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Molecular Identification and Genetic Diversity of *Avibacterium paragallinarum* Isolated from Chickens with Infectious Coryza in Bogor, Indonesia

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ABSTRACT

Infectious coryza, a respiratory disease caused by *Avibacterium paragallinarum* (*A. paragallinarum*), poses a major threat to poultry health and productivity, particularly in tropical countries such as Indonesia. This study aimed to isolate, molecularly identify, and characterize the genetic diversity of *A. paragallinarum* from chickens exhibiting clinical symptoms of coryza. A total of 60 infraorbital sinus swab samples were aseptically collected from commercial layer and broiler chickens in Bogor Regency, West Java, Indonesia. The method consists of phenotypic identification (culture on Nicotinamide Adenine Dinucleotide [NAD], Gram staining, and biochemical tests) and molecular confirmation using Polymerase Chain Reaction (PCR) HPG-2, Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR, and *16S rRNA* sequencing. Eight field isolates were successfully cultured on NAD-supplemented blood agar, Gram-negative, catalase and oxidase-negative. Molecular confirmation was conducted using species-specific HPG-2, with all isolates amplifying the expected 500 bp product. The evaluation of genetic diversity was conducted through ERIC-PCR, which identified five unique clusters among the isolates, demonstrating considerable genomic variation. Furthermore, partial *16S rRNA* gene sequences were amplified and analyzed through phylogenetic tree construction and BLAST comparison. Sequence analysis revealed 95.7–99.2% homology with reference *A. paragallinarum* strains in GenBank, and 96.5–99.2% homology among the study isolates themselves. The phylogenetic tree highlighted a close relationship among the local isolates, yet also indicated a distinct genetic lineage from several international reference strains, suggesting possible regional specificity. This study provided the first detailed genetic characterization of *A. paragallinarum* field isolates in Indonesia using *16S rRNA* sequencing. These findings highlighted the need for continuous molecular surveillance to guide accurate diagnosis.

Keywords: *16S rRNA* sequencing, *Avibacterium paragallinarum*, Field isolate, Molecular identification

INTRODUCTION

Coryza, also known as snot, is one of the infectious diseases that affects the respiratory system in chickens. This disease may affect all varieties of chickens, including laying hens, broilers, and native breeds (El-Gazzar et al., 2025). Coryza is induced by the bacterium *Avibacterium paragallinarum*, which was originally classified as *Haemophilus paragallinarum* (Blackall et al., 2005). *Avibacterium paragallinarum* is classified as a Gram-negative bacterium and a member of the *Pasteurellaceae* family (Liu et al., 2023). Clinical symptoms in infected chickens include conjunctivitis, unilateral or bilateral ocular inflammation in acute conditions, and serous exudate leading to caseous sinusitis in chronic conditions (Babazadeh and El-Ghany, 2023). Coryza has a significant economic impact on poultry farming.

The mortality rate in chickens is relatively low, around 1%-5% but may reach up to 30% under severe or uncontrolled conditions; however, the morbidity rate might reach 80%-100% (Kusumaningsih and Poernomo, 2000). In laying hens, the productivity decline might be as much as 10%-40% (Blackall and Soriano-Vargas, 2020). The eradication of coryza disease in developing countries is challenging due to multiple factors and the presence of mixed infections. This disease may rapidly spread from one chicken to another within a flock or across different flocks (Clothier et al., 2019). Direct transmission can occur between infected or carrier chickens and other susceptible chickens (Pierdon et al., 2025). Coryza may

infect, particularly during seasonal transitions or due to various types of stress, such as the cage environment, weather, nutrition, vaccination efforts, and diseases that reduce immunity (Moenek, 2016).

Diagnosis of coryza is acquired by visible clinical symptoms and confirmed by several tests, including bacterial isolation, biochemical characterization, and polymerase chain reaction (PCR) test; nevertheless, culturing *A. paragallinarum* bacteria faces significant challenges (Deresse et al., 2022). This bacterium has a slow growth rate, requires particular media and environmental conditions, and shows little reactivity in biochemical tests (Akter et al., 2016). This microorganism is frequently associated with co-infection involving other pathogens, such as *Escherichia coli, Pasteurella multocida, Bordetella avium, and Mycoplasma gallisepticum*, which also complicate the isolation of pure cultures and diagnosis of the disease (Sarika et al., 2019).

According to Page (1962), *A. paragallinarum* bacteria are categorized into three serogroups: A, B, and C. On the other hand, the Kume classification differentiates these bacteria into nine serovars: A-1, A-2, A-3, A-4, B-1, and C-1, C-2, C-3, C-4 (Kume et al., 1983; Buter et al., 2023). Both classifications derive from the results of the hemagglutination inhibition (HI) test. Subsequent development began using multiplex PCR for serotyping (Sakamoto et al., 2012). The amplification of *16S ribosomal ribonucleic acid* (*16S rRNA*) is often selected for genus and species identification due to its universal presence in all bacteria (Srinivasan et al., 2015). The *16S rRNA* gene serves as a conserved molecular marker widely employed for studying bacterial phylogeny and taxonomic relationships (Indraswari et al., 2021). Despite the economic burden caused by infectious coryza in Indonesia, studies into the molecular characteristics of *A. paragallinarum*, particularly its genetic diversity, remain limited. Notably, no studies have been published on the *16S rRNA* gene sequences of field isolates from Indonesia. This hinders the understanding of strain variation and its epidemiological consequences. The present study aimed to isolate, molecularly identify, and sequence the *16S rRNA* of *A. paragallinarum* bacteria from chickens exhibiting coryza symptoms.

MATERIALS AND METHODS

Ethical approval

All chicken samples and related procedures were ethically approved by the Animal Ethics Committee, School of Veterinary Medicine and Biomedical Sciences, Bogor, Indonesia, with approval number 293/KEH/SKE/II/2025.

Sample collection

A total of 60 swab samples were collected from four commercial farms located in Bogor Regency, West Java, Indonesia. Samples were taken from the layer and broiler chickens that showed symptoms of nasal discharge, lacrimation, and head swelling. The exudate was aseptically collected from the infraorbital sinus using a sterile swab.

Isolation and identification

All the sample swabs were inoculated onto 5% chicken blood agar (CBA; Oxoid Ltd., UK) supplemented with nicotinamide adenine dinucleotide (NAD; Oxoid Ltd., UK), and incubated at 37°C for 24 hours. The growth on the plates was subsequently evaluated. Afterward, the colonies underwent Gram staining and testing, followed by the observation of specific characteristics, which included a lack of growth on catalase and being oxidase negative, along with indole production, H_2S production, and being non-hemolytic on CBA. The suspected colonies from each sample were then passaged onto new CBA to isolate pure suspected colonies of *A. paragallinarum* (Tangkonda et al., 2019; Fauziah et al., 2021).

