and methods

Isolates identification

In the present study, we analyzed a total of 330 tracheal swab specimens randomly obtained from 33 flocks of broiler chicken, with/without respiratory symptoms, slaughtered in abattoirs of East Azerbaijan, Northwest of Iran, from April to September 2019. The specimens were sent to the Department of Research and Development of Razi Vaccine and Serum Research Institute (Northwest branch, Marand, Iran) in test tubes containing the Cary-Blair transport medium (Becton-Dickinson, USA) placed in sterile ice-filled containers to prevent swabs drying out and preserve the bacteria. Serotype-specific *O. rhinotracheale* strain ORT-R87-7 (serotype: A, accession number: JF810491) was prepared from the microbial culture collection of the Department of Avian Diseases Research and Diagnosis, Razi Vaccine and Serum Research Institute (Karaj, Iran), and was included in the analysis. They were streaked on 5% sheep blood agar (Merck, Germany) using gentamicin (5 µg/mL). Plates were incubated at 37 ˚C with 7.5% CO_2 in a moist chamber for 24-48 hours (Joubert *et al.* 1999). Circular, pinpoint was chosen along with opaque to gray, non-hemolytic colonies, and 1-3 mm diameter. Those with *O. rhinotracheale* characteristics were stained with gram staining followed by the identification for confirming the major *O. rhinotracheale* features (biochemically) (Joubert *et al.* 1999) and via polymerase chain reaction (PCR, genetically). Also, we conducted biochemical tests, including oxidase, catalase, growth on MacConkey agar (Merck, Germany), synthesis of H_2S in triple sugar iron (TSI) agar, indole generation, and urease, as well as nitrate reduction and motility. Carbohydrate fermentation ability was tested on the bacteria using sucrose, sorbitol, lactose, glucose, arabinose, and maltose (Van Empel *et al.* 1997, Tsai and Huang 2006). Pure cultures were preserved in skimmed milk at - 70 ˚C until use.

DNA extraction

DNA was extracted on colonies suspended in sterile distilled water (300 µL) followed by heating (100 ˚C/5 min) and centrifugation (15 min/13,000 rpm). The supernatant (150 µL) was collected and stored at - 20 ˚C for the next applications.

PCR

Primers were designed according to Van Empel and Hafez (Empel and Hafez 1999). Primers OR 16S-F1 (5´-GAGAATTAATTTACGGATTAAG-3´) and OR 16S-R1 (5´-TTCGCTTGGTCTCCGAAGAT-3´) were utilized to amplify a 784-base pair (bp) segment on the 16S rRNA gene of *O. rhinotracheale*. The mastercycler gradient thermocycler was used to perform PCR (Eppendorf AG) in a total volume of 25 µL, including 10 mM dNTP mix 0.50 µL,1.25 U Taq DNA polymerase 0.50 µL, 25 mM $MgCl_2$ 1.50 µL, 10 pM primers 1.50 µL, 2.50 µL PCR buffer 10X, and 50 ng DNA template 5 µL. The reagents were purchased from SinaClon Bioscience Co. (Tehran, Iran). The PCR program for amplification was as follows: initial denaturation at 95 ˚C for 5 minutes followed by 30 cycles of denaturation at 94 ˚C for 30 seconds, annealing at 53 ˚C for 1 minute, extension at 72 ˚C for 2 minutes, and the final extension at 72 ˚C for 5 minutes. PCR products (8 µL) were separated by agarose gel electrophoresis (85 volts within 45 min) on 1% agarose gel stained with safe stain (0.50 µg/mL) (SinaClon Bioscience Co., Tehran, Iran). UV transillumination was used to visualize DNA fragments (Kodak, New York, USA), and the visualized bands were detected in comparison with a 100-bp DNA ladder.

ERIC-PCR assay

Making some modifications, the ERIC-PCR was carried out based on the Ryll and colleagues (Ryll *et al.* 1996) and Thachil and colleagues (Thachil *et al.* 2007) methods. The oligonucleotides ERIC 1R (5´-ATGTAAGCTCCTGGGGATTCAC-3´) was applied for amplifying 50 ng of purified *O. rhinotracheale* genomic DNA regarding every ERIC-PCR

fingerprinting reaction. The 20-µL reaction mixture was prepared in PCR buffer (AccuPower PCR PreMix, Bioneer, South Korea) and contained 10 mM Tris-HCl (pH 9.0), 10 pM of primer (SinaClon Bioscience Co., Tehran, Iran), 1 U of Top DNA polymerase, 1.5 mM $MgCl_2$, 250 µM each dATP, dCTP, dGTP, and dTTP, and 30 mM KCl. The thermal cycler was used to perform reactions. The following PCR conditions were considered: the initial denaturation at 95 ˚C for 10 minutes followed by 35 cycles of denaturation at 94 ˚C for 45 seconds, annealing at 40 ˚C for 3 minutes, extension at 72 ˚C for 4 minutes, and the final extension at 72 ˚C for 10 minutes. We electrophoresed (75 volts for 120 min) 8 µL of PCR products in 2% agarose gel using 0.50 µg of safe stain per mL. DNA segments were detected using a 1,000-bp DNA ladder. For confirming the technique reproducibility, three individual reactions were performed per isolate, and all reactions provided the identical ERIC-PCR fingerprint model.

