Original Article

A phylogenetic landscape of *Ornithobacterium rhinotracheale* isolates from poultry in Iran presented based on 16S rRNA gene analysis

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ABSTRACT

Ornithobacterium rhinotracheale (ORT) is one of the most important bacterial agents of respiratory diseases causing significant economic losses to the poultry industry. The partial 16S rRNA gene sequences of ORT isolates originating from different geographical areas in Iran have been deposited in the GenBank database from 2011 to the present. The objectivepresent study was to evaluate the molecular evolution of ORT isolates. to this end, a dataset comprising 16S rRNA gene sequences of 48 field strains derived from outbreaks on poultry farms and one reference strain was subjected to phylogenetic and evolutionary analysis using a series of substitution pattern homogeneity, nucleotide diversity, and neutrality assays. The phylogenetic tree, based on the 16S rRNA gene, revealed the presence of a large clade, designated as A, and a distinct clade B, which included six sequences derived from the recent ORT isolates. The majority of the isolates exhibited homogeneityand clustered together,irrespective of their geographical origin. The evolutionary inference indicated that a molecular divergence influences the genetic diversity of ORT isolates. The Tajima's D statistic yielded a negative value of -1.46, while the R-value for transition/transversion bias was 1.399. Additionally, the dN/dS ratio was estimated to be higher for clade B. These findings suggest that recent ORT isolates in Iran are subjected to increasing selection pressure on the 16S rRNA gene of recent ORT isolates in Iran. The phylogeny analysis and evolutionary affiliations of the ORT 16S rRNA gene are expected to prove effective in the monitoring of the strains for antibiotic resistance and selection pressure. These phylogenetic relationships have the potential to be determinative in the selection of the best vaccine candidate strain.

Keywords: *Ornithobacterium* rhinotracheale, phylogenetic relationship, 16S rRNA, Iran

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1. Introduction

The poultry industry is perpetually susceptible to damage from respiratory infections caused by a variety of viruses and bacteria. The most significant respiratory pathogens in chickens, which result in considerable economic losses, have been identified as the avian influenza virus, Newcastle disease virus, infectious bronchitis virus, avian pneumovirus, *Mycoplasma gallisepticum*, *Pasteurella multocida*, *Haemophilus paragallinarum*, and *Escherichia col*i. These pathogens are considered as the most serious respiratory pathogens in chickens, and they can cause severe economic losses. In the early 1990s, a new respiratory disease emerged in broiler chickens in South Africa. The causative agent, *Ornithobacterium rhinotracheale* (ORT), is a pleomorphic, gram-negative, non-mobile, and non-sporulating bacillus. It is taxonomically classified in the *Flavobacteriaceae* family (1). ORT infection is now considered to be among the most important agents of avian respiratory disease. In addition to respiratory signs, the clinical picture of the infection is mainly associated with growth retardation, decreased egg production, and increased mortality (2). Over the past few decades, there has been a notable increase in the number of reports on the isolation of ORT from commercial poultry worldwide. This infection has also been reported with low frequency in wild bird species, including quail, ducks, geese, ostriches, pigeons, chukar partridges, and guinea fowl (3, 4).

Given the prevalence of the ORT infection in many areas, the prevention and control of the disease assume particular significance during the course ofpoultry breeding. The administration of antibiotics represents a viable treatment option for bacterial respiratory disease in poultry. However, choosing the appropriate drugs and the timing of their administration are of paramount importance. The main problem in managing respiratory diseases is the emergence of antimicrobial resistance and the advent of multidrug-resistant bacteria, which impairs the efficacy of treatment for infections in poultry. A number of studies have demonstrated that ORT isolates exhibit varying susceptibility and resistance profiles, which may be contingent on the phenotypic profile of the strains and their geographical origin (5-9). The significant discordance between sensitivity and resistance profiles and the inappropriate prescription of antimicrobial agents against ORT infections can contribute to the emergence of more resistant variant strains that will disseminate to other poultry farms (10). It is reasonable to conclude that the majority of ORT strains have been subjected to selection pressure due to the generation of multidrug-resistant strains and the increase in genetic variability.

