

## Intraspecies Genetic Variability of *Ornithobacterium rhinotracheale* in Commercial Birds in Peru

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**SUMMARY.** Strains of the bacterium *Ornithobacterium rhinotracheale* (ORT), a causal agent of respiratory diseases in birds, were microbiologically isolated, identified, and molecularly characterized. Blood-enriched culture media and biochemistry tests were used for microbiologic identification. Polymerase chain reaction (PCR) and repetitive extragenic palindromic PCR (rep-PCR) techniques were used for molecular identification and characterization, respectively, of the microorganism. ORT strains were isolated in enriched media from the trachea and air sacs of broilers, breeders, and layers from several geographic zones of Peru. Of the original 75 strains isolated from 75 clinical samples from which ORT was recovered during 1998–2000, 25 were selected for further study based on ORT as the primary pathogenic isolate (no other pathogens were detected). Selected isolates were molecularly identified and characterized by PCR using specific primers designed from the conserved zones of the 16S ribosomal genes. Primers used for the identification of ORT produced a specific fragment of 784 base pair (bp), which did not appear in *Haemophilus paragallinarum* or *Pasteurella multocida*, microorganisms with similar morphologic and biochemical characteristics that produce clinical signs identical to those of ORT. All 25 strains of ORT tested with rep-PCR had a genetic profile similar to that of ORT American Type Culture Collection 51463, indicating the presence of only one genotype in the ORT strains studied.

**RESUMEN.** Variabilidad genética entre especies de *Ornithobacterium rhinotracheale* en aves comerciales en Perú.

Se aislaron e identificaron, mediante técnicas microbiológicas, y se caracterizaron, mediante técnicas moleculares, varias cepas de la bacteria *Ornithobacterium rhinotracheale* (ORT), agente causal de enfermedades respiratorias en aves. En la identificación microbiológica de ORT se emplearon medios de cultivo enriquecidos con sangre y pruebas bioquímicas. En la identificación y caracterización molecular de ORT se emplearon las pruebas de reacción en cadena por la polimerasa y la reacción en cadena por la polimerasa repetitiva en zonas palindrómicas extragénicas, respectivamente. Mediante el uso de un medio enriquecido, se aislaron cepas de ORT a partir de la tráquea y sacos aéreos de pollos de engorde, reproductoras, y ponedoras comerciales de varias regiones geográficas de Perú. De las 75 cepas originales aisladas a partir de 75 muestras clínicas obtenidas durante los años 1998 al 2000 de las cuales se aisló ORT, se seleccionaron 25 cepas para realizar estudios adicionales con base en ORT como aislamiento patógeno primario (único agente patógeno detectado en la muestra). Los aislamientos seleccionados fueron identificados y caracterizados molecularmente mediante la reacción en cadena por la polimerasa empleando iniciadores específicos diseñados a partir de las zonas conservadas del gene ribosomal 16S. Los iniciadores empleados para la identificación de ORT produjeron un fragmento específico de 784 pares de bases, no observado en *Haemophilus paragallinarum* o *Pasteurella multocida*, microorganismos con características morfológicas y bioquímicas similares que producen signos clínicos idénticos a los observados en infecciones por ORT. Las 25 cepas de ORT evaluadas mediante la técnica de la reacción en cadena por la polimerasa repetitiva en zonas palindrómicas extragénicas mostraron perfiles genéticos similares al ser comparados con la cepa ATCC 51463 de ORT, indicando la presencia única de un solo genotipo en las cepas de ORT estudiadas.

**Key words:** *Ornithobacterium rhinotracheale*, respiratory disorders, commercial birds, polymerase chain reaction

**Abbreviations:** ATCC = American Type Culture Collection; ORT = *Ornithobacterium rhinotracheale*; PCR = polymerase chain reaction; rep-PCR = repetitive extragenic palindromic PCR

*Ornithobacterium rhinotracheale* (ORT) is a worldwide distributed bacterium that has been isolated in several countries, especially in Germany, Belgium, Israel, and the United States (2,4,15). When first isolated, it was described as a *Pasteurella*-like or *Kingella*-like bacterium (15), a pleomorphic gram-negative bacillus. The name *Ornithobacterium* was suggested for the new genera within the V rRNA superfamily and the name *rhinotracheale* for the species (13).