RAPD assay with M13 Primer

In this technique, PCR was done for bacterial genomic DNA (50 ng) using a Universal M13 primer (5´-TTATGTAAAACGACGGCCAGT-3´) (Sander *et al.* 1998, Thachil *et al.* 2007). All testing procedures were similar to the ERIC-PCR assay.

RAPD assay with OPG11 Primer

The OPG11 primer (5´-TGCCCGTCGT-3´) was applied based on the Leroy-Setrin and colleagues (Leroy-Setrin *et al.* 1998). PCR program was described elsewhere in fingerprinting assays. Amplifications in the used thermocycler were as follows: the initial cycle at 92 ˚C for 1 min, 35 ˚C for 30 seconds, and 72 ˚C for 90 seconds, followed by 39 cycles at 94 ˚C for 40 seconds, 35 ˚C for 30 seconds, 72 ˚C for 90 seconds, and the final extension at 72 ˚C for 10 minutes.

The 16S rRNA gene sequencing

Partial sequences of the 16S rRNA gene were assessed according to Amonsin and colleagues (Amonsin *et al.* 1997) with minor corrections. The 16S rRNA gene was amplified from *O. rhinotracheale* genomic DNA through primers of nucleotide positions 8 to 27 (5´-AGAGTTTGATCCTGGCTCAG-3´) and the reverse-complement of nucleotide positions 1467 to 1485 (5´-GGTTACCTTGTTACGACTT-3´). The 50-µL reaction mixture included 100 ng of genomic DNA, PCR buffer (AccuPower PCR PreMix, Bioneer, South Korea), 10 mM Tris-HCl (pH 9.0), 20 pM each primer (SinaClon Bioscience Co., Tehran, Iran), 1.5 mM $MgCl_2$, 250 µM each dATP, dCTP, dGTP, and dTTP, 2.5 U of Top DNA polymerase, and 30 mM KCl. Using a thermal cycler, mixtures were amplified based on the following program: the initial denaturation at 95 ˚C for 2 minutes, followed by 35 cycles of denaturation at 94 ˚C for 30 seconds, annealing at 50 ˚C for 1 minute, extension at 72 ˚C for 2 minutes, and the final extension at 72 ˚C for 7 minutes. Next, 7 µL of PCR products was electrophoresed (80 volts for 120 min) in 1.5% agarose gel. A comparison was made between DNA fragments and a 100-bp DNA ladder (Figure 4). PCR products were sequenced in two sides using an automatic sequencer via a commercially available sequencing facility (FAZA Biotech Co., Tehran, Iran). The obtained sequences (13 sequences) were compared with other 39 sequences available on GenBank. The BioEdit Sequence Alignment Editor Software version 7.2 was employed to edit, analyze, and align nucleotide sequences (Hall 1999). The phylogenetic tree was built from the 52 16S rRNA nucleotide sequences (nucleotides 780-1404 according to the nucleotide positions of DSM 15997, accession number: NR102940) by the maximum likelihood method and model of maximum composite likelihood in MEGA software version 7.0 (Tamura *et al.* 2013). The robustness of the phylogenetic tree was evaluated with 1,000 replicates and displayed as a percentage value on the branch nodes.

Analysis of ERIC-PCR and RAPD assay findings

The existence or lack of bands was assessed for amplification to generate a binary matrix. The genetic similarity of *O. rhinotracheale* strains was estimated by the Dice coefficient, and a dendrogram was depicted using the unweighted pair group approach by arithmetic mean (UPGMA). The NTSYS-pc 2.02 (Numerical Taxonomy and Multivariate Analysis System) was utilized to assess the robustness of the dendrogram topology using 500 bootstrap replicates (Rohlf 1997).

Accession numbers of nucleotide sequences

The GenBank accession numbers for sequences were MN900654, MN960065, MN960068, MN960070, MN960081, MN960082, MN960083, MN960087, MN960088, MN960089, MN960091, MN960092 and MN960093.

Results

Identification

The findings of this study indicated that 14 isolates of tracheal swabs related to five flocks, including the ORT-R87-7 strain, were identified as *O. rhinotracheale*.

Pinpoint, grey to grey/white colonies grew to their maximum sizes 48 hours after culturing on blood agar. Non-hemolytic colonies found on blood agar indicated that the bacteria are non-motile and do not grow on MacConkey and TSI agar. Gram-negative, pleomorphic, rod-shaped bacteria were identified by Gram staining. The bacteria were negative for urease, catalase, indole, and gelatinase but positive for oxidase and fermented glucose, sucrose, lactose, maltose, and arabinose but not sorbitol (data not shown). The PCR results confirmed biochemical findings, and the isolates biochemically identified as *O. rhinotracheale* were also confirmed by PCR based on a 784-bp amplified fragment related to the estimated length. No amplified yields were produced by the negative control. In this research, the samples infected with *O. rhinotracheale* belonged to Tabriz (5 isolates, 35.71%), Khosrowshahr (4 isolates, 28.57%), Ajabsheer (3 isolates, 21.42%) and Soofian (2 isolates, 14.28%) cities in Northwest of Iran. Table I represents the origins and geographic areas of the used *O. rhinotracheale* isolates.

