NOTE

Bacteriology

Phylogenetic relationship of
Ornithobacterium rhinotracheale strains

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ABSTRACT. The bacterium Ornithobacterium rhinotracheale is associated with respiratory disease in wild birds and poultry. In this study, the phylogenetic analysis of nine reference strains of O. rhinotracheale belonging to serovars A to I, and eight Mexican isolates belonging to serovar A, was performed. The analysis was extended to include sequences from another 23 strains available in the public domain. The analysis showed that the 40 sequences formed six clusters, I to VI. All eight Mexican field isolates were placed in cluster I. One of the reference strains appears to present genetic diversity not previously recognized and was placed in a new genetic cluster. In conclusion, the phylogenetic analysis of O. rhinotracheale strains, based on the 16S rRNA gene, is a suitable tool for epidemiologic studies.

KEY WORDS: Ornithobacterium rhinotracheale, phylogenetic analysis, reference strains

The Gram-negative bacterium Ornithobacterium rhinotracheale has been associated with respiratory disease and mortality in wild-birds and poultry and has a worldwide distribution in many avian species [3]. Isolates of O. rhinotracheale can be classified into 18 agar gel precipitation (AGP) serovars (A through R), and serovar A is the most prevalent among chicken and turkey isolates [3, 5, 14, 17]. Genotyping studies of O. rhinotracheale isolates obtained from poultry throughout the world have shown a small group of closely related clones [2, 4, 7, 10]. Six electrophoretic types (ET) obtained by multi-locus enzyme electrophoresis have been recognized in O. rhinotracheale isolates from eight countries around the world [2]. The six ETs were confirmed by 16S rRNA gene sequencing and rep-PCR analysis [2].

The phylogenetic relationships amongst a number of reference strains as well as Mexican isolates of O. rhinotracheale are unknown. Hence, the aim of the present study was to perform a phylogenetic analysis of O. rhinotracheale using a number of serovar reference strains and Mexican field isolates.

In the present study, a total of nine reference strains of O. rhinotracheale were included: B 3263/91, GGD 1261, ORV K91-201, ORV 94108 no. 2, O-95029 no. 12229, ORV 94084 K858, O-95029 no. 16279, E-94063 4.2, and BAC 96-0334 #MINN 18, representing serovars A through I [12, 17], respectively (Table 1). All reference strains were sourced from the culture collection held at the University of Queensland, Australia. A total of eight well-characterized Mexican isolates of O. rhinotracheale [10], were included in the study. Bacteria were cultivated on 10% sheep blood agar at 37°C in a candle jar. Brain-heart infusion broth was used for propagation and maintenance of bacterial cultures. For improved growth, this medium was supplemented with 1% (v/v) filter-sterilized and heat-inactivated horse serum [13]. The DNA was extracted directly from cell biomass cultured in brain-heart infusion broth and purified by using DNeasy® Blood and Tissue kit (QIAGEN, Austin, TX, U.S.A.), according to the manufacturer’s protocols. The 16S ribosomal RNA (rRNA) gene was amplified from the nine reference strains and the eight Mexican isolates of O. rhinotracheale by using primers and conditions as elsewhere reported [2]. The sequencing of the 16S rRNA gene was performed by Macrogen Inc. (Seoul, Republic of Korea) using the Sanger dideoxy terminator sequencing method. The 16S rRNA gene sequences were obtained in the region covering Escherichia coli positions 27-1492. A Basic Local Alignment Search Tool (BLAST) search was performed in GenBank [1]. Pairwise comparisons for similarity were performed by the program WATER included in European Molecular Biology Open Software Suite (EMBOSS) [11]. The phylogenetic analysis was performed...
by construction of a multiple alignment by ClustalX, removal of gapped columns, and analysis by the maximum likelihood method
[16] and genetic distances obtained by Kimura's 2-parameter model, conducted using MEGA5 [6]. Additional sequences of
well-characterized reference strains and isolates from GenBank and Green Genes data-bases were included in the analysis (Fig. 1)
[2, 18, 19]. Shorter sequences (less than 700 nucleotides) were not included in the analysis [4, 8].

Sequences for the 16S rRNA gene of the nine reference strains and eight Mexican isolates of O. rhinotracheale were obtained
and deposited in GenBank (accession numbers KY612254 and KY809786 to KY809801). Pairwise sequence comparisons
revealed 99.1 to 100% sequence identity among the 40 sequences included in the study. The analysis of polymorphic nucleotides
in the 16S rRNA gene showed differences from 1 to 10 nucleotides in comparison to a consensus 16S rRNA gene sequence of O.
rhinotracheale (Table 2). The 40 sequences included into the phylogenetic analysis were clustered into six genetic clusters (I to VI;
Fig. 1).