The first occurrence of ORT infection in Iran was reported in 2000 ,describing a commercial broiler flock with high mortality rate (11). Subsequently, field serological surveys revealed the pervasive prevalence of ORT in commercial layers and broiler breeders across the majority of regions of the country. The prevalence of ORT antibodies has been observed to vary across different regions of Iran. These include in West Azerbaijan (12), Kerman (13), Mazandaran (14, 15), Kermanshah (16), Guilan (17), Chaharmahal Va Bakhtiari (18), Khuzestan (8), Fars (19), and Isfahan (20). The results of these studies indicate that there is no complete correlation between seropositivity and the clinical manifestations, isolation of the ORT, and antimicrobial resistance profile in all of the cases. In recent decades, the amplification of the 16S rRNA gene has been employed in the classification of ORT isolates in a limited number of studies (1, 21). Despite the existence of knowledge regarding serotyping, pathogenicity, antibiotic resistance/susceptibility, and the extent of bacterial spread, there is a lack of information concerning phylogenetic relationships and adaptive evolution among the isolates has not been elucidated. In this study, we conducted a dynamic analysis of the 16S rRNA gene sequences of Iranian ORT isolates to gain insight into the evolutionary outcomes and selection pressures acting on these bacteria. This analysis aimed to highlight the need for better strategies to combat the disease.

2. Materials and Methods

2.1. Data Collection

A dataset includes 50 16S rRNA sequences of ORT isolated from Iran and a reference strain isolated in 1991 (Acc.No. KX998703) deposited in the GenBank sequence database (https://www.ncbi.nlm.nih.gov/genbank/) was collected.

2.2. Phylogeny and Molecular Evolutionary Analysis

A total of 51 sequences underwent multiple sequence alignment using ClustalW. Two complete gene sequences in dataset 2 were excluded from the alignment. A Neighbor-Joining phylogenetic tree was constructed using evolutionary distances computed by the Maximum Composite Likelihood method, with units of the number of base substitutions per site in MEGA 7 (22). The tree was searched using the Close-Neighbor-Interchange algorithm at a search level of 1. To ascertain the confidence of the tree topologies, a bootstrap analysis of 1,000 replicates was performed. The rate variation among sites was modeled using a gamma distribution (shape parameter = 1).

In order to estimate the approximate divergence times of the ORT isolates, a linearized tree was constructed under the assumption of rate constancy was constructed. The evolutionary distances were calculated using the Maximum Composite Likelihood method. The branch-length test hypothesis was employed to examine the deviation of the branch length between the tree root and a tip from the average length.

A disparity index test of substitution pattern homogeneity was conducted on 16S rRNA nucleotide sequences. The difference in evolutionary patterns was measured by a Disparity Index (DI), based on the Monte Carlo procedure with 1,000 replicates, was employed to quantify the discrepancy in evolutionary patterns. All positions

containing gaps and missing data were excluded from the dataset.

Tajima's D statistic test was employed to test the null hypothesis in determination of nucleotide diversity in the context of positive selection or purifying selection. In order to calculate the frequency of nucleotide variants, two statistics are employed: the mean pairwise difference (π, representing the average number of nucleotide differences per site between two sequences) and the number of segregating (S) sites, which were considered at $P<0.01$.

The distribution of individual nucleotides across the three codon positions in all the annotated ORT isolates was analyzed. The nucleotide frequencies, the transition/transversion rate ratios for purines (k1) and pyrimidines (k2), and the overall transition/transversion bias (R), where $R = [A\times G\times k1 + T\times C\times k2]/[(A+G) \times (T+C)]$, were determined.

Synonymous (dS) and/or nonsynonymous (dN) nucleotide substitutions involved in the potential phylogenetic divergence of the isolates were identified for statistically significant evidence of positive selection (ratio of dN/dS) at $P < 0.05$. The probability of rejecting the null hypothesis of strict neutrality in favor of stabilizing selection was calculated based on the dN/dS ratio. The variance of the difference was calculated using the 500 replicates.

In order to get some insight into the evolutionary pressure acting on the 16S rRNA gene in ORT, the evolutionary rate, defined as the speed of divergence between sequences was calculated using the Tajima' relative rate test. Two sequences, including the first and the most recent ORT isolates were subjected to testing in conjunction with KX998703, which served as the outgroup. The locations of the divergent sites in all three sequences and the unique differences in each sequence were identified. Based on the aforementioned data, it may be posited that the molecular clock hypothesis is untenable with respect to this particular set of sequences.