ERIC-PCR

We found two obvious ERIC patterns. The commonest model (type 1) included 13 isolates (92.85%) (Table I, Figure 1). All ERIC type 1 isolates showed approximately seven bands between 340 and 2,400 bp, with 700 and 2,400 bp were the most important that can show that these bands are available in the genome in over than a copy or that the DNA fragments are slightly different regarding their nucleotides slightly. ERIC type 2 isolate (1 isolate, 7.15%) showed approximately two bands at 700 and 2,050 bp. There was no alteration in the DNA

Table I. Ornithobacterium rhinotracheale *strains isolated and used in this study.*

Isolation				Type			
Isolate number	Year of isolation	Geographic origin	Source	ERIC	M13	OPG11	16S rRNA cluster
ORT-R98-1	2019	Iran/East Azarbaijan province/Tabriz	Chicken, trachea	1	1	1	1
ORT-R98-2	2019	Iran/East Azarbaijan province/Khosrowshahr	Chicken, trachea	1	1	1	-[a]
ORT-R98-3	2019	Iran/East Azarbaijan province/Tabriz	Chicken, trachea	1	1	1	1
ORT-R98-4	2019	Iran/East Azarbaijan province/Soofian	Chicken, trachea	1	1	1	1
ORT-R98-5	2019	Iran/East Azarbaijan province/Soofian	Chicken, trachea	1	1	1	1
ORT-R98-6	2019	Iran/East Azarbaijan province/Ajabsheer	Chicken, trachea	1	1	1	1
ORT-R98-7	2019	Iran/East Azarbaijan province/Ajabsheer	Chicken, trachea	1	1	1	1
ORT-R98-8	2019	Iran/East Azarbaijan province/Tabriz	Chicken, trachea	1	1	1	1
ORT-R98-9	2019	Iran/East Azarbaijan province/Khosrowshahr	Chicken, trachea	2	1	2	1
ORT-R98-10	2019	Iran/East Azarbaijan province/Khosrowshahr	Chicken, trachea	1	1	1	1
ORT-R98-11	2019	Iran/East Azarbaijan province/Tabriz	Chicken, trachea	1	1	1	1
ORT-R98-12	2019	Iran/East Azarbaijan province/Khosrowshahr	Chicken, trachea	1	1	1	1
ORT-R98-13	2019	Iran/East Azarbaijan province/Tabriz	Chicken, trachea	1	1	1	1
ORT-R98-14	2019	Iran/East Azarbaijan province/Ajabsheer	Chicken, trachea	1	1	1	1
ORT-R87-7	2009	Iran/Guilan province/NI[b]	Chicken, NI[b]	1	1	1	1

[a]No sequencing and analysis; [b]NI = No information available.

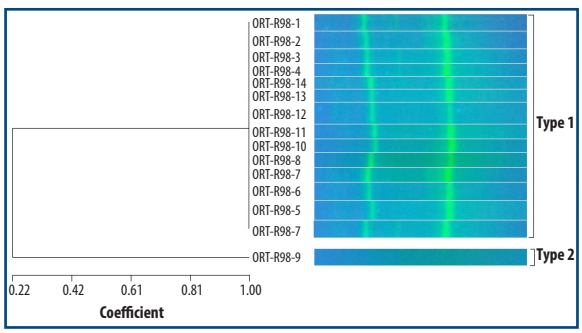

Figure 1. *Dendrogram grouping the studied* O. rhinotracheale *isolates based on the ERIC-PCR results using the unweighted pair group method with the arithmetic average method (UPGMA).* All the isolates were apparently grouped into two genetic types (1 and 2).

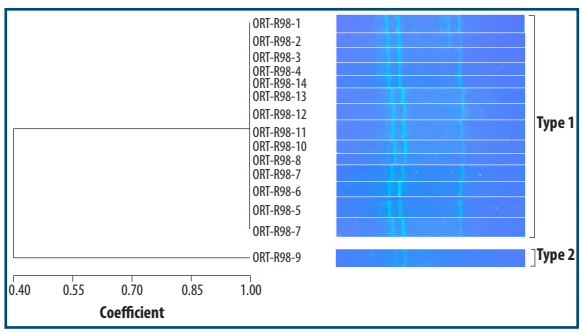

Figure 2. *Dendrogram grouping the studied* O. rhinotracheale *isolates based on RAPD assay with the OPG11 primer using the unweighted pair group method with the arithmetic average method (UPGMA).* All the isolates were apparently grouped into two genetic types (1 and 2).