DNA extraction

The DNA from bacteria was extracted using the boiling method. Around 2-3 well-separated colonies of *A. paragallinarum*, which corresponded to an estimated cell mass of about 10^8 colony-forming units (CFU), were placed into 500 µL of nuclease-free water within an Eppendorf tube. The mixture was then heated to 98°C for 10 minutes using a dry bath incubator, followed by a freeze for 5 minutes. The tube was centrifuged at 10,000 rpm for one minute. After the DNA was extracted, 50 µL of the supernatant was collected and stored at -20°C until further analysis (Dashti et al., 2009).

Species-specific polymerase chain reaction (PCR)

The polymerase chain reaction test adheres to the method reported by Chen et al. (1996) and Putra et al. (2023) with some modifications. The final volume was 50 µL, which contained 25 µL MyTaq HS Red Mix, 2 µL of both forward and reverse HPG-2 primers (Table 1), 16 µL nuclease-free water, and 5 µL of DNA template. The PCR process entails pre-denaturation at 95°C for one minute, followed by denaturation, annealing, and extension at 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 10 seconds, for as many as 30 cycles using a thermal cycler. Amplicons were visualized using agarose gel electrophoresis with Ethidium bromide staining in 100 mL of 1 × TAE buffer.

Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

The PCR kit utilized for ERIC-PCR was obtained from the KAPA2G Fast Hotstart Readymix PCR Kit (Kapa Biosystem, Wilmington, MA, USA). The amplification consists of three minutes at 95°C and 35 cycles each consisting of 95°C for one minute, annealing at 52°C for 30 seconds, and 72°C for 6 minutes, followed by a final extension step of five minutes at 72°C. The

amplified products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The fragment sizes were estimated with a 100 bp molecular weight marker (VC 100 bp Plus DNA Ladder, Vivantis, Selangor, Malaysia). Banding patterns were assessed using PyElph software version 1.4 to create a binary matrix. Hierarchical clustering was then conducted employing the Ward's linkage method in Minitab version 18. The primer pair ERIC1R as reverse primer and ERIC2 as forward primer used is shown in Table 1.

Table 1. The PCR primers used in this present study for the identification of *Avibacterium paragallinarum*

Gene	Primer sequence	Reference
HPG-2	(F) 5′-TGAGGGTAGTCTTGCACGCGAAT-3′ (R) 5′-CAAGGTATCGATCGTCTCTCTACT-3′	Chen et al. (1996)
ERIC	(F) 5′-AAGTAAGTGACTGGGGTGAGCG-3′ (R) 5′-ATGTAAGCTCCTGGGGATTCAC-3′	Soriano et al. (2004)
16S rRNA	(F) 5′-AGAGTTTGATCMTGGCTCAG-3′ (R) 5′-TACGGYTACCTTGTTACGACTT-3′	Jeong et al. (2020)

HPG-2: *Haemophilus paragallinarum*, ERIC: Enterobacterial Repetitive Intergenic Consensus, *16S rRNA*: *16S ribosomal RNA*

16S rRNA sequencing

The isolates that had been identified as *A. paragallinarum* were tested for the presence of the *16S rRNA* gene using *16S rRNA* primers (Table 1). The PCR process was conducted with MyTaq HS Red Mix (Bioline), comprising a total reaction volume of 50 µl and an annealing temperature set at 56°C. First BASE Laboratories, Malaysia, performed Sanger sequencing, which separated the PCR products using electrophoresis, and the target band was purified for sequencing. The nucleotide and amino acid sequences of a recently sequenced *16S rRNA* gene were determined using Bioedit v.7 (https://bioedit.software.informer.com/7.0/), and ClustalW was used for alignment. A modern phylogenetic tree was constructed using MEGA v 7.0 (https://www.megasoftware.net/) with the neighbor-joining method and 1,000 bootstrap replicates. The phylogenetic tree was compared the the present study isolates and other prototype *A. paragallinarum* isolates from GenBank. The genetic distance between isolates and the topology of the phylogenetic tree were used to compare strains. The genetic similarity percentage among all study strains was calculated using the maximum composite likelihood model available in MEGA v 7.0.

Nucleotide sequence accession numbers

The partial coding sequence (CDS) of the *16S rRNA* gene from all *A. paragallinarum* isolates examined in this study has been submitted to GenBank and assigned the accession numbers PV653197, PV653198, PV653199, PV653200, PV653201, PV653202, PV653203, and PV653204.

RESULTS

Phenotypic identification

Only eight isolates were successfully cultured on agar media and subjected to further characterization. Every isolate demonstrated growth on NAD-CBA, and non-hemolysis was observed (Figure 1A). The characteristics of the bacterial cells were Gram-negative with a coccoid bipolar structure (Figure 1B). Furthermore, all isolates were found to be negative for catalase and oxidase, indole negative, and none were able to produce H_2S (Table 2).

Molecular identification

Species-specific PCR was conducted on all samples to verify their identification as *A. paragallinarum.* All samples produced a 500 bp amplicon, confirming the presence of *A. paragallinarum* (Figure 2).

Avibacterium paragallinarum diversity based on Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) displayed differing patterns of DNA fragments across the eight *A. paragallinarum* isolates examined in this study. There were 3-8 bands generated in these fragment patterns, with sizes ranging from 300–2800 bp (Figure 3A). Hierarchical clustering analysis of DNA fragment patterns was conducted using the Dice similarity coefficient and Ward's linkage method, applying a 95% similarity threshold to define genetic clusters (Figure 3B). Based on this analysis, five clusters were identified: Cluster I (Apg-6), Cluster II (Apg-5 and Apg-8), Cluster III (Apg-2 and Apg-3), Cluster IV (Apg-1), and Cluster V (Apg-4 and Apg-7).

Homology and phylogenetic investigation

The amplification of the partial PCR product for *16S rRNA* is illustrated in Figure 4A. Using the BLAST algorithm to analyze the nucleotide sequences of *16S rRNA* indicated that the present *A. paragallinarum* isolates exhibit a high degree of sequence similarity, ranging from 95.7–99.2% with the NCBI database reference strains. When compared among the recent study isolates (Apg-1 to Apg-8), the sequences obtained from all eight isolates demonstrated a homology of 96.5-99.2% (Figure 4B). A phylogenetic analysis of *16S rRNA* sequences,

incorporating 33 sequences, which included the eight isolates identified in this study and 25 sequences available in GenBank, representing strains isolated from various geographical regions such as Japan, China, Mexico, and the United States. A phylogenetic tree created using the neighbor-joining method was shown in Figure 5.