In Peru, it was first isolated in 1998 from broiler chickens experiencing respiratory disease (7) that was worsened by the simultaneous presence of Newcastle disease virus or infectious bronchitis virus. The affected birds were negative for *Haemophilus paragallinarum* and *Pneumovirus* isolation, suggesting the presence of a new pathogenic agent. Morbidity and mortality rates due to this microorganism are as high as 50% in the most severe cases, causing economic losses because of confiscation of the most affected birds by veterinary public health authorities, as well as decreased productivity in layers and breeders and increased use of antibiotics for treatment.

Although microbiologic isolation and identification have been done by several investigators (5,11,12,14), there are few reports using molecular identification techniques such as polymerase chain reaction (PCR) (6,8,16) and 16S ribosomal gene sequencing (1). Repetitive extragenic palindromic PCR (rep-PCR) is a simple and highly reproducible method that distinguishes closely related strains and enables the determination of phylogenetic relationships between strains and the study of their genetic diversity. The rep-PCR technique uses DNA complementary primers that naturally occur in highly repetitive and conserved DNA sequences in the genome of many gram-negative and some gram-positive bacteria that, when amplified, give rise to a specific genetic fingerprinting (1,9,10,15). This technique has been successfully used to genotype different ORT strains in the United States (1). In Peru, genetic characterization of current ORT strains has not been reported, to our knowledge.

The objective of the present study was to determine the genetic variability of ORT strains isolated from birds by rep-PCR. This study may help to determine the origin and likely spreading

Table 1. Strains of ORT recovered from avian sources.

Strain <sup>A</sup>	Geographic zone	Source	Age of bird
1	Lima	Chicken (trachea)	50 days
2	Lima	Chicken (trachea)	35 days
3	Lima	Chicken (air sacs)	40 days
4	Lima	Chicken (vitellus)	1 day
5	Lima	Breeder (trachea, orbital sinus)	40 wk
6	Lima	Breeder (trachea, air sacs)	30 wk
7	Lima	Breeder (trachea, air sacs)	29 wk
8	Loreto	Breeder (trachea)	35 wk
9	Ica	Layer (trachea)	22 wk
10	Loreto	Chicken (trachea, orbital sinus)	35 days
11	Ica	Chicken (trachea, air sacs, wattles)	35 days
12	La Libertad	Chicken (trachea, air sacs)	42 days
13	La Libertad	Chicken (trachea, air sacs)	40 days
14	Lima	Layer (trachea, orbital sinus)	16 wk
15	Lima	Chicken (trachea, air sacs)	42 days
16	Ica	Layer (trachea, air sacs)	35 wk
17	Lima	Chicken (trachea)	36 days
18	Lima	Chicken (trachea, air sacs)	25 days
19	Lima	Chicken (orbital sinus, air sacs, trachea)	47 days
20	Lima	Chicken (orbital sinus, air sacs, wattles)	47 days
21	Arequipa	Layer (trachea)	37 wk
22	Ica	Chicken (air sacs)	38 days
23	Lima	Breeder (trachea, orbital sinus, air sacs, lungs)	37 wk
24	Lima	Chicken (trachea)	32 days
25	Lima	Chicken (orbital sinus, trachea, wattles)	49 days

<sup>A</sup>Most strains (16/25) were isolated from broiler chickens, followed by breeders (5/25) and layer hens (4/25). From these 25 strains, 64% (16/25) were isolated from birds in the department of Lima geographic zone, which has approximately 60% of the commercial bird population in Peru.

mechanisms of ORT within peruvian flocks and to facilitate the implementation of appropriate control measures.

## MATERIALS AND METHODS

**Sample collection.** From October 1998 to December 2000, samples from broiler chickens, layers, breeders, and turkeys were submitted to our laboratory and coded with individual clinical cards according to their origin. Submitted birds showed respiratory signs such as dyspnea, nasal and ocular secretion, sneezing, swollen head, weakness, and reduced feed and water intake. In some cases, when the complete bird was not accessible, the trachea, air sacs, and lung samples were submitted in sterile containers with ice. Nasal and ocular swabs were transported to the laboratory in thioglycolate medium to be subsequently processed.