3. Results

The phylogenetic analysis of the 16S rRNA genome sequences of the ORT revealed a high degree of relatedness, with a similarity ranging from closely related 98.8% to100% at a scale of 0.002 substitutions per site. The evolutionary history between these sequences was inferred using the Neighbor-Joining method. All positions containing gaps and missing data were eliminated,and a total of 1420 positions in the final dataset were evaluated. As shown in Figure 1, the ORT sequences were found to be arranged in at least two clades and multiple clusters. The most probable allocation of these sequences, based on the confidence probability of each node, was found to be clustered together with minimal diversity. In contrast, while the OP006257, OP006260-OP006262, OP006266, and OP006267 sequences were observed to be distant from the remaining isolates and to have formed a distinct clade. Despite the very low genetic diversity observed within the isolates, the existence of several branches in the tree was supported by high bootstrap values.

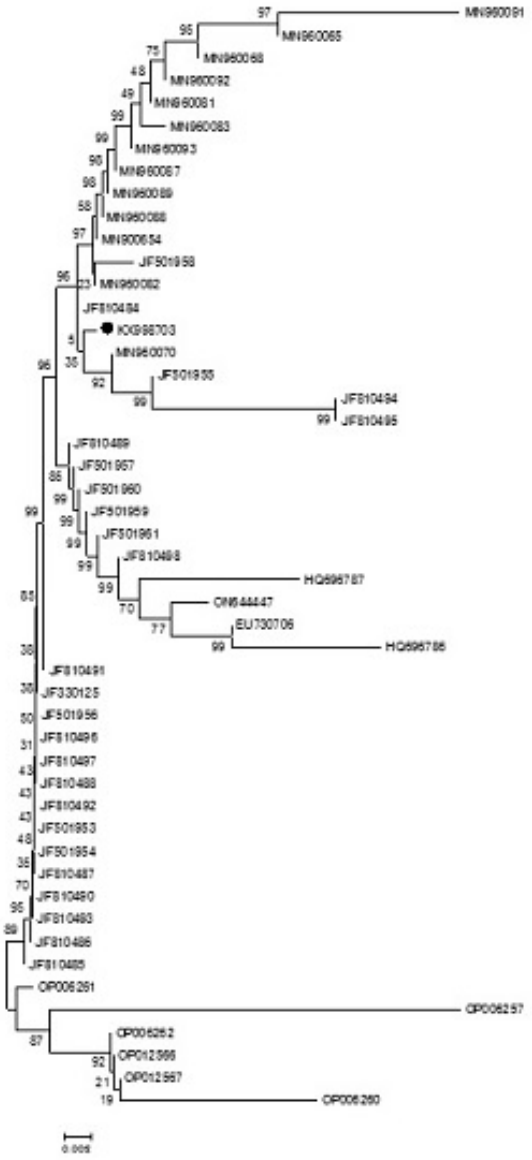

Figure 1. The evolutionary history of *Ornithobacterium rhinotracheale* isolates based on the partial sequence of the 16S rRNA gene. The optimal tree with the sum of branch length = 0.07011222 was constructed by the Neighbor-Joining method. The confidence probability of each node (multiplied by 100) that estimated using the bootstrap test is shown next to the branches. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1420 positions in the final dataset. The reference sequence is indicated by the black circle.

In order to gain a deeper insight into the topology of the phylogenetic tree, the evolutionary history was inferred using the Neighboring-Joining method, and the tree was linearized. Cumulative results of the phylogenetic analyses are presented in Figure 2. Once more, the, sequences situated within the compact clade (clade B) exhibited greater divergence than those in the other clade (clade A). This suggests the possibility of an alternative point of entry for ORT in Iran or positive selection.

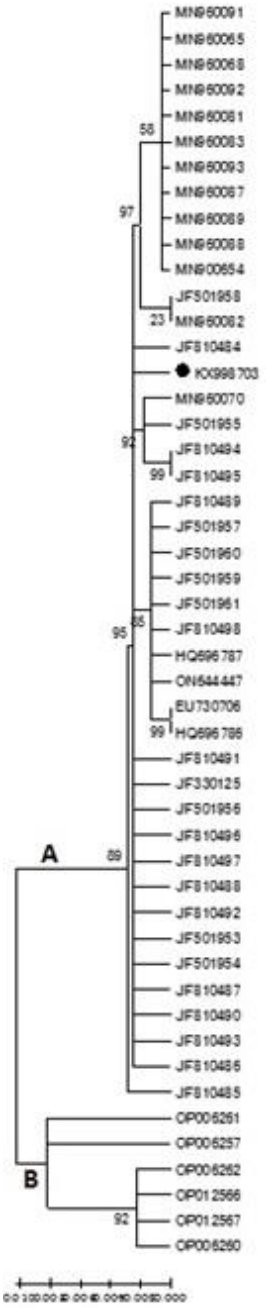

Figure 2. This study presents an evolutionary history of *Ornithobacterium rhinotracheale* isolates based on the partial sequence of the 16S rRNA gene. The minimum evolution trees (sum of branch length = 0.07011222) was constructed and linearized assuming equal evolutionary rates. The reference sequence is indicated by the black circle.