Table 2. Biochemical and morphological characteristics of *Avibacterium paragallinarum* study isolates

Test Performed \ Isolate code	Apg-1	Apg-2	Apg-3	Apg-4	Apg-5	Apg-6	Apg-7	Apg-8
Gram nature	Negative Rods	Negative Rods	Negative Rods	Negative Rods	Negative Rods	Negative Rods	Negative Rods	Negative Rods
Catalase	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-
Hemolysis on BA	-	-	-	-	-	-	-	-
NAD dependence	+	+	+	+	+	+	+	+

Apg: *Avibacterium paragallinarum* isolate, H_2S: Hydrogen Sulphide, BA: Blood agar, NAD: Nicotinamide adenine dinucleotide

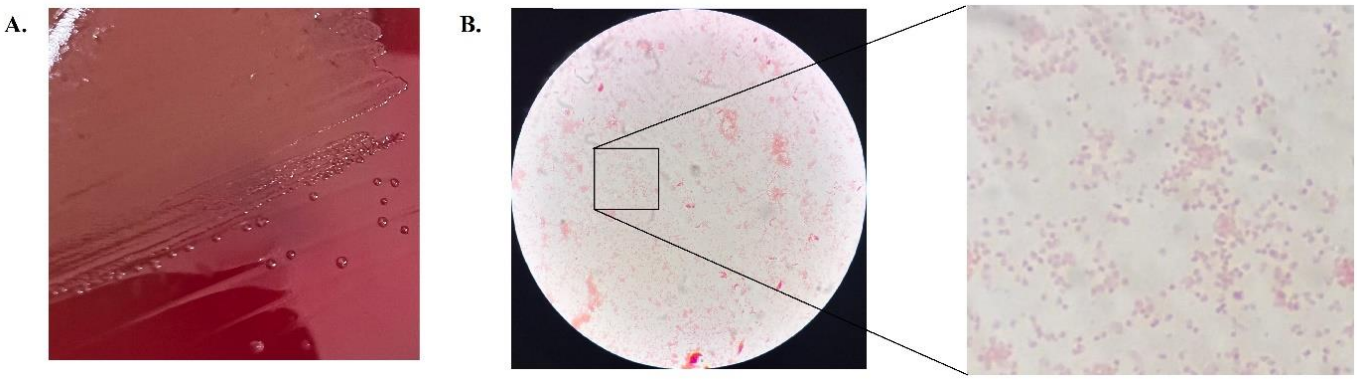


Figure 1. Satellite colonies of *Avibacterium paragallinarum* (**A**) and coccoid bipolar structure of gram-negative bacteria isolated from infraorbital sinus swabs of chickens in Indonesia (Gram staining, ×1000, **B**).

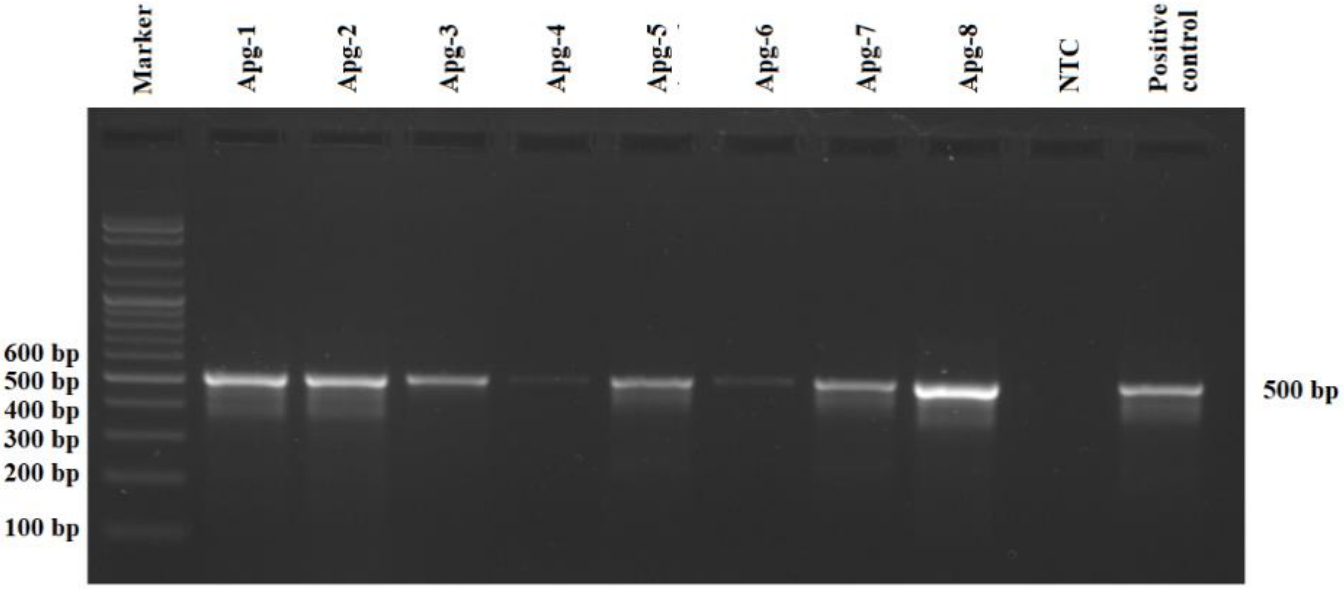


Figure 2. Molecular detection of HPG-2 PCR from Indonesian field isolates of *Avibacterium paragallinarum*

