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Molecular identification of *Avibacterium paragallinarum* isolates from backyard and commercial layer chickens in Iran

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Abstract

Infectious coryza, caused by *Avibacterium paragallinarum*, poses a significant economic threat to the global poultry industry. Although infectious coryza is present in Iran, there is limited molecular data characterizing the circulating strains, particularly regarding key hemagglutinin genes such as *hagA*. This study aimed to investigate the genetic diversity and phylogenetic relationships of the *Av. paragallinarum* isolates recovered from commercial and backyard poultry in Iran by sequencing the *hagA* gene and comparing the findings with those of global strains. Twenty-three *Av. paragallinarum* isolates collected from Iranian poultry farms underwent PCR amplification and sequencing of the *hagA* gene. Nucleotide and deduced amino acid sequences were analyzed for homology, subjected to BLAST searches, and used for phylogenetic reconstruction. The isolates showed high sequence identity, ranging from 94.10 - 99.90% at the nucleotide level and 92.10 - 100% at the protein level. Phylogenetic analysis classified the isolates into five distinct clusters (Iran-1 to Iran-5). The largest cluster (Iran-1) showed a strong affiliation with strains from China and India, whereas the other isolates clustered with strains from South Korea, Germany, and Japan. Notably, the Iran-5 group exhibited high similarity to a non-typable strain from USA. Additionally, eight isolates encoded truncated HagA proteins, each consisting of 344 rather than 345 amino acids. This study demonstrates the circulation of genetically diverse *Av. paragallinarum* strains in Iran. These findings highlight the need for further molecular investigations to improve control measures and assess the efficacy of the vaccines currently used in the country.

Introduction

Avibacterium paragallinarum, a gram-negative bacterium, is the etiological agent of infectious coryza (IC), an acute respiratory disease primarily affecting chickens. This pathogen significantly impacts poultry production by reducing egg yield, impairing weight gain, and increasing vulnerability to secondary infections. The economic ramifications are considerable, particularly in regions with developing poultry industries, where inconsistencies in biosecurity measures and vaccination practices exacerbate the disease burden.[1,2]

The pathogenicity of *Av. paragallinarum* is attributed to various virulence factors, including adhesins and capsule-like structures, which facilitate host colonization and immune evasion. The *Av. paragallinarum* is classified into serovars based on hemagglutinin antigens, with notable geographical variations in serovar prevalence reflecting regional epidemiological patterns.[3] Accurate identification of this pathogen is critical for implementing effective control measures, including tailored vaccination programs and biosecurity protocols.

In Iran, poultry production constitutes a vital component of the agricultural sector, with backyard and industrial flocks both contributing to national food security. However, infectious diseases, including IC, pose a continuous threat to the productivity and profitability of this sector.[4,5] Given the critical role of the poultry industry in ensuring food security and the ongoing threat posed by infectious diseases, comprehensive and accurate

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investigations into pathogens are imperative. Although previous research has documented the prevalence of *Av. paragallinarum* in Iranian poultry, there remains a critical need for use of advanced molecular diagnostic techniques for its detection and characterization. These methods offer greater precision and reliability compared to traditional approaches, thereby facilitating more accurate assessments of the current epidemiological status of IC in Iran.

Traditional serotyping methods for *Av. paragallinarum* are primarily based on two schemes: the Page scheme and the Kume scheme. The Page scheme classifies *Av. paragallinarum* into three serovars, A, B, and C, using a plate agglutination assay. The Kume scheme, on the other hand, originally classified *Av. paragallinarum* into three groups based on the hemagglutination inhibition (HI) test. However, Blackall *et al.*, later regrouped and renamed the Kume protocol to match the Page serovars of A, B, and C, subdividing it further into nine serovars (A-1 to A-4, B-1, and C-1 to C-4).[6] Despite these methods, both schemes present limitations. The Page scheme, while more commonly used because inactivated vaccines only protect against the Page serovar present in the vaccine, depends on the availability of three serovar-specific antibodies. The Kume scheme, in its extended form, requires nine serovar-specific antibodies, which are complicated and necessitate chicken erythrocytes fixed with glutaraldehyde and antigens treated with potassium thiocyanate or hyaluronidase. Consequently, serovar classification using these methods is complex, making it achievable only in specialized laboratories. Moreover, some isolates fail to display hemagglutination activity even after hyaluronidase treatment, rendering them unsuitable for the HI test.[7,8]

