

Amplified 16S Ribosomal DNA Restriction Analysis for Identification of *Avibacterium paragallinarum*

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SUMMARY. A molecular technique based on the restriction fragment length polymorphism of the 16S ribosomal genes amplified by a polymerase chain reaction (PCR), referred to as amplified 16S ribosomal DNA restriction analysis (ARDRA), was designed to identify 19 *Avibacterium paragallinarum* strains isolated from infraorbital sinus and nasal turbinate bone samples of broiler chickens, breeders, and laying hens from different regions of Peru. The 16S rDNA was amplified by PCR using a pair of bacterial universal primers and restriction analysis of 16S rDNA sequences was done to select endonucleases with the highest number of cutting points inside the 16S rDNA. The DNA patterns with *DdeI* and *RsaI* endonucleases were identical for the 19 *A. paragallinarum* strains, but differed from those obtained for *Ornithobacterium rhinotracheale*, a bacterium with a high genetic and phenotypic resemblance to *A. paragallinarum*, as well as from *Escherichia coli*, a bacterium associated with infectious coryza. The ARDRA method could prove to be valuable for molecular identification of *A. paragallinarum*, a microorganism implicated in respiratory diseases in commercial birds.

RESUMEN. Análisis de restricción del DNA ribosomal 16S amplificado para la identificación de *Avibacterium paragallinarum*.

Se desarrolló una técnica molecular basada en la prueba de polimorfismo de la longitud de los fragmentos de restricción del gen 16S ribosomal amplificado por medio de la prueba de reacción en cadena por la polimerasa, denominada análisis de restricción del DNA 16S ribosomal amplificado (ARDRA, por su sigla en Inglés). Esta prueba fue diseñada para identificar 19 cepas de *Avibacterium paragallinarum* aisladas de muestras de seno infraorbitario y huesos de las fosas nasales de pollos de engorde, reproductoras pesadas y ponedoras comerciales de diferentes regiones de Perú. El DNA 16S ribosomal fue amplificados por medio de la prueba de la reacción en cadena por la polimerasa (PCR, por si sigla en Inglés), usando un par de iniciadores universales para bacterias y el análisis de las secuencias del DNA 16S ribosomal se realizó seleccionando endonucleasas con el más alto número de puntos de corte dentro del DNA ribosomal 16S. Los patrones DNA con las endonucleasas *DdeI* y *RsaI* fueron identificados para 19 cepas de *A. paragallinarum*, pero diferían de las obtenidas para *Ornithobacterium rhinotracheale*, una bacteria con altas similitudes genéticas y fenotípicas con *A. paragallinarum*, así como con *Escherichia coli*, una bacteria asociada con coriza infecciosa. El método ARDRA podría ser una herramienta valiosa para la identificación molecular de *A. paragallinarum*, un microorganismo implicado en enfermedades respiratorias de aves comerciales.

Key words: *Avibacterium paragallinarum*, *Ornithobacterium rhinotracheale*, infectious coryza, 16S ribosomal genes, ARDRA

Abbreviations: ARDRA = amplified 16S ribosomal DNA restriction analysis; EDTA = ethylenediaminetetraacetic acid; IC = infectious coryza; NADH = nicotinamide adenine dinucleotide; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; Sv = serovar; TE = Tris-EDTA

Avibacterium paragallinarum, a gram-negative, nonmotile, pleomorphic bacterium, is the causative agent of infectious coryza (IC), an upper-respiratory disease of breeders, laying hens, and broiler chickens characterized by nasal secretion, sneezing, and head swelling. Clinical disease is exacerbated when concomitant infections with *Ornithobacterium rhinotracheale* and *Escherichia coli* are present (2,3,13).

Some of the factors that contribute to the spread of IC in Peru include failure of vaccination programs, bird heaping, limited ventilation in avian farms or houses, and the use of nonchlorinated water. IC causes significant economic losses in the avian industry because of delayed growth, weight loss, increased number of discarded birds, and decreased egg production of up to 40% in laying hens (3,13).