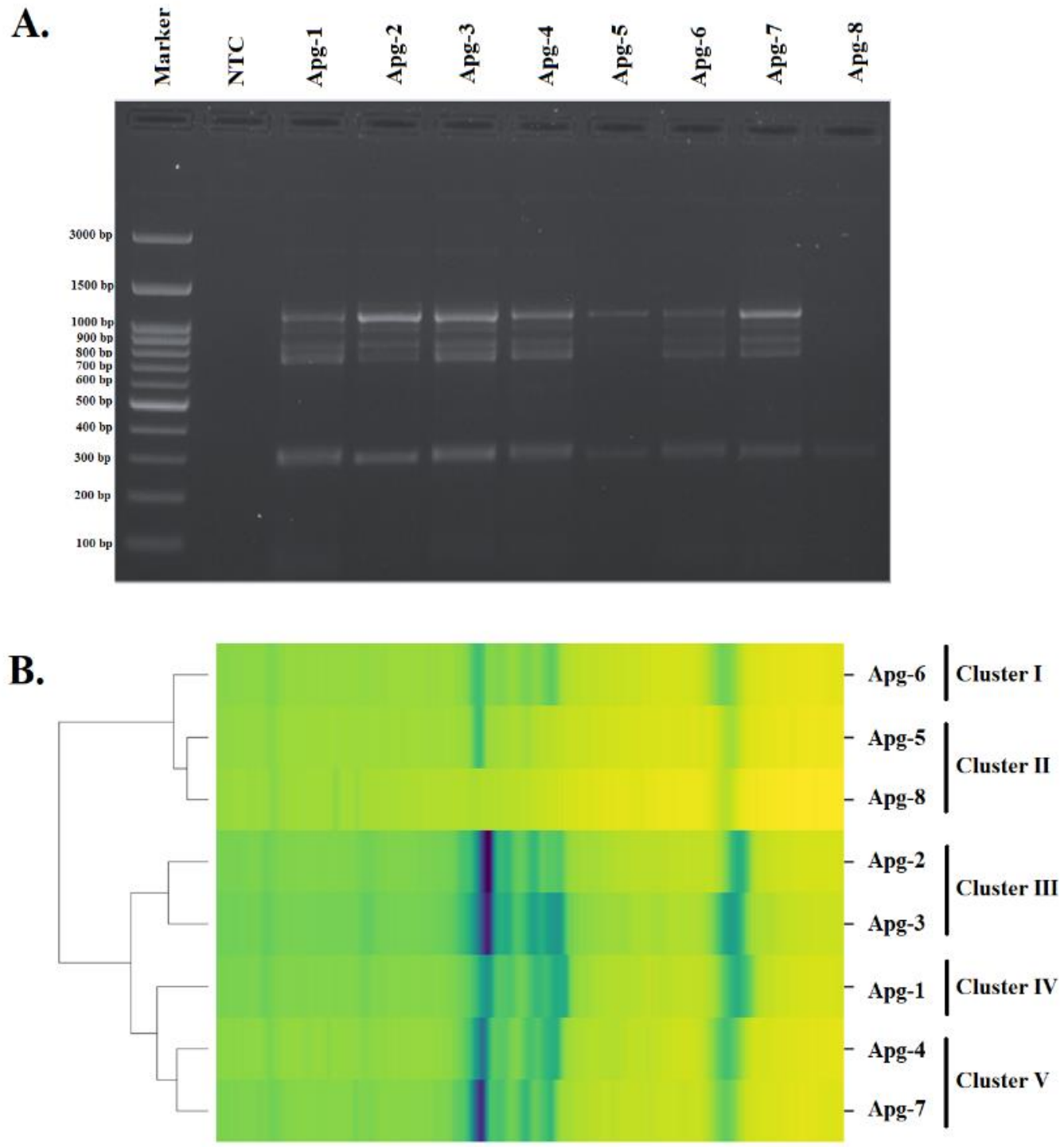
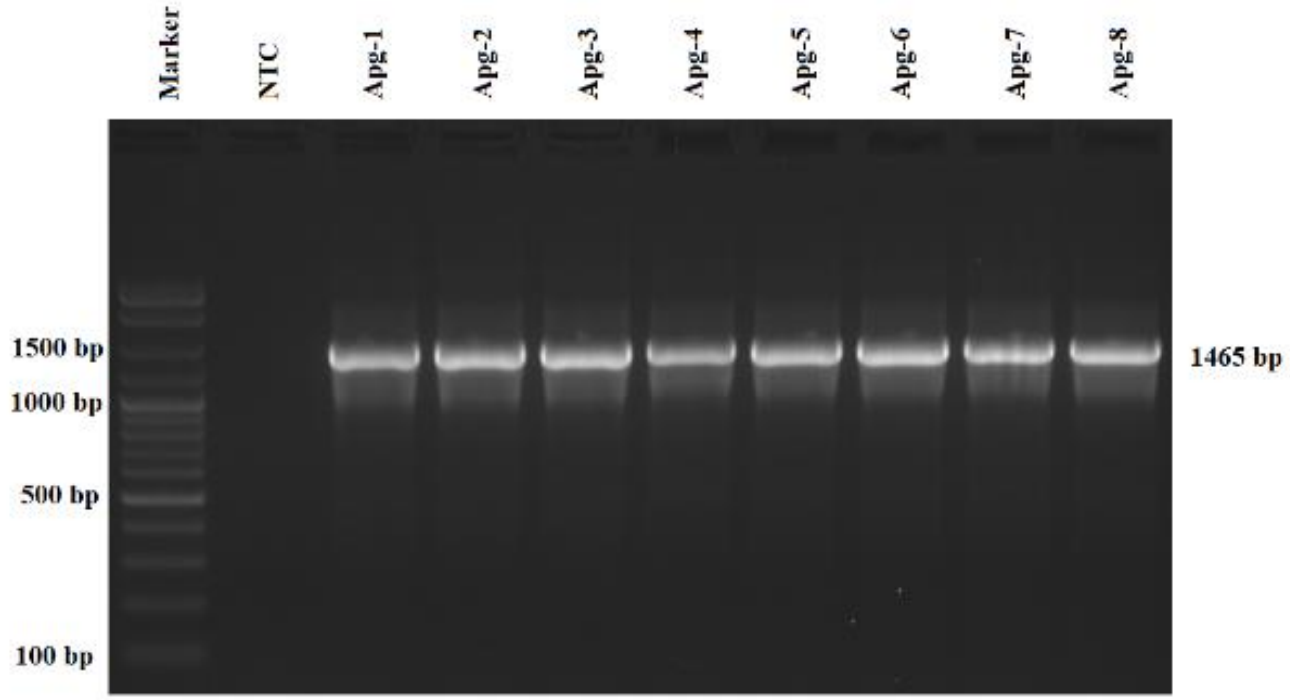


Figure 3. ERIC-PCR banding patterns obtained for eight *Avibacterium paragallinarum* field isolates collected from chickens in Indonesia (**A**), showing 3-8 DNA bands ranging from 300-2800 bp. The dendrogram generated using Ward's linkage method (**B**) revealed five distinct clusters, indicating genetic diversity among the isolates. NTC: No template control.

A.