**Macroscopic and microscopic lesions.** Birds with infections consistent with ORT were euthanatized and necropsied for detection of macroscopic lesions in the orbital sinus, trachea, bronchus, lungs, air sacs, and reproductive organs of production birds.

**Bacteriologic isolation and identification.** Samples were cultured in 5%–10% blood-enriched media and incubated at 37 C for 24–48 hr with 5%–10% carbon dioxide. Agar plates were checked for suspected colonies (small, grey, and nonhemolytic). Suspected isolates were identified using a commercial kit (Sensident EM ident NF; Merck Research Labs, West Point, PA) and confirmed by catalase and oxidase reactions.

All ORT-positive samples were subcultured three consecutive times in 5 to 20 ml of trypticase soy agar at 37 C for 24 hr until the bacteria adapted to the media. After the last passage, media containing the bacteria were dispensed in aliquots of 2 ml with 20% glycerine and maintained at –20 C during 1 day and transferred to liquid nitrogen until they were processed.

**Genomic DNA extraction.** Selected isolates were grown overnight at 37 C in biosynthetic human insulin broth and subjected to genomic DNA extraction by the phenol-chloroform method (1,3). The concentration and purity of the DNA obtained were estimated by using known concentrations of phage molecular DNA Lambda/HindIII on 0.9% agarose Tris acetate-EDTA gel electrophoresis at 80 V.

**PCR.** Primers OR16S-F1 (5'-GAGAATTAATTTACGGATTAAG) and OR16S-R1 (5'-TTCGCTTGGTCTCCGAAGAT) designed from the RNA 16S sequence (14) were used. Amplification reactions were performed in 0.6-ml tubes in a final volume of 50 µl containing 1× PCR buffer, 1.5 mM magnesium chloride, 200 mM deoxyribonucleotide triphosphate, 0.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), 20 pmol of each primer, and 100 ng of genomic DNA. The reactions were amplified in a 480 Perkin-Elmer thermocycler with an initial denaturation step at 94 C for 7 min, followed by 45 cycles at 94 C for 30 min, 52 C for 60 min, and 72 C for 90 min, with one final cycle at 72 C for 90 min.

DNA of local strains of *H. paragallinarum* or *Pasteurella multocida* and ORT American Type Culture Collection (ATCC) 51463 (Rockville, MD) were used as negative and positive controls, respectively. All PCR products were separated on 1% agarose TAE gel using phage Phi X174RF DNA as a molecular weight marker.

**Rep-PCR.** Complementary oligonucleotides of the conserved DNA sequences belonging to the 124- to 127-bp enterobacteria repetitive intergenic consensus ERIC 1R (5'-ATGTAAGCTCCTGGGGATT-CAC-3') and ERIC 2 (5'-AAGTAAGTACTGGGGTGGAGCG-3') were used (13). The final volume of 25 µl contained 1× PCR buffer, 3.5 mM magnesium chloride, 200 mM deoxyribonucleotide triphosphate, 2.5 U of *Taq* DNA polymerase, 20 pmol of each primer, and 100 ng of genomic DNA. The reactions were amplified in a 2400 Perkin-Elmer thermocycler, as described (1), with modifications in the hybridization temperature and time of reaction (50 C for 1.5 min). PCR products (8 µl) were size-separated on 2% agarose gel by electrophoresis with 1× Tris borate EDTA buffer using the 100-bp DNA ladder as a molecular weight marker, colored with ethidium bromide and visualized with an ultraviolet light transilluminator. Serotype A strain of ORT isolated from European broiler chickens (ATCC 51463) was used as a standard control.

The DNA patterns generated by rep-PCR were considered identical on the basis of similar numbers and matching positions of all bands. ORT strains were tested on at least three separate occasions to assess the reproducibility of the typing method.

## RESULTS

**Macroscopic and microscopic lesions.** Hemorrhagic tracheitis, unilateral or bilateral pneumonia, fibrinous material in the lungs, and abdominal aerosaculitis with spumous, whitish, or caseous material in the abdominal cavity were observed at necropsy. Histopathologic changes included lymphocytic tracheitis with congestion and focal hemorrhages, congestion of the lungs, and hyperplasia of peribronchial lymphoid follicles with macrophages. Infiltration and severe purulent air sacculitis were also seen. Necropsied hens showed peritonitis and hemorrhagic and atresic ovarian follicles.