The DI per site was calculated for all sequence pairs, resulting in an overall average of 0. A value of 0 indicates that the homogeneity assumption is satisfied, whereas a value greater than 0 denotes a greater degree of divergence in base composition biases than expected based on evolutionary divergence between sequences and by chance alone. Accordingly, the index indicates that a homogeneity pattern of substitution has been maintained for all the ORT sequences. This finding demonstrated that the sequences located in the clades exhibited no genetic distance.

In order to distinguish between the neutral evolution of the ORT isolates and non-random evolution, the polymorphism of the sequences was analyzed based on the Tajima's D test. A total of49 genome sequences were subjected to a test, which yielded two polymorphic sites (Table 1). The nucleotide diversity equals to -1.466603, while the nucleotide variants (12%) with low frequency reflect a moderate level of genetic variation.

Distribution of nucleotides pattern over the three codon positions in ORT isolates estimated by Maximum Composite Likelihood is shown in Table 2. The nucleotide frequencies are 0.125 (A), 0.19 (T/U), 0.436 (C), and 0.249 (G). The transition/transversion rate ratios were determined as k1 = 0 and k2 = 2.997, and R value of 1.399 was obtained in case of Transition/Transversion bias. On the evolutionary bias, if the distribution of transition and transversion mutations are random R would expect to be 0.5 because there are twice as many possible transversions as transitions. Here, the R ratio >0.5 suggests that this bias is caused by a biological influence. On the other hand, the likelihood values support the selective pressure acting on these sequences.

To infer the evolutionary selective pressure on the 16S rRNA gene, a pairwise comparison approach was applied using the Nei-Gojobori method. The coefficients dS and dN were calculated for all the isolates, and the mean pairwise dN/dS ratio for the ORT isolates in clade A was found to be 0.42. For all six isolates in clade B, the ratio of 0.76 was estimated. A ratio greater than 1 indicates positive selection, less than 1 implies purifying or stabilizing selection, and a ratio of exactly 1 corresponds to neutral selection. Since the average dN/dS ratio was less than 1, a purifying selection is suggested in all of the ORT isolates, though the corresponding ratio value in the recent isolates was higher than the others. To further investigate selection pressure on the 16S rRNA of ORT, the evolutionary rate was calculated. The equality of evolutionary rate between JF330125 as the first ORT isolate (clade A) and OP006257 as the recent and divergent isolate (clade B) was tested using KX998703 as an outgroup (Table 3). The $\chi 2$ test statistic was 18.62 and the P = 0.00002 with 1 degree of freedom. The result indicated that the OP006257 isolated in 2022 had a significantly faster substitution rate than the old isolate. According to the null hypothesis, a P value less than 0.05 is used to reject the hypothesis of equal rates between sequences.

Table 1. Tajima's neutrality test for *Ornithobacterium rhinotracheale* 16S rRNA gene sequences

m	S	p_s	Θ	π	D
49	2	0.125000	0.028034	0.005102	-1.466603

m = number of sites, S = Number of segregating sites, p_s = S/m, Θ = p_s/a_1, and π = nucleotide diversity, D = Tajima test statistic

Table 2. The nucleotide substitution pattern of *Ornithobacterium rhinotracheale* 16S rRNA gene

	A	T	C	G
A	-	*4.9*	*11.25*	0
T	*3.22*	-	33.72	*6.41*
C	*3.22*	14.69	-	*6.41*
G	0	*4.9*	*11.25*	-

Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics.

Table 3. The equality of evolutionary rate analysis between *Ornithobacterium rhinotracheale* 16S rRNA gene sequences

Configuration	Count
Identical sites in all three sequences (miii)	728
Divergent sites in all three sequences (mijk)	1
Unique differences in Sequence A (mijj)	2
Unique differences in Sequence B (miji)	24
Unique differences in Sequence C (miij) χ^2 test statistic P value	3 18.62 0.00002

The sequence A (JF501958) and sequence B (OP006257) were subjected to testing tested using KX998703 as outgroup in a Tajima' relative rate test. A p-value of less than 0.05 is frequently employed to reject the null hypothesis.