Carbohydrate fermentation tests are commonly used for identification of *A. paragallinarum* (6), although their use is very laborious because this fastidious microorganism requires special growth conditions such as blood or chocolate agar supplemented with nicotinamide adenine dinucleotide (NADH) and a microaerophilic

atmosphere. Rapid DNA techniques include oligonucleotide fingerprinting analysis, profiles of proteins in denaturalized gels (5), restriction fragment length polymorphism (RFLP) (4), and polymerase chain reaction (PCR) (7). The first three techniques are used for strain characterization, and the last one for bacterial identification.

Because the nucleotide sequences found in 16S ribosomal genes vary in an orderly fashion throughout the phylogenetic tree, these sequences have been useful targets for taxon-specific oligonucleotide probes. When no specific primers are designed, the 16S rDNA genes could be amplified using universal bacterial primers and digested with restriction enzymes (1,9,10,11,14,15,16). Therefore, in the present study, an alternative rapid technique for *A. paragallinarum* identification based on amplified 16S ribosomal DNA restriction analysis (ARDRA) is shown.

MATERIALS AND METHODS

Bacterial strains and cultivation. A total of 19 *A. paragallinarum* strains were tested. All of the isolates were obtained from outbreaks of IC in laying hens, breeders, and broiler chickens from different poultry

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Table 1. Strains used in the present study.^A

Species	Strain	Source ^B
<i>Ornithobacterium rhinotracheale</i>	51463	ATCC
<i>Ornithobacterium rhinotracheale</i>	ORT 07	Bioservice S.R.L.
<i>Ornithobacterium rhinotracheale</i>	ORT 16	Bioservice S.R.L.
<i>Ornithobacterium rhinotracheale</i>	ORT 58	Bioservice S.R.L.
<i>Ornithobacterium rhinotracheale</i>	ORT 73	Bioservice S.R.L.
<i>Escherichia coli</i>	15222	ATCC
<i>Escherichia coli</i>	Ec 02	Bioservice S.R.L.
<i>Avibacterium paragallinarum</i>	29545 (Sv A)	ATCC
<i>Avibacterium paragallinarum</i>	Sv B	Australia
<i>Avibacterium paragallinarum</i>	Sv C	Australia
<i>Avibacterium paragallinarum</i> ^C	Apg-01	Trujillo
<i>Avibacterium paragallinarum</i> ^C	Apg-02	Huacho
<i>Avibacterium paragallinarum</i> ^C	Apg-03	Cañete
<i>Avibacterium paragallinarum</i> ^C	Apg-04	Chincha
<i>Avibacterium paragallinarum</i> ^C	Apg-05	Ate-Vitarte
<i>Avibacterium paragallinarum</i> ^C	Apg-06	Iquitos
<i>Avibacterium paragallinarum</i> ^C	Apg-07	Huacho
<i>Avibacterium paragallinarum</i> ^C	Apg-08	Ate-Vitarte
<i>Avibacterium paragallinarum</i> ^C	Apg-09	Ate-Vitarte
<i>Avibacterium paragallinarum</i> ^C	Apg-10	Ate-Vitarte
<i>Avibacterium paragallinarum</i> ^C	Apg-11	Iquitos
<i>Avibacterium paragallinarum</i> ^C	Apg-12	Pacasmayo
<i>Avibacterium paragallinarum</i> ^C	Apg-13	Ate-Vitarte
<i>Avibacterium paragallinarum</i> ^C	Apg-14	Ate-Vitarte
<i>Avibacterium paragallinarum</i> ^C	Apg-15	Chancay
<i>Avibacterium paragallinarum</i> ^C	Apg-16	Puente Piedra
<i>Avibacterium paragallinarum</i> ^C	Apg-17	Trujillo
<i>Avibacterium paragallinarum</i> ^C	Apg-18	Trujillo
<i>Avibacterium paragallinarum</i> ^C	Apg-19	Cañete

^ASv = serovar; ATCC = American Type Culture Collection.

^BThe Australian strains were provided by Dr. Backall.

^C*Avibacterium paragallinarum* isolates.

farms in Peru between January 2001 and December 2002. The collected strains of *A. paragallinarum* and *O. rhinotracheale* are listed in Table 1.

The *A. paragallinarum* isolates were grown in chocolate agar supplemented with 0.0025% NADH in 5%–10% CO₂ atmosphere for 24–48 hr (6). After three sequential cultures, a single colony of *A. paragallinarum* was grown in a modified brain–heart infusion with 30% sterile glycerol and conserved at –20 C for further examination.