B.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	
1		99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.7	99.6	99.7	99.6	99.7	99.6	99.7	99.5	99.8	99.7	99.6	99.7	99.5	99.6	99.7	99.5	99.7	99.4	99.4	99.4	99.5	99.3	99.4	99.3	99.3	1 JN592515.1_Avibacterium_endocarditidis_strain_B11/9902
2	99.8		99.9	99.9	99.9	99.9	99.9	99.9	99.7	99.6	99.7	99.6	99.7	99.6	99.7	99.6	99.8	99.7	99.7	99.7	99.5	99.6	99.7	99.6	99.7	99.5	99.4	99.4	99.5	99.4	99.4	99.4	99.3	2 M75068.1_Avibacterium_volantium_strain_NCTC_4101
3	99.9	99.9		100.0	99.9	99.9	99.9	99.9	99.7	99.6	99.7	99.6	99.7	99.6	99.7	99.6	99.8	99.7	99.6	99.7	99.5	99.6	99.7	99.6	99.7	99.5	99.4	99.4	99.5	99.4	99.4	99.4	99.3	3 NR_025937.1_Avibacterium_volantium_strain_NCTC_3438
4	99.9	99.9	100.0		99.9	99.9	99.9	99.9	99.7	99.6	99.7	99.6	99.7	99.6	99.7	99.6	99.8	99.7	99.6	99.7	99.5	99.6	99.7	99.6	99.7	99.5	99.4	99.4	99.5	99.4	99.4	99.4	99.3	4 NR_115217.1_Avibacterium_volantium_strain_CCUG_3713
5	99.8	99.8	99.9	99.9		100.0	99.9	99.9	99.7	99.6	99.7	99.6	99.7	99.6	99.7	99.6	99.8	99.7	99.7	99.7	99.5	99.6	99.7	99.6	99.7	99.5	99.4	99.4	99.5	99.4	99.4	99.4	99.3	5 NR_044750.1_Avibacterium_avium_strain_NCTC_11297
6	99.8	99.8	99.9	99.9	100.0		99.9	99.9	99.7	99.6	99.7	99.6	99.7	99.6	99.7	99.6	99.8	99.7	99.7	99.7	99.5	99.6	99.7	99.6	99.7	99.5	99.4	99.4	99.5	99.4	99.4	99.4	99.3	6 NR_116216.1_Avibacterium_avium_strain_CCUG_12833
7	99.8	99.8	99.9	99.9	99.8	99.8		100.0	99.7	99.6	99.7	99.6	99.7	99.5	99.7	99.5	99.7	99.7	99.6	99.7	99.5	99.6	99.7	99.5	99.7	99.4	99.4	99.4	99.5	99.3	99.4	99.3	99.3	7 NR_118754.1_Avibacterium_gallinarum_strain_NCTC_11188
8	99.8	99.8	99.9	99.9	99.8	99.8	100.0		99.7	99.6	99.7	99.6	99.7	99.5	99.7	99.5	99.7	99.7	99.6	99.7	99.5	99.6	99.7	99.5	99.7	99.4	99.4	99.4	99.5	99.3	99.4	99.3	99.3	8 JN592516.1_Avibacterium_sp._RB410
9	98.5	98.7	98.6	98.6	98.7	98.7	98.5	98.5		99.8	99.7	99.8	100.0	99.7	100.0	99.7	99.7	99.9	99.8	99.9	99.7	99.7	100.0	99.7	99.6	99.6	99.5	99.5	99.6	99.5	99.5	99.4	99.4	9 AY498870.1_Haemophilus_paragallinarum_strain_Modesto
10	98.1	98.2	98.2	98.2	98.2	98.2	98.1	98.1	99.3		99.7	99.9	99.8	99.8	99.8	99.8	99.6	99.7	99.7	99.7	99.8	99.8	99.8	99.8	99.7	99.7	99.5	99.6	99.7	99.5	99.5	99.5	99.5	10 AY498871.1_Haemophilus_paragallinarum_strain_SA7177
11	98.6	98.6	98.7	98.7	98.8	98.8	98.6	98.6	99.2	98.7		99.6	99.7	99.6	99.7	99.6	99.6	99.7	99.7	99.7	98.6	99.6	99.7	99.6	99.5	99.6	99.4	99.4	99.6	99.4	99.4	99.4	99.3	11 GJ737687.1_Avibacterium_paragallinarum_strain_SCDY_1
12	97.8	98.0	97.9	97.9	98.0	98.0	97.8	97.8	99.2	99.7	98.4		99.8	99.9	99.8	99.9	99.6	99.7	99.7	99.7	99.9	99.8	99.8	99.9	99.7	99.7	99.6	99.6	99.7	99.6	99.6	99.5	99.5	12 GU561543.1_Avibacterium_paragallinarum_strain_HP105
13	98.5	98.7	98.6	98.6	98.7	98.7	98.5	98.5	100.0	99.3	99.2	99.2		99.7	100.0	99.7	99.7	99.9	99.8	99.9	99.7	99.7	100.0	99.7	99.6	99.6	99.5	99.5	99.6	99.5	99.5	99.4	99.4	13 GU561544.1_Avibacterium_paragallinarum_strain_HP107
14	97.7	97.9	97.8	97.8	97.9	97.9	97.7	97.7	99.2	99.6	98.4	99.8	99.2		99.7	100.0	99.6	99.7	99.7	99.7	100.0	99.8	99.7	100.0	99.7	99.7	99.6	99.6	99.7	99.6	99.6	99.5	99.5	14 KC951265.1_Avibacterium_paragallinarum_strain_ESV-129
15	98.5	98.7	98.6	98.6	98.7	98.7	98.5	98.5	100.0	99.3	99.2	99.2	100.0	99.2		99.7	99.7	99.9	99.8	99.9	99.7	99.7	100.0	99.7	99.6	99.6	99.5	99.5	99.6	99.5	99.5	99.4	99.4	15 KC951275.1_Avibacterium_paragallinarum_strain_HP60
16	97.7	97.9	97.8	97.8	97.9	97.9	97.7	97.7	99.2	99.6	98.4	99.8	99.2	100.0	99.2		99.6	99.7	99.7	99.7	100.0	99.8	99.7	100.0	99.7	99.7	99.6	99.6	99.7	99.6	99.6	99.5	99.5	16 KM361708.1_Avibacterium_paragallinarum_strain_FARPER-107