Here, the null hypothesis of neutrality was rejected for clade B due to the highly significant deviation in evolutionary rate between the clades. Thus the molecular clock hypothesis was also rejected for two clades. The nucleotide substitution rate in clade B deviated from the test assumption and may followed the positive selection.

4. Discussion

Given that ORT strains have been isolated from poultry species, it is crucial to gain insight into the evolutionary dynamics of these isolates in order to effectively control the disease on farms. The results of several studies demonstrated that serum antibody levels were present in poultry infected with ORT, despite the absence of any clinical signs. This observation suggests that ORT strains may undergo neutral evolution in their natural host population. In the present study, we sought to quantify positive selection in ORT isolates by identifying sequences that do not align with the neutral theory model.

A total of 48 sequences for the 16S rRNA gene of ORT isolated from Iran and one consensus strain deposited in GenBank were subjected to analysis. Pairwise sequence comparisons revealed a high level of homogeneity, with similarity values ranging from 98.8% to 100% among the sequences. Although there was limited information available on the disease status of some ORT strains in this study, it is evident that almost all of the isolates clustered together on the phylogenetic tree. The majority of the isolates along with the reference strain, were grouped together in one clade and separated from another clade that had recently been isolated from poultry in Mazandaran province (7). Given the generation of two clades, the net base composition bias disparity between sequences was estimated on the assumption that the sequences within each clade have evolved with the same pattern of nucleotide substitution. The Tajima's D value was attributed to the neutral or weak purifying selection that has occurred recently in ORT outbreaks. As indicated by prior serological studies, the prevalence of ORT antibodies has been observed to increase in certain geographic regions (12, 13, 15-19). However, the present study was constrained by limitations in sample size and the availability of data on disease status. Considering the negative value of Tajima's D, the null hypothesis that the sequences have evolved with the same pattern of substitutions was rejected. This suggests that the 16S

rRNA in clade B sequences has evolved via a different evolutionary process from that observed in clade A. Consequently, it can be inferred that the quality of prevention and control strategies, the occurrence of resistance to the main drugs used in poultry farming, and the lack of antimicrobial susceptibility profile in recent ORT isolates have had an impact on the evolution of the gene.

The issue of antibiotic resistance represents a significant challenge for the poultry industry. The frequent and inappropriate use of antibiotics can impose selective pressure on bacteria, thereby facilitating the spread and adaptation of resistant bacteria (23). As evidenced by field reports, there is considerable inconsistency in the sensitivity and resistance of ORT to recommended antibiotics, including tiamulin, florfenicol, gamithromycin, ampicillin, ceftiofur, tetracycline, cotrimoxazole, enrofloxacin, gentamicin, and fosfomycin. This variability is largely attributed to the geographical origin of the samples (3, 5, 10, 17). As resistance to different classes of drugs, especially the newer generation agents, increases, the hypothesis that resistance can be readily transferred between ORT strains is strengthened. The generation of resistant multidrug strains and an increase in genetic diversity indicate that ORT strains have been subjected to the selection pressure.

Based on previous serological studies the prevalence of ORT antibodies has increased in certain geographic regions of Iran or remained constant (12, 13, 15, 16, 19). However, there are significant gaps in knowledge gaps regarding the generation of the multidrug-resistant strains. To date, a few studies have sequenced the 16S rRNA gene of ORT isolates that are available at GenBank (6-8, 11). From an epidemiological standpoint, it is highly advantageous to know if multidrug resistance is present in ORT. Consequently, the identification of antimicrobial resistance genes and the determination of their frequencies in isolates is crucial for distinguishing between resistances resulting from genetic drift or acquired antimicrobial resistance genes, tracking interspecies transmission of antimicrobial resistance, and selecting the best vaccine candidate strain.

The objective of this study was to gain a deeper comprehension of the molecular evolution landscape of ORT strains isolated from Iran over the past two decades. The results of this study provide evidence for the generation of a divergent phylogenetic clade of ORT isolates in Iran, which emphasizes the need for continued monitoring of ORT isolates for antibiotic resistance and selection pressure.

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Authors' Contribution

Study concept and design: SS, NG, MM E
Acquisition of data: SS, NG
Analysis and interpretation of data: SS
Drafting of the manuscript: SS
Critical revision of the manuscript for important intellectual content: NG, MM E, RG, VK

Ethics

As a non-interventional experiment, this study does not involve the inclusion of human or animal subjects.

Conflict of Interest

The authors declare that they have no conflict of interests.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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