**Bacteriologic isolation and identification.** Of the 75 clinical samples tested during 1998–2000, 75 strains were isolated and identified as ORT based on their morphologic characteristics of gram-negative nonsporulated nonmotile pleomorphic rods. Colonies were rounded, small (0.2–0.6 µm in diameter), greyish-white, and nonhemolytic, with a butyric odor. Only 25 demonstrated ORT as a single causal agent implicated in respiratory disease and, therefore, were considered for genetic variability studies.

ORT culture and isolation were done mostly from air sacs and trachea of broiler chickens, followed by layers and breeders. Table 1 shows the origin, source, and age of birds for the 25 ORT isolates studied. Biochemically, ORT was positive to urea, 2-nitrophenyl-β-D-galactopyranoside, lactose,

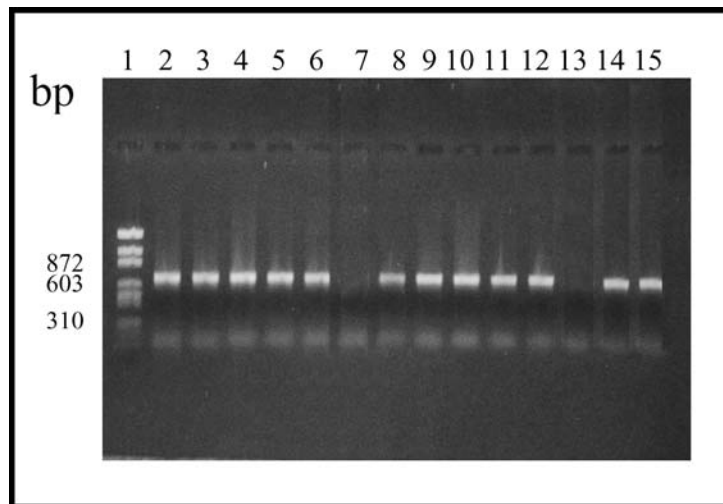


Fig. 1. ORT PCR products in 1% agarose gel. Line 1, X174RF DNA *Hae*III molecular weight marker. Line 2, ATCC 51463 standard strain. Lines 3–6, 8–12, and 14–15, local ORT strains. Line 7, local strain of *H. paragallinarum*. Line 13, local strain of *P. multocida*.

maltose, and galactose and was negative to indole, ornithine decarboxylase, and catalase.

**PCR.** To verify the reproducibility of the test, each sample was done in triplicate at different times, obtaining similar results every time. A 784-bp fragment was amplified in all samples identified microbiologically as ORT but not in *H. paragallinarum* and *P. multocida* (Fig. 1). Molecularly, the PCR reaction is a sensitive and specific test that identifies diverse microorganisms through their specific DNA band and has allowed rapid identification of ORT strains isolated from different sources in Peru.

**Rep-PCR.** All of the ORT strains showed approximately 19 bands between 0.1 and 2.6 kb, with 1.3 kb being the most prominent, which may indicate that this band is present in the genome in more than one copy or that the DNA fragments differ in their nucleotides only slightly. Three bands were also evident between 0.6 and 0.8 kb. The genetic patterns of 25 strains were all similar, demonstrating no differences for the type of bird, geographic zone, or comparison with ORT ATCC 51463 (Fig. 2). The reproducibility of the rep-PCR method was examined in all isolates by three independent amplification reactions and agarose gel electrophoresis. No change in the DNA fingerprints was observed in any of the three independent experiments (data not shown). Negative control reactions were negative. The rep-PCR technique used identified all strains (25/25) of ORT isolated from different geographic areas producing commercial birds in Peru as having the same genetic pattern, similar to that of ORT ATCC 51463.