17	99.3	99.5	99.4	99.4	99.5	99.5	99.3	99.3	98.9	98.2	98.2	98.2	98.9	98.1	98.9	98.1		99.7	99.7	99.7	99.6	99.6	99.7	99.6	99.7	99.5	99.4	99.4	99.5	99.3	99.4	99.3	99.3	17 KT371940.1_Avibacterium_paragallinarum_strain_ESV-142
18	98.5	98.7	98.6	98.6	98.7	98.7	98.5	98.5	99.8	99.1	99.2	99.0	99.8	99.0	99.8	99.0	98.9		99.9	100.0	99.7	99.7	99.9	99.7	99.6	99.6	99.5	99.5	99.6	99.4	99.5	99.4	99.4	18 KT371941.1_Avibacterium_paragallinarum_strain_ESV-184
19	98.4	98.6	98.5	98.5	98.6	98.6	98.4	98.4	99.7	99.0	99.1	99.0	99.7	99.0	99.7	99.0	98.8	99.9		99.9	99.7	99.7	99.8	99.7	99.6	99.5	99.4	99.5	99.6	99.4	99.4	99.4	99.4	19 KT379998.1_Avibacterium_paragallinarum_strain_ESV-244
20	98.5	98.7	98.6	98.6	98.7	98.7	98.5	98.5	99.8	99.1	99.2	99.0	99.8	99.0	99.8	99.0	98.9	100.0	99.9		99.7	99.7	99.9	99.7	99.6	99.6	99.5	99.5	99.6	99.4	99.5	99.4	99.4	20 KT379999.1_Avibacterium_paragallinarum_strain_ESV-346
21	97.7	97.9	97.8	97.8	97.9	97.9	97.7	97.7	99.2	99.6	98.4	99.8	99.2	100.0	99.2	100.0	98.1	99.0	99.0	99.0		99.8	99.7	100.0	99.7	99.7	99.6	99.6	99.7	99.6	99.6	99.5	99.5	21 KT380001.1_Avibacterium_paragallinarum_strain_Lavetec_02
22	97.9	98.1	98.0	98.0	98.1	98.1	97.9	97.9	99.0	99.6	98.6	99.7	99.0	99.7	99.0	99.7	98.1	99.0	99.0	99.0	99.7		99.7	99.8	99.7	99.7	99.6	99.6	99.7	99.6	99.6	99.5	99.5	22 KX722532.1_Avibacterium_paragallinarum_strain_IND102
23	98.5	98.7	98.6	98.6	98.7	98.7	98.5	98.5	100.0	99.3	99.2	99.2	100.0	99.2	100.0	99.2	98.9	99.8	99.7	99.8	99.2	99.6		99.7	99.6	99.6	99.5	99.5	99.6	99.5	99.5	99.4	99.4	23 MT706900.1_Avibacterium_paragallinarum_strain_PL-06
24	97.7	97.9	97.8	97.8	97.9	97.9	97.7	97.7	99.2	99.6	98.4	99.8	99.2	100.0	99.2	100.0	98.1	99.0	99.0	99.0	100.0	99.7	99.2		99.7	99.7	99.6	99.6	99.7	99.6	99.6	99.5	99.5	24 MT706902.1_Avibacterium_paragallinarum_strain_PL-09
25	98.7	98.9	98.8	98.8	98.9	98.9	98.7	98.7	98.2	98.7	97.6	99.0	98.2	99.0	98.2	99.0	99.0	98.2	98.2	98.2	99.0	98.9	98.2	99.0		99.6	99.5	99.5	99.6	99.5	99.5	99.5	99.4	25 KT371936.1_Avibacterium_paragallinarum_strain_LSV-58
26	97.0	97.2	97.1	97.1	97.2	97.2	97.0	97.0	98.2	98.8	97.6	98.9	98.2	98.9	98.2	98.9	97.2	98.1	98.0	98.1	98.9	99.0	98.2	98.9	98.0		99.6	99.6	99.7	99.7	99.7	99.5	99.6	26 Apg-1_(PV653197)
27	96.4	96.5	96.4	96.4	96.5	96.5	96.4	96.4	97.5	98.1	97.0	98.2	97.5	98.2	97.5	98.2	96.5	97.4	97.4	97.4	98.2	98.4	97.5	98.2	97.4	98.6		99.6	99.5	99.6	99.6	99.6	99.5	27 Apg-2_(PV653198)
28	96.2	96.4	96.3	96.3	96.4	96.4	96.2	96.2	97.4	98.0	97.0	98.1	97.4	98.1	97.4	98.1	96.4	97.3	97.2	97.3	98.1	98.3	97.4	98.1	97.2	98.3	98.2		99.5	99.6	99.6	99.6	99.5	28 Apg-3_(PV653199)
29	97.1	97.3	97.2	97.2	97.3	97.3	97.1	97.1	98.4	99.0	97.8	99.0	98.4	99.0	98.4	99.0	97.3	98.2	98.2	98.2	99.0	99.2	98.4	99.0	98.1	98.7	98.0	97.7		99.5	99.6	99.5	99.5	29 Apg_4_(PV653200)
30	96.2	96.4	96.3	96.3	96.4	96.4	96.2	96.2	97.4	98.0	96.8	98.1	97.4	98.1	97.4	98.1	96.4	97.3	97.2	97.3	98.1	98.3	97.4	98.1	97.2	99.2	98.4	98.2	97.9		99.7	99.5	99.5	30 Apg-5_(PV653201)
31	96.3	96.4	96.4	96.4	96.4	96.4	96.3	96.3	97.5	98.1	96.9	98.2	97.5	98.2	97.6	98.2	96.4	97.4	97.3	97.4	98.2	98.3	97.5	98.2	97.3	99.1	98.4	98.3	98.0	98.8		99.6	99.5	31 Apg-6_(PV653202)
32	96.0	96.2	96.1	96.1	96.2	96.2	96.0	96.0	96.9	97.4	96.4	97.5	96.9	97.5	96.9	97.5	96.2	96.9	96.8	96.9	97.5	97.9	96.9	97.5	97.0	97.6	98.1	98.1	97.2	97.7	98.0		99.4	32 Apg-7_(PV653203)
33	95.7	95.9	95.8	95.8	95.9	95.9	95.7	95.7	97.0	97.5	96.4	97.6	97.0	97.6	97.0	97.6	95.9	96.8	96.7	96.8	97.6	97.8	97.0	97.6	96.7	98.3	97.6	97.4	97.9	97.9	98.1	96.5		33 Apg-8_(PV653204)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	