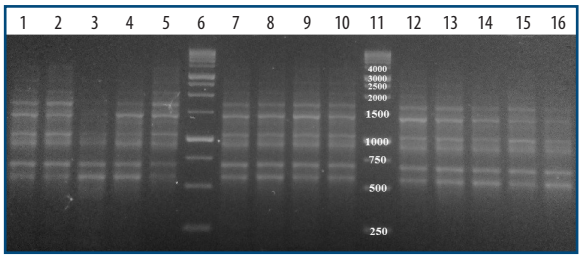

Figure 3. *Results of M13 primer-based RAPD analysis of* 0. rhinotracheale *isolates in 2% agarose gel.* Lanes 1-5, 7-9 and 12-16, studied *O. rhinotracheale* isolates; Lane 10, the ORT-R87-7 strain (accession number: JF810491); Lanes 6 and 11, 1000-bp DNA ladder. This technique grouped all of the isolates in the same genetic pattern.

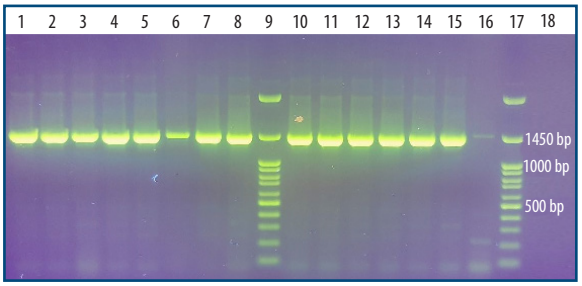

Figure 4. *Electrophoresis of PCR products on 1.5% agarose gel stained with safe stain.* Lanes 9 and 17, 100-bp DNA ladder; Lanes 1-8, 10-15, *O. rhinotracheale* specific 1450 bp band; Lane 16, the ORT-R87-7 strain (accession number: JF810491); Lane 18: negative control.

fingerprints in three individual experiments (data not shown). The ERIC-PCR technique used identified most of the strains (13/14) of *O. rhinotracheale* isolated from different geographic areas of East Azerbaijan province in Northwest of Iran. They have the same genetic model, same as that of ORT-R87-7.

RAPD assays using OPG11 and M13 primers

The tested isolates were categorized into two main profiles based on the results of PCR via the OPG11 primer. The most common profile (type 1) included 13 isolates (92.85%) (Table I, Figure 2). All RAPD type 1 isolates showed approximately five bands between 580 and 1,770 bp, with the most prominent 580, 680, and 1,770 bp. RAPD type 2 isolate (1 isolate, 7.15%) showed approximately two bands at 580 and 680 bp.

The RAPD test using the M13 primer determined all studied *O. rhinotracheale* isolates (14/14) to the same genetic pattern (type 1) (Table I, Figure 3). All isolates showed approximately 10 bands between 500 and 3,900 bp.

16S rRNA gene sequence analysis

In the current study, we used the Clustal W technique

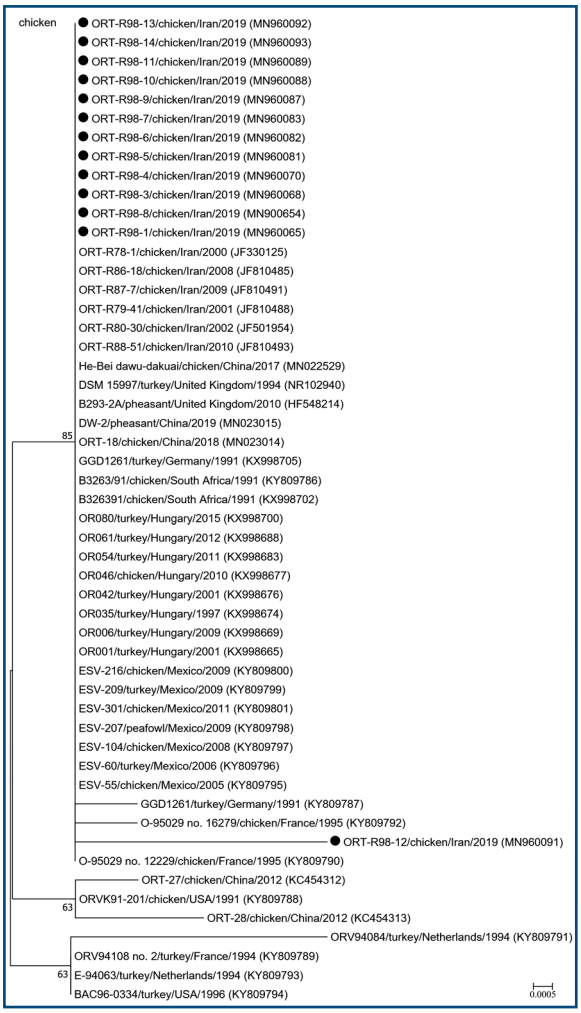

Figure 5. *Phylogenetic tree constructed from the partial 16S rRNA nucleotide sequences of 13* 0. rhinotracheale *(nucleotides 780-1404 based on the nucleotide positions of DSM 15997, accession number: NR102940) studied isolates, the ORT-R87-7 strain (accession number: JF810491), and the 38 sequences obtained from GenBank.* The tree was generated by MEGA7 software using the maximum likelihood method. The isolates that were analyzed are marked with filled circles.