## DISCUSSION

Although the presence of viral, bacterial, fungal, or toxic factors may stimulate the activity of ORT as a secondary pathogenic agent (14,19), there is evidence that this bacterium may cause disease without predisposing factors, acting as a primary pathogen in producing respiratory diseases (17). ORT has been isolated and identified from avian tracheal and air sac samples from several geographic zones, and its wide range of pathogenicity varied among zones and birds. Mortality rates represented 2%–3% of the total population, reaching 30%–40% in some cases, especially when interacting with other microorganisms (12,14).

Isolation of ORT from biological samples is not an easy task because it requires enriched media such as blood agar in a micro-

aerophilic condition (5%–10% carbon dioxide) and biochemical identification (14,16). Morphologically, it can be confounded with related bacteria such as *H. paragallinarum* and *P. multocida*, agents that cause infections similar to those produced by ORT. Moreover, polymicrobial infections with these pathogenic microorganisms have been reported.

Few studies have evaluated and compared the usefulness of the rep-PCR method for typing ORT. Amonsin *et al.* (1) studied 55 avian strains from several countries and found seven genotypes, with genotype A predominant in 60% (33/55). In this study, only eight isolates, originally from Belgium and South Africa, were obtained from chickens, including ORT ATCC 51463, all belonging to

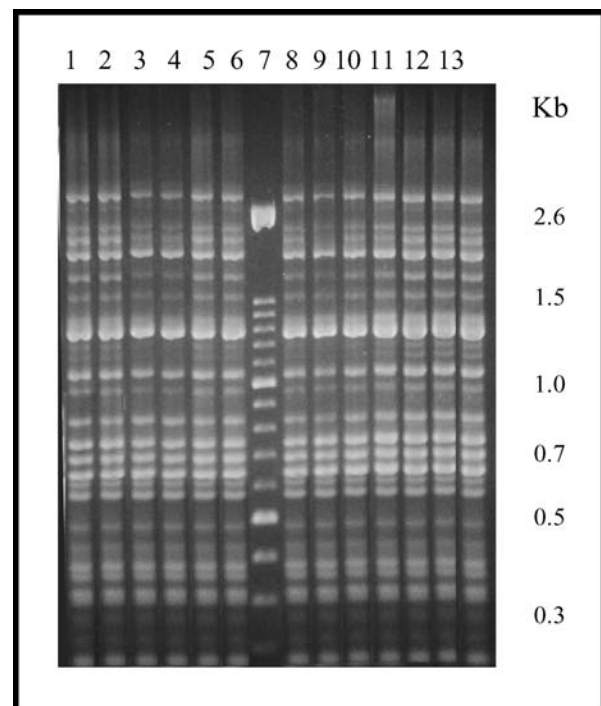


Fig. 2. Rep-PCR genetic patterns of ORT genomic DNA in 2% agarose gel. Lines 1–6 and 8–13, local ORT strains. Line 14, ATCC 51463 standard strain. Line 7, 100-bp DNA ladder molecular weight marker.

genotype A. To our knowledge, no previous chicken, breeder, or layer hen isolates from South American origin were molecularly typed by rep-PCR. Using this method, our results support the theory that genotype A could be the most common genotype circulating in diverse types of birds worldwide.

PCR and rep-PCR are fast, sensitive, and specific techniques to identify and characterize bacterial strains (9,10,14,18). However, the cost and availability of the equipment, as well as incorrect manipulation of the reagents with the amplification of contaminants, may lead to a misinterpretation of the results and, in some instances, could be a limiting factor for use of these techniques. Nevertheless, despite these features, PCR and rep-PCR are alternative methods for determining etiologic agents, especially during epidemics of respiratory disease in poultry.

Few molecular studies have been performed with respect to ORT. Therefore, it is important to continue investigations to further elucidate the genetic aspect of this bacterium and to determine the role it may play in the presentation of diverse respiratory infectious processes in birds.

There are still many unsolved questions in relation to ORT presence in Peru, such as where it came from and when it was introduced. Was it introduced by importation of birds or avian products? What role may migrating birds play in spreading the bacterium? Further investigations are planned to answer these intriguing questions.

In conclusion, we have shown that PCR and rep-PCR are efficient techniques to identify and characterize ORT strains and that there are no differences between the genetic patterns of all strains of ORT isolated from hens and broiler chickens from diverse zones of Peru and the strain ATCC 51463.

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