DNA extraction. Genomic DNA from *A. paragallinarum* was extracted by phenol–chloroform method (12,17). Briefly, *A. paragallinarum* strains were cultivated in chocolate agar for 24 hr. One colony was then suspended in phosphate saline buffer, and the cells were collected by centrifugation at 12,000 rpm for 5 min. The pellet was resuspended in 600 µl of STE buffer (50 mM Tris, 10 mM ethylenediaminetetraacetic acid [EDTA], 100 mM NaCl), 1% (w/v) sodium dodecyl sulfate, and proteinase K (20 mg/ml) and incubated at 55 C for 4 hr. Equal volumes of phenol and chloroform were used for purification. DNA was precipitated with 3 M sodium acetate and an equal volume of isopropanol. The precipitated sample was washed with 70% ethanol and finally resuspended with Tris-EDTA (TE) buffer. Purity and concentration of extracted DNA was estimated by electrophoresis in 1% agarose gel with 1× Tris acetate EDTA buffer and compared with a λDNA/*Hind*III molecular weight marker. All chemical reagents were obtained from Amresco (Solon, OH).

PCR Test. Nineteen field isolates were tested using F1/R1 and N1/R1 primers with a heminested PCR test known to be specific for *A. paragallinarum* as described by Cheng *et al.* (7). Heminested PCR products of *A. paragallinarum* strains were verified by restriction enzyme analysis with *Alu*I, *Rsa*I, and *Mbo*I endonucleases (Invitrogen Life Technologies, Carlsbad, CA).

Amplification of 16S rDNA. Universal bacterial primers 16SF (5'-AGAGTTTGATCATGGCTCAG-3') and 16SR (5'-GGTTACCTT

GTTACGACTT-3') were used to amplify 16S ribosomal genes from all strains of *A. paragallinarum* (1). Approximately 20 ng of total DNA were subjected to PCR amplification in a total volume of 25 µl containing 1× PCR buffer, 1.5 mM MgCl₂, 200 µM deoxyribonucleotide, 1.25 U *Taq* DNA polymerase (Invitrogen Life Technologies), and 0.2 µM of each primer (16SF and 16SR). Amplification conditions were 94 C for 4 min, 35 cycles of 94 C for 45 sec, 55 C for 1 min, and 72 C for 45 sec, with a final extension of 72 C for 3 min, using a 2400 Perkin Elmer thermal cycler (Norwalk, CT).

Two microliters of PCR products were analyzed by electrophoresis in 1% agarose gel electrophoresis using 1× TAE buffer, stained with ethidium bromide, and visualized in a transilluminator. All assays included *A. paragallinarum* DNA of the ATCC 29545 strain as a positive control and *E. coli* and *O. rhinotracheale* DNA as negative controls.

Restriction enzyme analysis. Approximately 1 µg of amplified DNA was digested with 4 U of *Dde*I, *Rsa*I, and *Eco*RI (Invitrogen Life Technologies) in a volume of 25 µl at the optimal conditions as described by the manufacturer. The Web cutter program (<http://Tools.NEB.com//NEBcutter2/index.PHP>; New England Biolabs, Ipswich, MA) was used to select endonucleases with the highest number of cutting points inside the 16S rDNA. The *Dde*I and *Rsa*I enzymes were selected because they had several cutting points in 16S ribosomal DNA, gave unique restriction patterns for each species, and demonstrate DNA fragments greater than 100 bp, which allows for easier visualization and interpretation. The *Eco*RI enzyme was also used because there was one cutting point in the *A. paragallinarum* 16S rDNA and none in *O. rhinotracheale*.

Digestion products were separated by electrophoresis using 3% agarose gel in 0.5× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8), stained with ethidium bromide and visualized in a transilluminator.

Data analysis. The molecular size of the DNA fragments was estimated using DNA molecular weight markers and DNA collection strain patterns.

RESULTS

Identification of bacteria. After the incubation period, small grayish colonies were observed in chocolate agar. Gram-negative, filamentous, pleomorphic bacteria were identified on microscopic observation. A total of 19 *A. paragallinarum* strains were identified by PCR test described by Cheng *et al.* (7). Specific DNA fragments of approximately 1600 bp and 500 bp were amplified using F1/R1 and N1/R1 primers from *A. paragallinarum* strains. No DNA fragments of this size were amplified for *O. rhinotracheale*. Restriction patterns of heminested PCR products were made to determine the specificity of the test, and similar DNA patterns were observed in *A. paragallinarum* isolates (Fig. 1).