via the BioEdit software to assess the 16S rRNA nucleotide sequences of 13 *O. rhinotracheale* strains isolated from broiler chickens compared to the ORT-R87-7 strain and further 38 sequences available on GenBank, and the results confirmed a 99.3-100% similarity. The 16S rRNA sequence of 52 ORT strains is categorized as three genetic clusters (I–III), based on the obtained phylogenetic tree. All the studied strains, the ORT-R87-7 strain, and 31 other sequences (isolated from chicken, turkey, pheasant, and peafowl) were achieved by GenBank fell in cluster I; however, the other seven isolates fell in clusters II and III (Table I, Figure 5).

Discussion

O. rhinotracheale can co-infect birds and other

respiratory pathogens; thus, the colonies of *O. rhinotracheale* were almost masked by other strong bacteria due to its low growth rate and the small size of colonies. It is difficult to isolate *O. rhinotracheale* from biological samples since enriched media, such as blood agar, is necessary in a microaerophilic environment (5-10% CO_2) and biochemical recognition (Van Empel *et al.* 1997). Our isolation frequency in chickens was higher than its frequency announced by Erganis and colleagues (Erganis *et al.* 2002) (0.4%) and Ozbey and colleagues (Ozbey *et al.* 2004) (1.5%) in Turkey, and Hassanzadeh and colleagues (Hassanzadeh *et al.* 2010) (0.6%) and Asadpour and colleagues (Asadpour *et al.* 2011) (1.03%) in Iran; however, little has been reported by Chansiripornchai and colleagues (Chansiripornchai *et al.* 2007) in Thailand (63%), Canal and colleagues (Canal *et al.* 2003) in Brazil (63.83%), Turan and Ak (Turan and Ak 2002) in Turkey (64.4%), and Allymehr (Allymehr 2006) (82%) in Iran. The recently reported high rate of bacterial isolation from chickens might be attributed to screening samples for the presence of *O. rhinotracheale* by ELISA. On the other hand, in cultural technique, the chance of *O. rhinotracheale* isolation is higher in the first phases of infection, whereas its recovery at the next phases has almost not occurred. Also, in contaminated specimens, *O. rhinotracheale* is hidden simply via the overgrowth of other bacteria leading to undetectable, ordinary assessments (Hafez 2002).

For the first time, we analyzed the ERIC of *O. rhinotracheale* isolates in Iran. The effectiveness of ERIC-PCR for typing *O. rhinotracheale* has not been widely considered. Amonsin and colleagues (Amonsin *et al.* 1997) investigated 55 avian strains in many countries and discovered seven genotypes, of which genotype A was dominant in 60%. They announced that the strains of chickens and turkeys could not be distinguished by ERIC-PCR; however, the ERIC fingerprints of wild bird isolates were distinguishable from poultry isolates. Using ERIC-PCR, Koga and Zavaleta (Koga and Zavaleta 2005) in Peru found a similar pattern in 25 *O. rhinotracheale* strains isolated from chickens. They concluded that genotype A is possibly the most typical genotype circulating in different birds all over the world. Also, Thachil and colleagues (Thachil *et al.* 2007) evaluated 58 *O. rhinotracheale* strains (50 fields and eight references), using the ERIC 1R primer for genetic variations and 58 *O. rhinotracheale* strains belonged to eight fingerprinted serotypes, six different fingerprints were achieved by ERIC 1R fingerprinting.

Similarly, Szabo and colleagues (Szabo *et al.* 2017) analyzed 37 *O. rhinotracheale* field strains in Hungary by ERIC-PCR, identifying 13 distinct patterns with this technique. Accordingly, the ERIC pattern had no correlations with the isolation time and serotype. A higher heterogeneity was found in chicken-isolated strains based on ERIC-PCR compared to those obtained from turkeys. They reported ERIC-PCR as the best technique to discriminate *O. rhinotracheale* strains in terms of genetic variation. According to the present study, the isolates were distinguished into two types 1 and 2 genetic patterns, most of which were type 1. In agreement with Amonsin and colleagues (Amonsin *et al.* 1997) and Koga and Zavaleta (Koga and Zavaleta 2005), most *O. rhinotracheale* isolates belong to genotype A and circulate in diverse types of birds. Our findings also show that most of the studied isolates from commercial poultry in the northwest of Iran are genetically similar and dependent on a unique genetic profile.