Figure 4. The PCR amplification targeting the *16S rRNA* (**A**) and homology comparison of eight Indonesian field isolates with GenBank database reference strains of *A. paragallinarum* (**B**).

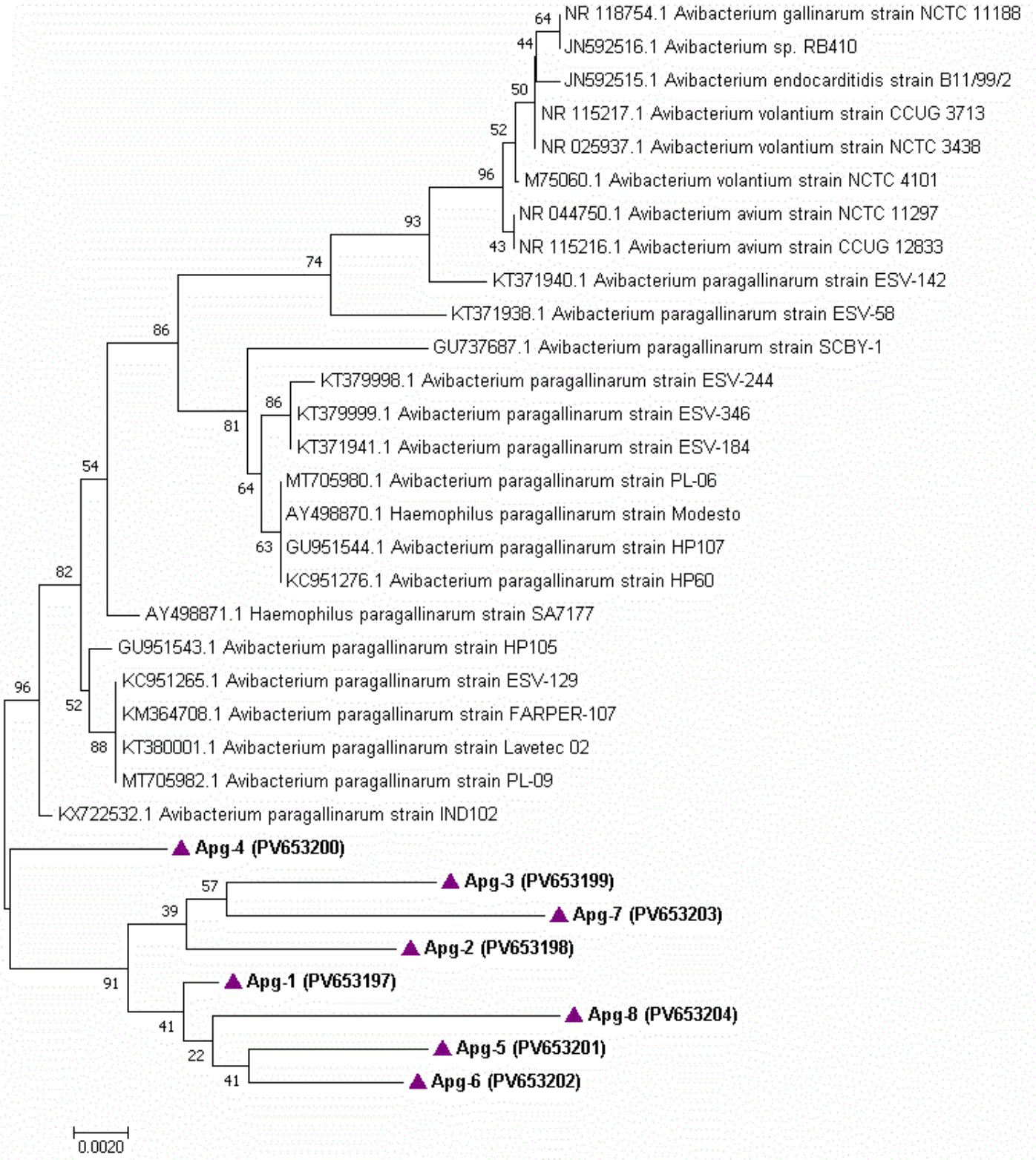


Figure 5. Phylogenetic tree based on the nucleotide sequences of the *16S rRNA*. The tree includes eight *Avibacterium paragallinarum* isolates from this study (▲) and reference strains retrieved from GenBank. The local isolates clustered in a separate clade, indicating genetic divergence from several international reference strains.

DISCUSSION

Avibacterium paragallinarum is a bacterium that is challenging to cultivate in media. The growth of *A. paragallinarum* bacteria requires a temperature of 37°C under aerobic or anaerobic environments, together with elevated CO_2 pressure levels (5%-10%) for a duration of 24-48 hours (Akter et al., 2014). Because nicotinamide adenine dinucleotide (NAD; V-factor) is essential for *in vitro* growth, *Staphylococcus aureus* is also inoculated in the same medium to produce additional NAD (Akter et al., 2014; Deresse et al., 2022). Although, along with the advancement, it has been discovered that *A. paragallinarum* could multiply in the absence of NAD (Deshmukh, 2015). In this study, *A. paragallinarum* was successfully cultivated on blood agar supplemented with NAD and 5% chicken serum. The colonies that grew were recultured until pure colonies of *A. paragallinarum* were obtained. The study by Badr et al. (2022) used the infraorbital swab results to grow bacteria on blood agar medium containing 10% sheep blood and added *Staphylococcus aureus* as an NAD feeder. Satellite growth of bacterial colonies was also shown in the blood medium.

Avibacterium paragallinarum is classified as a Gram-negative bacterium, resulting in a red hue during Gram staining. The envelope of a Gram-negative cell is made up of three distinct layers, namely the outer membrane, the peptidoglycan cell wall, and the inner or cytoplasmic membrane (Fivenson et al., 2024). According to the cell wall permeability theory, Gram-negative bacteria possess thin cell membranes that allow the primary stain to be removed, enabling absorption of the counterstain (Vijayakumar et al., 2023). Meanwhile, the biochemical assays conducted were catalase, oxidase, and indole tests, revealing negative results. The catalase test identifies microorganisms that produce the enzyme catalase, which neutralizes hydrogen peroxide and forms bubbles in positive results (Chandra, 2023). The oxidase test determines the ability of bacteria to manufacture the enzyme (Hafezi and Khamar, 2024). The indole test demonstrated the ability of the microorganisms to produce the tryptophanase enzyme, while the bacteria neither produced Hydrogen Sulphide (H_2S) nor exhibited hemolytic activity (Roy et al., 2023). Previous studies have also reported Gram-negative results in the form of coccobacilli, biochemical tests showed negative results for catalase, oxidase, motility, urease, and indole, followed by positive results for lactose, maltose, mannitol, and sorbitol (Akter et al., 2016; Wahyuni et al., 2022).