As the study of Amonsin et al. [2] had identified a system for correlating the ET typing results with single nucleotide
polymorphisms (SNP) in the 16S rRNA gene sequence, we were able to predict that these 13 strains/isolates would be ET 1
(Table 2). As 17 of the 19 strains and isolates allocated to cluster I aligned with the scheme suggested by Amonsin et al. [2]
for correlating polymorphisms in the 16S rRNA gene sequence, we were able to predict that these 13 strains/isolates would be ET 1
(Table 2). A further six strains/isolates within cluster I had the same SNP pattern as assigned by Amonsin et al. [2] to ET 1 but also
had additional SNP sites not noted by Amonsin et al. [2] and were thus predicted to be variants of ET 1 (Table 2). Cluster II consisted of strain LMG-11553 (GenBank accession number U87102). Cluster III consisted of strain LMG-15870
(GenBank accession number U87103) of known ET 5 and four strains that could not be assigned to a predicted ET. Cluster IV
consisted of strain LMG-11554 (GenBank accession number U87104) of known ET 6 and one isolate that could not be assigned to a
predicted ET. Cluster V consisted of strain LMG-14578 (GenBank accession number U87105) of known ET 4 and three reference
strains that were assigned to a predicted ET. Cluster VI had only a single reference strain that could not be assigned to an ET.

In this study, we report the sequencing of 16S rRNA gene from nine reference strains of Ornithobacterium rhinotracheale
belonging to serovars A to H which have been previously reported [9], the sequences are not available in public data-bases.

Amonsin et al. [2] assigned 90.9% (50 of 55) of isolates of O. rhinotracheale to ET 1 and ET 2, comprising the ET 1
complex. In the present study, phylogenetic analysis of 16S rRNA gene sequences resulted in a cluster (cluster I) that consisted
predominantly of strains and isolates that were known to be ET 1 or ET 2 or predicted to be ET 1 or a variant of ET 1, confirming
the ET 1 complex. All of the Mexican serovar A isolates and reference strains of serovars A, B, E and G were clustered in cluster
I (Fig. 1). Clearly, this complex (16S rRNA gene cluster I/ET complex 1) is a cluster of strains and isolates that are commonly
distributed around the world. In the current study, more variation in the 16S rRNA gene was found than was used by the earlier
study of Amonsin et al. [2] to correlate ET with 16S rRNA sequences. Despite this increased diversity, there was still a good

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**Table 1.** Bacterial strains used in the study

| Strain   | Source       | Origin        | Year of isolation | Serovar |
|----------|--------------|---------------|-------------------|---------|
| B 3263/91 | Broiler      | South Africa  | 1991              | A       |
| GGD 1261  | Turkey       | Germany       | 1991              | B       |
| ORV K91-201 | Broiler    | U.S.A.        | 1991              | C       |
| ORV 94108 no. 2 | Turkey    | France        | 1994              | D       |
| O-95029 no. 12229 | Broiler | France        | 1995              | E       |
| ORV 94084 K858 | Turkey     | The Netherlands | 1994             | F       |
| O-95029 no. 16279 | Broiler | France        | 1995              | G       |
| E-94063 4.2 | Turkey      | The Netherlands | 1994             | H       |
| BAC 96-0334 MINN 18 | Turkey | U.S.A.       | 1996              | I       |
| ESV-55   | Broiler      | Mexico        | 2005              | A       |
| ESV-60   | Turkey       | Mexico        | 2006              | A       |
| ESV-104  | Broiler      | Mexico        | 2008              | A       |
| ESV-207  | Peacock      | Mexico        | 2009              | A       |
| ESV-209  | Turkey       | Mexico        | 2009              | A       |
| ESV-216  | Layer        | Mexico        | 2010              | A       |
| ESV-301  | Hobby chicken| Mexico        | 2011              | A       |
| ESV-305  | Hobby chicken| Mexico        | 2012              | A       |

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correlation between ET results (predicted or known) and the phylogeny.

Reference strain ORV 94084 K858 of serovar F of *O. rhinotracheale* was allocated to cluster VI as the sole member of that cluster. Ten nucleotide changes were recorded in this strain (Table 2). It is clear that this strain represents significant new diversity that has not been previously recognized and association with antigenic and pathogenic traits need to be studied. This reference strain was included in a multilocus sequence typing (MLST) analysis that included seven housekeeping genes, and showed the most nucleotide polymorphisms [15, 16]. Two distinct phylogenetic clusters were identified in the phylogenetic tree generated from MLST sequences and reference strain ORV 94084 K858 of *O. rhinotracheale* was allocated, along with other two strains, to cluster A and differed considerably from the vast majority of *O. rhinotracheale* strains included in that study [16]. Further studies comparing phylogenetic analysis based on 16S rRNA gene and MLST are needed.