RAPD-PCR technique is a simple and applicable method to characterize *O. rhinotracheale* providing a good level of discrimination. For the first time in Iran, RAPD was applied to determine genetic heterogeneity of *O. rhinotracheale* strains collected from chickens. Typing by RAPD assays with the OPG11 primer classified the isolates into two molecular patterns: types 1 and 2. Most of which the isolates belonged to type 1; however, fingerprinting by M13 primer-based RAPD technique, clustered all the isolates in the same pattern. In a study by Leroy-Setrin and colleagues (Leroy-Setrin *et al.* 1998) in France, 23 *O. rhinotracheale* strains with different origins were evaluated using three methods: ribotyping, plasmid profiles, and RAPD. They evaluated eight various RAPD primers and found the highest discriminatory potential with OPG11 and OPG19. They also categorized the strains into three clusters by combining RAPD and ribotyping, though they found no correlations in the origin of isolates. RAPD showed the highest discriminatory potential power compared with two others regarding the subtyping of *O. rhinotracheale* strains. Similarly, in Turkey, Ozbey and colleagues (Ozbey *et al.* 2005) used the RAPD assay with the OPG11 primer and reported a great genetic variation in chicken-isolated *O. rhinotracheale* strains.

In this regard, Thachil and colleagues (Thachil *et al.* 2007) stated that out of 58 isolates of *O. rhinotracheale*, which belonged to eight serotypes fingerprinted with M13 primer-based RAPD assay and ERIC-PCR technique, 10 different molecular models were achieved by M13 fingerprinting, whereas six models were achieved by ERIC 1R fingerprinting. Also, Chou and colleagues (Chou *et al.* 2009) studied 93 Taiwanese *O. rhinotracheale* strains from various origins via RAPD with the OPG11 primer and single-enzyme amplified fragment length polymorphism (SE-AFLP) method. They concluded that RAPD and SE-AFLP had high discrimination and classified all of the *O. rhinotracheale* strains into seven and four genetic clusters based on 60%

genetic similarity, respectively. In addition, Erganis and colleagues (Erganis *et al.* 2013) analyzed 30 Turkish *O. rhinotracheale* isolates from poultry and turkeys by RAPD assay (with OPG11 primer), and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) technique and fingerprinted the isolates into eight genetic profiles and 19 protein profiles, respectively. In Hungary, Szabo and colleagues (Szabo *et al.* 2017) assessed 37 *O. rhinotracheale* field strains via the RAPD-PCR method with OPG11, OPH19, and M13 primers. The results showed that the utilized method could assign isolates into 10 various patterns via the M13 primer, but two other RAPD methods showed no effectiveness in distinguishing and categorizing the isolates. There was no correlation of the RAPD pattern with the time and site of isolation. Our results indicated that in *O. rhinotracheale* strains fingerprinting, the ERIC 1R and OPG11 primers were more useful than the M13 and distinguished the isolates into two different genetic patterns.

The fingerprints originated from ERIC-PCR, and the OPG11 primer-based RAPD assay coincided entirely, and all isolates belonged to one ERIC profile (type 1) as indicated by the RAPD pattern (type 1). Only one isolate (ORT-R98-9) with a specific ERIC pattern (type 2) belongs to a unique RAPD type (type 2). In contrast, the M13 primer-based RAPD technique fingerprinted all of the tested isolates in the same molecular pattern (type 1). In general, most *O. rhinotracheale* strains isolated from chickens in Northwestern Iran were genetically similar and had a unique pattern.

All the isolated *O. rhinotracheale* strains were genetically similar to the bacterial sequences available on GenBank (similarity: 99.3%-100%), based on the 16S rRNA sequence analysis. In other words, *O. rhinotracheale* strains with various origins and hosts have a similar 16S rRNA sequence (Amonsin *et al.* 1997, Van Empel and Hafez 1999, Tsai and Huang 2006, Szabo *et al.* 2017). Accordingly, the 16S rRNA sequencing did not have a high potential to genetically discriminate *O. rhinotracheale* subtypes. In a study, a defined cluster was obtained from strains isolated from Taiwanese pigeon, whereas sequences of 16S rRNA from strains isolated from chicken and others available in GenBank belonged to two different clusters using a shorter fragment of the 16S rRNA gene (Tsai and Huang 2006). The strains isolated from turkey and pigeon in Iran were categorized into a defined cluster (e.g., Taiwanese pigeon isolates). In contrast, isolates collected from quail and partridge of Iran were found to have a close relationship with chicken native isolates and also some foreign isolates from other areas in the GenBank (Mirzaie and Hassanzadeh 2013). Szabo and colleagues (Szabo *et al.* 2017), in a phylogenetic study indicated that the 16S rRNA sequence analysis could not differentiate bacterial strains isolated from various hosts and generated two clusters. In our research, the 16S rRNA sequence analysis was not able to differentiate the tested into isolates distinct clusters and placed all in one cluster (cluster I).

The 16S rRNA analysis findings showed no complete corresponding to those obtained by RAPD or ERIC-PCR assays. The isolate slightly different from the remaining field isolates in their 16S rDNA sequence (ORT-R98-12) belonged to the dominant ERIC or RAPD pattern (type 1). Using the 16S rRNA sequence analysis, however, the isolate that had a unique ERIC or OPG11 primer-based RAPD profile (type 2) (ORT-R98-9) fells in the largest genetic cluster (cluster I). Accordingly, all three techniques can recognize the distinct types.