ARDRA. A PCR product of approximately 1400 bp was obtained for each strain of *A. paragallinarum*, indicating that 16S rDNA size is highly similar in all the operons from *A. paragallinarum* strains studied. PCR amplifications with the same oligonucleotide primers were performed with *E. coli* and *O. rhinotracheale* and a single band of similar size was obtained.

PCR products of the 16S rDNA from all strains of *A. paragallinarum* were digested with *Dde*I, *Rsa*I, and *Eco*RI endonucleases. The 19 strains showed identical RFLP patterns for each enzyme (Fig. 2).

To differentiate *A. paragallinarum* from other phylogenetically related species such as *O. rhinotracheale*, digestions of the PCR products of the 16S ribosomal genes with the same three endonucleases were performed. Point mutations along of amplified 16S ribosomal genes of *A. paragallinarum*, *O. rhinotracheale*, and *E. coli* were detected by *Dde*I enzyme; specific DNA patterns were observed for each one after agarose gel electrophoresis (Fig. 3).

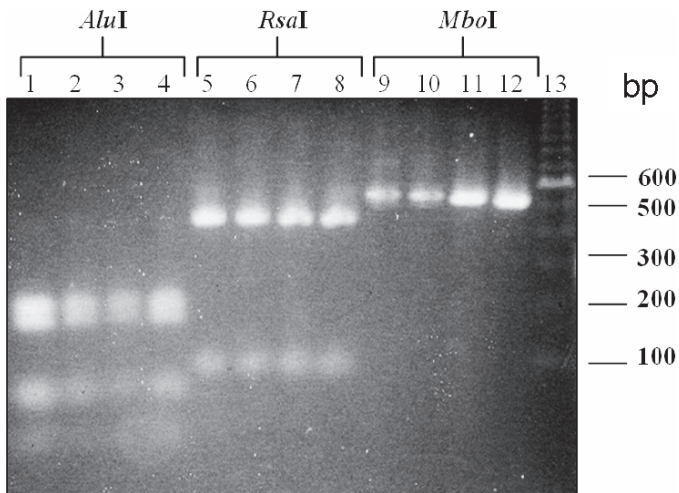


Fig. 1. Heminested PCR products from *A. paragallinarum* strains using F1/R1 and N1/R1 primers (7) digested by restriction enzymes in 3% agarose gel. *AluI*: lane 1, ATCC 29545; 2, strain Apg-18; 3, Apg-17; 4, Apg-01; *RsaI*: 5, ATCC 29545; 6, Apg-18; 7, Apg-17; 8, Apg-01; *MboI*: 9, ATCC 29545; 10, Apg-18; 11, Apg-17; 12, Apg-01; 13, ladder 100-bp (molecular weight marker).

DISCUSSION

The ARDRA technique, as applied to bacteria, is based on 16S ribosomal genes as DNA targets because they are generally present in numbers ranging from one to 11 copies within the genome, which may increase the sensitivity of this molecular biotechnique.

ARDRA has been used for the identification of different bacterial species (9,13,14,15). In this study, different DNA profiles were obtained for *E. coli*, *A. paragallinarum*, *O. rhinotracheale*, *Pasteurella multocida*, *Pasteurella gallinarum*, and *Gallibacterium anatis* (data not shown), all of which are routinely encountered in the analysis of upper respiratory tract disease in birds, demonstrating that the ARDRA technique may not only allow the identification of *A. paragallinarum*, but also of other bacteria implicated in respiratory infections in birds, and become a suitable test method for genotyping. Of the 19 analyzed strains from different avian regions of Peru, five were identified by traditional bacteriologic techniques as *O. rhinotracheale*, indicating that conventional techniques do not differentiate microorganisms with similar phenotypic characteristics and that the development of specific methods are required.