Molecular analysis using PCR was performed on eight samples collected from an Indonesian field. All analyzed samples were declared positive for *A. paragallinarum*. According to Chen et al. (1996), HPG-2 PCR gave equivalent results as conventional culture techniques and was recommended as a confirmatory and species-specific test for *A. paragallinarum*. Polymerase chain reaction (PCR) detection in Indonesia is still limited due to the difficulty in isolating this bacterium; nevertheless, a few published reports include Fauziah et al. (2021) and Putra et al. (2023), who successfully identified *A. paragallinarum* from swab samples of layer hens that had coryza symptoms in the Sleman district, Yogyakarta. Numerous further investigations across various countries have used HPG-2 PCR to detect this bacterium with a target band of 500 bp (Nabeel Muhammad and Sreedevi, 2015; Feberwee et al., 2019; Guo et al., 2022).

The Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR method has proven to be a valuable molecular tool for assessing genomic diversity among bacterial isolates, including *Avibacterium paragallinarum* (Hellmuth et al., 2017). In the present study, ERIC-PCR successfully differentiated eight *A. Paragallinarum* isolates obtained from chickens with infectious coryza in Indonesia. The banding patterns generated by ERIC-PCR revealed substantial variation, with each isolate displaying between 3 to 8 distinct bands ranging from 300 bp to 2,800 bp. This heterogeneity underscores the genomic diversity present among field strains of *A. paragallinarum*, which may contribute to differences in virulence, transmission dynamics, and vaccine responsiveness (Morales-Erasto et al., 2014).

The application of hierarchical clustering analysis with Ward's linkage approach divided the isolates into five separate clusters at a 95% similarity level. This finding is significant as it indicates that despite originating from a relatively narrow geographical area, the *A. paragallinarum* population in the study site exhibits considerable genotypic variability. Such clustering patterns reflect the existence of multiple circulating strains or subpopulations within a region, which may arise from genetic recombination, mutation, or evolutionary pressure exerted by antimicrobial usage and host immune responses.

The presence of several unique ERIC profiles among the isolates supports the notion that *A. paragallinarum* possesses a dynamic genome, likely influenced by mobile genetic elements such as plasmids or phages that are commonly targeted by ERIC primers. This aligns with previous studies that have demonstrated the discriminatory power of ERIC-PCR in distinguishing between bacterial strains with high resolution, making it a useful method for epidemiological surveillance and outbreak investigations (Bakhshi et al., 2018). Moreover, the clustering patterns observed may have epidemiological implications. For example, isolates that grouped closely within the same cluster could represent transmission events within or between locations, whereas isolates in separate clusters may indicate multiple sources of infection (Aljindan et al., 2018). The diversity revealed by ERIC-PCR should be considered when designing control strategies, including the development of autogenous vaccines tailored to the circulating strains.

According to Li et al. (2024), *16S rRNA* gene sequencing is a more sensitive method for detecting

pathogens and adequate to identify novel bacteria not only in human and animal diseases. Similarity tests from sequencing results showed that the eight samples were *A. paragallinarum* bacteria. Sequence analysis revealed 95.7-99.2% homology with reference *A. paragallinarum* strains in GenBank, and 96.5-99.2% homology among the study isolates themselves. The test sequence may be categorized as having similarities with the sequence in GenBank if its query cover and percent identity values are above 96% or as having a higher similarity if it approaches 100% (Bahri et al., 2023). The study conducted by Patil et al. (2017) employed *16S rRNA* sequencing to detect *A. paragallinarum* isolated from Indian field conditions, and the BLAST analysis demonstrated 96% to 99% similarity with the reference strain *A. paragallinarum*. The *16S rRNA* is located in bacterial ribosomes with a total length of approximately 1540 nucleotides (Yang et al., 2024). This gene is remarkably conserved in structure and function, making it often used to reflect phylogenetic relationships between bacteria (Idris et al., 2020).

The findings of *16S rRNA* sequencing in this study were used to generate a phylogenetic tree. All eight local isolates clustered within the same clade, indicating a close genetic relationship among them. However, these isolates formed a separate clade from the reference strains, suggesting the presence of distinct genetic diversity between the local Indonesian isolates and the reference *A. paragallinarum* strains. This result is contrary to the phylogenetic tree derived from *16S rRNA* sequencing of isolates from chickens in Korea between 2011-2016, which formed several clades sharing the same branch as the reference isolate (Jeong et al., 2020). However, the phylogenetic cluster of several *A. Paragallinarum* samples isolated from China displayed a distant genetic relationship, indicating significant geographical specificity (Cui et al., 2025). The observed genetic variation may result from factors such as genetic mutations, international transmission, or inherent local diversity, which necessitate further epidemiological analysis and tracing the origin strain is required (Guo et al., 2024).

CONCLUSION

Based on the findings of this study, *Avibacterium paragallinarum* was successfully isolated and identified from chickens showing clinical symptoms of infectious coryza in Indonesia. Phenotypic characterization and species-specific PCR confirmed all eight field isolates as *A. paragallinarum*. ERIC-PCR analysis revealed considerable genetic diversity among the isolates, indicating the presence of multiple circulating strains. The *16S rRNA* sequencing further confirmed the species identity and demonstrated high homology with global reference strains, while also showing distinct phylogenetic clustering. The present results underscored the importance of molecular surveillance for accurate diagnosis and effective control of infectious coryza in poultry flocks. Future studies should consider larger sample sizes across different regions in Indonesia and incorporate antimicrobial resistance profiling to enhance disease control strategies.

DECLARATIONS

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Author's contributions

Nia Karunia, Ryan Septa Kurnia, Muhammad Ade Putra, Christian Marco Hadi Nugroho, Agustin Indrawati, and Safika contributed to writing the original text, the experiment, carried out the experiments, and conducted the statistical analysis. Nia Karunia, Christian Marco Hadi Nugroho, Agustin Indrawati, and Safika analyzed and reviewed the results and then composed the final essay. All authors have reviewed and approved the final version of the manuscript for publication.

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Competing interests

The authors declared that there are no competing interests.

Ethical considerations

The authors declare that this manuscript is original and is not being considered elsewhere for publication. The authors have reviewed additional ethical concerns, such as research misconduct, data fabrication, and redundancy. The authors confirmed they have not assisted the AI in conducting and preparing the present study.

Availability of data and materials

All data from the current study are available upon reasonable requests from the authors.

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