It has shown that the limited heterogeneity in a bunch of samples can be attributed to the collection of *O. rhinotracheale* isolates from a wide geographical region, consisting of the closely-related clones during a long period of time (Amonsin *et al.* 1997, Chou *et al.* 2009, Thieme *et al.* 2016, Szabo *et al.* 2017). Although the number of isolates was small and the geographical region was limited, this theory was supported by the ERIC-PCR and RAPD assays.

In summary, in the current study, the 16S rRNA sequence analysis, the ERIC-PCR and RAPD assays with different primers were performed for comparing the genetic variation among the *O. rhinotracheale* strains, for the first time in Iran. Both RAPD and ERIC-PCR assay were highly precise techniques for detecting intraspecies diversity in *O. rhinotracheale* strains, which can be used for the epidemiological investigations of the bacteria. No differences were found among the genetic patterns of most *O. rhinotracheale* strains isolated from broiler chickens in Northwestern Iran, based on the results of RAPD and ERIC-PCR assay. The high genetic similarity among tested isolates and homology between the used isolates and those from other areas from different birds demonstrated that vaccination is an appropriate technique to manage this disease in commercial flocks.

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References

Allymehr M. 2006. Seroprevalence of *Ornithobacterium rhinotracheale* infection in broiler and broiler breeder chickens in West Azerbaijan province, Iran. *J Vet Med A Physiol Pathol Clin Med*, **53** (1), 40-42.

Amonsin A., Wellehan J.F., Li L.L., Vandamme P., Lindemann C., Edman M., Robinson R. & Kapur V. 1997. Molecular epidemiology of *Ornithobacterium rhinotracheale. J Clin Microbiol*, **35**, 2894-2898.

Asadpour Y., Banani M. & Pourbakhsh S.A. 2011. Isolation, identification and antibiotic sensitivity determination of *Ornithobacterium rhinotracheale* in slaughtering broiler chicken flocks of Guilan province. *Iranian J Vet Res*, **12**, 345-349.

Banani M., Khaki P., Goodarzi H., Vandyousefi J. & Pourbakhsh S.A. 2000. Isolation and identification of *Ornithobacterium rhinotracheale* from a broiler and a pullet flock. *Pajouhesh and Sazandegi*, **46**, 106-109. [In Persian].

Canal C.W., Leão J.A., Ferreira D.J., Macagnan M., Pippi Salle C.T. & Back A. 2003. Prevalence of antibodies against *Ornithobacterium rhinotracheale* in broilers and breeders in southern Brazil. *Avian Dis*, **47**, 731-737.

Canal C.W., Leao J.A., Rocha S.L., Macagnan M., Lima-Rosa C.A., Oliveira S.D. & Back A. 2005. Isolation and characterization of *Ornithobacterium rhinotracheale* from chickens in Brazil. *Res Vet Sci*, **78**, 225-230.

Chansiripornchai N., Wanasawaeng W. & Sasipreeyajan J. 2007. Seroprevalence and identification of *Ornithobacterium rhinotracheale* from broiler and broiler breeder flocks in Thailand. *Avian Dis*, **51**, 777-780.

Chaslus-Dancla E., Lesage-Descauses M.C., Leroy-Setrin S., Martel J.L., Coudert P. & Lafont J.P. 1996. Validation of random amplified polymorphic DNA assays by ribotyping as tools for epidemiological surveys of *Pasteurella* from animals. *Vet Microbiol*, **52**, 91-102.

Chou C.H., Lin S.Y., Chen C.L. & Tsai H.J. 2009. Use of random amplified polymorphic DNA analysis and single-enzyme amplified fragment length polymorphism in molecular typing of *Ornithobacterium rhinotracheale* strains. *Avian Dis*, **53**, 108-114.

Erganis O., Hadimli H.H., Kav K., Çorlu M. & Ozturk D. 2002. A comparative study on detection of *Ornithobacterium rhinotracheale* antibodies in meat type turkeys by dot immunobinding assay, rapid agglutination test and serum agglutination test. *Avian Pathol*, **31**, 201-204.

Erganis O., Hadimli H.H., Kav K. & Sayin Z. 2013. Phenotypic and molecular characterization of strains of *Ornithobacterium rhinotracheale* isolated from poultry in Turkey. *African J Microbiol Res*, **7**, 82-88.

Hafez H. 2002. Diagnosis of *Ornithobacterium rhinotracheale*. *Int J Poultry Sci*, **1**, 114-118.

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95-98.

Hassanzadeh M., Karimi V., Fallah N. & Ashrafi I. 2010. Molecular characterization of *Ornithobacterium rhinotracheale* isolated from broiler chicken flocks of Iran. *Turkish J Vet Animal Sci*, **34**, 373-378.