The PCR test (7) and the ARDRA technique developed in this study identified all *A. paragallinarum* strains listed in Table 1. Therefore, the ARDRA technique may be used as an alternative approach for identification of *A. paragallinarum* because the RFLP patterns of amplified 16S ribosomal genes are characteristic for the causative agent of IC. It is remarkable that nucleotide changes were found in 16S ribosomal genes from *A. paragallinarum* sv A, sv B, Hpg 08, and Hpg 19. These four strains of *A. paragallinarum* demonstrate a *DdeI* cutting point in position 623 of 16S ribosomal gene, which was not present in the other 17 strains and *A. paragallinarum* sv C. Nevertheless, this finding did not affect the species-specific identification profiles. These types of polymorphisms in stable molecules such as 16S ribosomal genes have been previously reported in *Helicobacter pylori*, *Ureaplasma urealyticum*, *E. coli*, *Vibrio cholerae*, and *Bacillus subtilis* (8). Variation between pairs of sequences of the 16S ribosomal genes in one bacterial strain or in

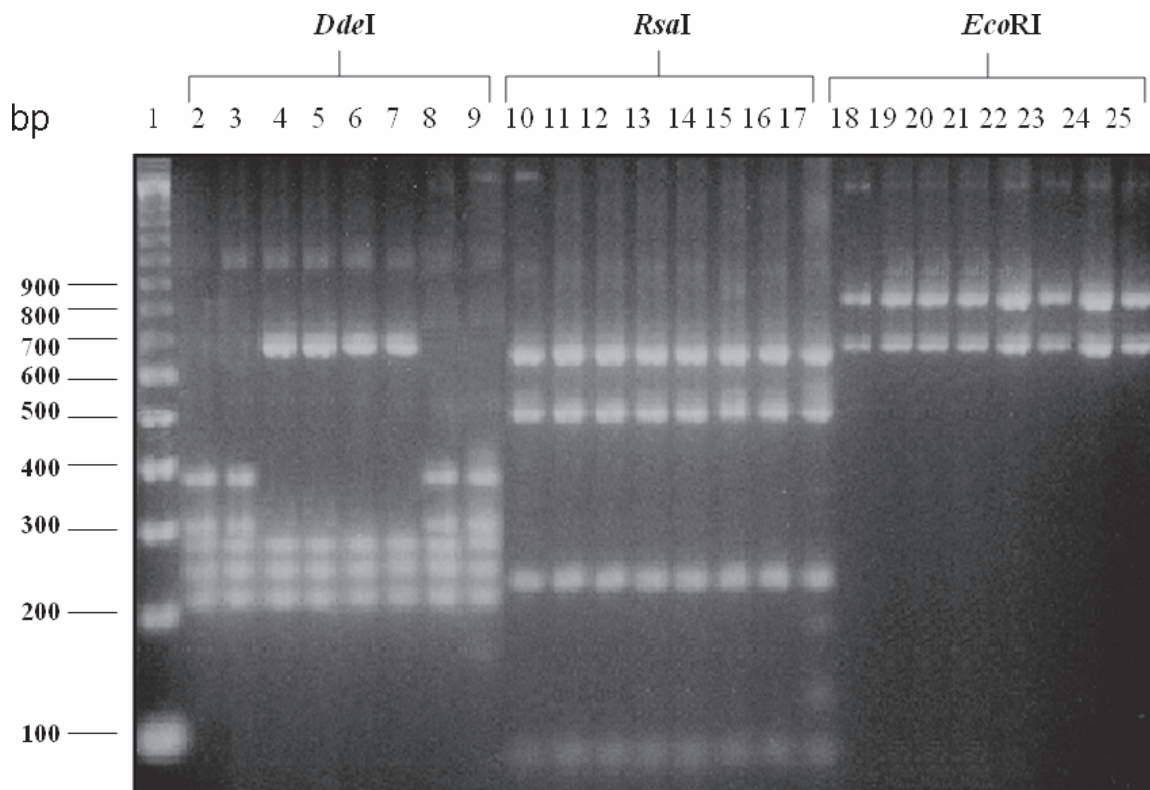


Fig. 2. 16S ribosomal genes digestion products of *A. paragallinarum* in 3% agarose gel. *DdeI*: lane (strain): 1, ladder 100-bp molecular weight marker; 2, ATCC 29545; 3, Sv B; 4, Sv C; 5, Apg-05; 6, Apg-04; 7, Apg-13; 8, Apg-08; 9, Apg-19; *RsaI*: 10, ATCC 29545; 11, Sv B; 12, Sv C; 13, Apg-05; 14, Apg-04; 15, Apg-13; 16, Apg-08; 17, Apg-19; *EcoRI*: 18, ATCC 29545; 19, Sv B; 20, Sv C; 21, Apg-05; 22, Apg-04; 23, Apg-13; 24, Apg-08; 25, Apg-19.