Identical genotypes (16S rRNA gene phylogeny and ERIC-PCR pattern) of Mexican field isolates included in the study, recovered over a long time period in two main Mexican geographic areas (Puebla and Jalisco), support that *O. rhinotracheale* population is predominantly clonal as has been previously suggested [2, 19].

In conclusion, new information was added to the knowledge of population structure of *O. rhinotracheale* by the phylogenetic analysis of the 16S rRNA gene, allowing a worldwide comparison of sequence data and evidencing the existence of greater genetic variability among strains than previously contemplated.

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Table 2. Polymorphic sites in *O. rhinotracheale* in 16S rRNA gene sequences

| Strain(a) | ET or predicted ET(b) | ERIC-PCR genotype(c) / AGP serovar | Cluster(d) | Base at the following nucleotide position(e) |
|-----------|------------------------|-----------------------------------|------------|--------------------------------------------|
| consensus |                        |                                   |            |                                            |
| D95-16426 | 1                      | I                                 |            | A 164 G 181 G 186 G 187 276 AG 309 G 374 432 G 443 558 570 G 598 819 A 984 985 A 996 1002 A 1136 A 1197 A 1229 A 1229 |
| B 3263-91 | 1                      | I                                 |            |                                           |
| O-95029 no. 12229 | 1*            | I / A                             |            |                                           |
| ORT-UMN 88 | 1*                     | I / IV                            |            |                                           |
| ESV-55 | 1*                     | I / VII                           |            |                                           |
| ESV-60 | 1*                     | I / VII                           |            |                                           |
| ESV-104 | 1*                     | I / VII                           |            |                                           |
| ESV-207 | 1*                     | I / VII                           |            |                                           |
| ESV-216 | 1*                     | I / VII                           |            |                                           |
| ESV-301 | 1*                     | I / VII                           |            |                                           |
| ESV-305 | 1*                     | I / VII                           |            |                                           |
| B238-11 | 1*                     | I                                 |            |                                           |
| R293-2A | 1*                     | I                                 |            |                                           |
| OR064 | 1*                     | A                                 |            |                                           |
| OR067 | 1*                     | A                                 |            |                                           |
| OR082 | 1*                     | B                                 |            |                                           |
| ORT-1 | 1*                     |                                  |            |                                           |
| GGD 1261 | 1*                     | I / B                             |            |                                           |
| O-95029 no. 16279 | 1v*           | I / VI                            |            |                                           |
| ORT-7 | 1v*                    | I                                 |            |                                           |
| ORT-18 | 1v*                    |                                   |            |                                           |
| ORT-16 | 1v*                    |                                   |            |                                           |
| ORT-26 | 1v*                    |                                   |            |                                           |
| LMG-9086T | 1                        | I                                 |            | 128 A            |
| ES-209 | 1                        | I                                 |            | 164 A            |
| LMG-15511 | 2                     | I                                 |            | 181 A            |
| FARPER-108 | ?                    | I                                 |            | 186 A            |
| LMG-11553 | 2                     | I                                 |            | 187 G            |
| LMG-155270 | 5                    | I                                 |            | 276 A            |
| OR063 | 5                       | D (C)                             |            | 309 G            |
| B-293-2B | ?                      | I                                 |            | 374 G            |
| ORT-11 | ?                      | II / C                            |            | 432 A            |
| LMG-11554 | 5                     | I                                 |            | 443 A            |
| ORT-14 | 5*                     | I                                 |            | 558 A            |
| LMG-14578 | 4                     | I                                 |            | 570 A            |
| ORV 94108 no. 2 | 4*                 | I                                 |            | 598 A            |
| E-98063-4.2 | 4*               | I                                 |            | 819 A            |
| BAC 96-0334 MINN 18 | 4*              | I                                 |            | 984 A            |
| ORV 94084 K858 | ?                | I                                 |            | 1136 A           |

- a) Strains with underlining were examined in the study of Amonsin et al. [2]. The polymorphisms reported for these strains are based on the Genbank sequence. b) ET=Electrophoretic type. The results that are underlined are those reported by Amonsin et al. [2]. The ET results that are underlined are those reported by Amonsin et al. [2]. The ET results with an asterisk (*) are predicted results based on the current sequencing data and an exact alignment with the polymorphism pattern reported by Amonsin et al. [2]. c) ERIC-PCR genotype—the enterobacterial repetitive intergenic consensus-type as previously defined [10]. d) Cluster assignment is based on Fig. 1. e) Base positions that are underlined are the base positions predicted by 16S rDNA polymorphisms. The other base positions are those found in the current study. f) ORT-26, further base nucleotide positions at 65 (G), 74 (G), 76 (G), 165 (T) and 172 (G). g) ORT-14, further base nucleotide positions at 408 (T), 418 (C), 420 (A) and 428 (A). h) Dots indicate no change from the consensus sequence.
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