Joubert P., Higgins R., Laperle A., Mikaelian I., Venne D. & Silim A. 1999. Isolation of *Ornithobacterium rhinotracheale* from turkeys in Quebec, Canada. *Avian Dis*, **43**, 622-626.

Koga Y. & Zavaleta A.I. 2005. Intraspecies genetic variability of *Ornithobacterium rhinotracheale* in commercial birds in Peru. *Avian Dis*, **49**, 108-111.

Leroy-Setrin S., Lesage M.C., Chaslus-Dancla E. & Lafont J.P. 1995. Clonal diffusion of EPEC-like *Escherichia coli* from rabbits as detected by ribotyping and random amplified polymorphic DNA assays. *Epidemiol Infect*, **114**, 113-121.

Leroy-Sétrin S., Flaujac G., Thénaisy K. & Chaslus-Dancla E. 1998. Genetic diversity of *Ornithobacterium rhinotracheale* strains isolated from poultry in France. *Letters Appl Microbiol*, **26**, 189-193.

Markey B.K., Leonard F.C., Archambault M., Cullinane A. & Maguire D. 2013. Clinical Veterinary Microbiology. 2nd ed. The United Kingdom, CV Mosby Company, London, 404-405.

Mirzaie S. & Hassanzadeh M. 2013. Molecular and phylogenetic characterization of *Ornithobacterium rhinotracheale* isolates from turkey, quail, partridge and domestic pigeon in Iran. *J Anim Poultry Sci*, **2**, 79-84.

Numee S., Hauck R. & Hafez H.M. 2012. Detection and typing of *Ornithobacterium rhinotracheale* from German poultry flocks. *Avian Dis*, **56**, 654-658.

Olive D.M. & Bean P. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol*, **37**, 1661-1669.

Ozbey G., Ongor H., Balik D.T., Celik V., Kilic A. & Muz A. 2004. Investigations on *Ornithobacterium rhinotracheale* in broiler flocks in Elazig province located in the East of Turkey. *Veterinární medicína*, **49**, 305-311.

Ozbey G., Ertas H.B. & Muz A. 2005. Random amplified polymorphic DNA (RAPD) analysis of *Ornithobacterium rhinotracheale* strains isolated from chickens in Turkey. *Vet Med*, **50**, 526-530.

Rohlf F.J. 1997. NTSYS-pc: Numerical taxonomy and multivariate analysis system version 2.02i. Applied Biostatistics Inc. Exeter Software, Setauket, New York.

Ryll M., Hinz K.H., Salisch H. & Kruse W. 1996. Pathogenicity of *Ornithobacterium rhinotracheale* for turkey poults under experimental conditions. *Vet Rec*, **139**, 19.

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

Sander A., Ruess M., Bereswill S., Schuppler M. & Steinbrueckner B. 1998. Comparison of different DNA fingerprinting techniques for molecular typing of *Bartonella henselae* isolates. *J Clin Microbiol*, **36**, 2973-2981.

Schuijffel D.F., Van Empel P.C., Pennings A.M., Van

Putten J.P. & Nuijten P.J. 2005. Successful selection of cross-protective vaccine candidates for *Ornithobacterium rhinotracheale* infection. *Infect Immun*, **73**, 6812-6821.

Szabó R., Wehmann E., Makrai L., Nemes C., Gyuris E., Thuma A. & Magyar T. 2017. Characterization of *Ornithobacterium rhinotracheale* field isolates from Hungary. *Avian Pathol*, **46**, 506-514.

Tamura K., Stecher G., Peterson D., Filipski A. & Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*, **30**, 2725-2729.

Thachil A.J., Velayudhan B.T., Lopes-Berkas V.C., Halvorson D.A. & Nagaraja K.V. 2007. Application of polymerase chain reaction fingerprinting to differentiate *Ornithobacterium rhinotracheale* isolates. *J Vet Diagnost Invest*, **19**, 417-420.

Thieme S., Mühldorfer K., Lüschow D., Hafez M.H. & Woo P.C.Y. 2016. Molecular characterization of the recently emerged poultry pathogen *Ornithobacterium rhinotracheale* by multilocus sequence typing. *PLoS ONE*, **11**, e0148158.

Tsai H.J. & Huang C.W. 2006. Phenotypic and molecular characterization of isolates of *Ornithobacterium rhinotracheale* from chickens and pigeons in Taiwan. *Avian Dis*, **50**, 502-507.

Turan N. & Ak S. 2002. Investigation of the presence of *Ornithobacterium rhinotracheale* in chickens in Turkey and determination of the seroprevalence of the infection using the enzyme-linked immunosorbent assay. *Avian Dis*, **46**, 442-446.

Van Empel P., Van den Bosch H., Loeffen P. & Storm P. 1997. Identification and serotyping of *Ornithobacterium rhinotracheale*. *J Clin Microbiol*, **35**, 418-421.

Van Empel P. & Hafez M.H. 1999. *Ornithobacterium rhinotracheale*: a review. *Avian Pathol*, **28**, 217-227.