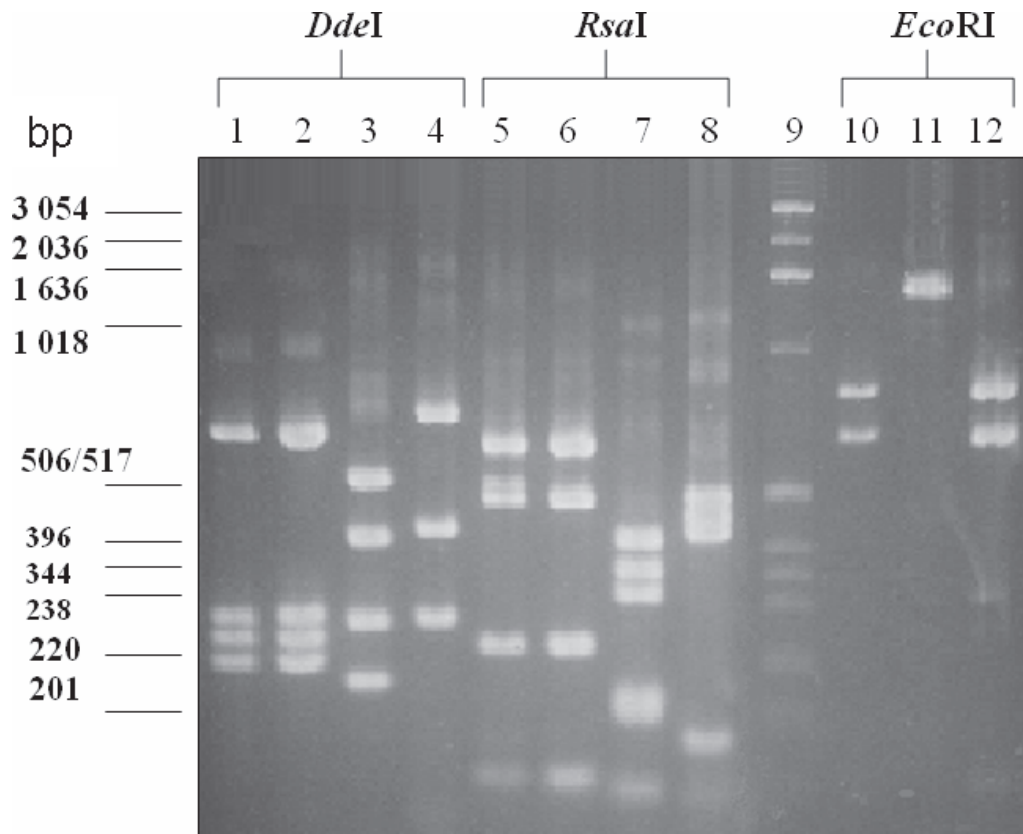


Fig. 3. Digestion fragments of the 16S ribosomal genes of *A. paragallinarum*, *O. rhinotracheale*, and *E. coli* in 3% agarose gel. Lanes: *DdeI* 1, Apg-17; 2, Apg-01; 3, *O. rhinotracheale* ATCC 51463; 4, *E. coli* ATCC 15222. *RsaI*: 5, Apg-17; 6, Apg-01; 7, *O. rhinotracheale* ATCC 51463; 8, *E. coli* ATCC 15222; 9, Ladder 1Kb; *EcoRI*: 10, Apg-17; 11, *O. rhinotracheale* ATCC 51463; 12, *E. coli* ATCC 15222.

different strains of the same species ranges from 1% to 5% (8). Although the 16S ribosomal gene sequences of these bacteria may be conserved, the nine serovariants of *A. paragallinarum* existing in the world must be investigated to determine if there is any correlation with the polymorphism found with the *DdeI* restriction enzyme.

In conclusion, ARDRA is a reliable and highly specific technique because it identifies a unique genetic profile for *A. paragallinarum*, which is distinctly different from the genetic profiles of *O. rhinotracheale* and *E. coli*.

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