Molecular diagnostic techniques, such as polymerase chain reaction (PCR) and sequencing, offer high precision in detecting and characterizing *Av. paragallinarum*. These methods not only enhance diagnostic accuracy but also facilitate strain differentiation and support robust epidemiological investigations. Furthermore, they provide valuable insights into the genetic diversity and evolutionary patterns of the pathogen within and across poultry populations.[9]

Among the potential virulence factors contributing to the pathogenicity of *Av. paragallinarum*, the hemagglutinin encoded by the *hagA* gene holds particular significance. Hemagglutinins are often crucial for bacterial adhesion, representing an essential early step in infection, and the HagA protein is also a likely major target for the host immune response and key to serotyping and vaccine development efforts.[10] Variations within the *hagA* gene or its expressed protein could thus influence strain virulence, antigenicity, and the efficacy of current control measures. Given the limitations of traditional serotyping methods, molecular approaches targeting the *hagA* gene offer a valuable alternative for accurate genotype identification.[11] This study aimed to provide better knowledge of the circulating *Av. paragallinarum* genotypes in Iran by analyzing isolates collected since 2018 from both backyard and industrial poultry flocks. By focusing on the *hagA* gene, we sought to assess the genetic diversity of the isolates and contribute to a better understanding of IC epidemiology in the country.

Materials and Methods

Bacteria. Some *Av. paragallinarum* isolates were obtained from the repository of the Bacterial Poultry Diseases Section at the Razi Vaccine and Serum Research Institute in Karaj, Iran (Table 1), while others were isolated during this project. All specimens were originally collected from the trachea, choanal clefts, and infraorbital sinuses of chickens suspected of having IC, using sterile cotton swabs. These samples were not part of a routine or systematic surveillance program; rather, they were collected from backyard and industrial poultry showing overt clinical respiratory signs. For bacterial isolation, the swabs collected from suspected IC cases were placed in Amies transport medium (HiMedia Laboratories Pvt. Ltd., Thane, India) and transported to the laboratory within 24 hr. Upon arrival, the swabs were streaked onto blood agar (Merck, Darmstadt, Germany) plates and cross-streaked with *Staphylococcus spp.* to supply nicotinamide adenine dinucleotide (NAD), a required growth factor. The cultures were incubated at 37.00 ˚C under 5.00% CO_2. A presumptive diagnosis of *Av. paragallinarum* was based on satellitic growth, characterized by dewdrop-like colonies forming adjacent to the *Staphylococcus spp.* streak.

DNA extraction. Following subculture in brain heart infusion broth (Merck) supplemented with 1.00% chicken serum and 0.0025% oxidized NAD for 24 - 48 hr, the bacterial suspensions were transferred to 1.50 mL microcentrifuge tubes and centrifuged at 3,000 *g* for 5 min. The resulting bacterial pellets were resuspended in 200 µL of phosphate-buffered saline. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's guidelines. The extracted DNA was stored at – 20.00 ˚C until further analysis.

Polymerase chain reaction and sequencing. The primers used in this study are listed in Table 2. To confirm *Av. paragallinarum* isolates, an HPG-2 PCR assay was performed, which specifically amplifies a fragment of approximately 510 base pairs.[12] Briefly, the PCR was carried out in a 50.00 µL reaction mixture containing 4.00 µL of extracted DNA, 5.00 µL of 10.00 X reaction buffer, 4.00 µL of 25.00 mM $MgCl_2$, 2.00 µL of 10.00 mM deoxynucleoside triphosphate (dNTP) mix, 2.00 µL of 10.00 µM primer MB-ICN1, 2.00 µL of 10.00 µM primer MB-ICR1, 0.50 µL of 5.00 U μL^{-1} Taq DNA polymerase, and 30.50 µL of nuclease-free water. The PCR cycling conditions were as

follows: an initial denaturation at 94.00 ˚C for 2 min, followed by 35 cycles of denaturation at 94.00 ˚C for 20 sec, annealing at 56.00 ˚C for 30 sec, and extension at 72.00 ˚C for 1 min, with a final extension at 72.00 ˚C for 10 min. PCR products were visualized by electrophoresis on a 1.00% agarose gel containing SYBR™ Safe DNA Gel Stain (ThermoFisher Scientific, Waltham, USA) under ultraviolet illumination. Amplification of the complete *hagA* gene was conducted according to a previously described method.[11] Briefly, PCR was conducted using 2.00X PCRBIO VeriFi™ Mix (PCR Biosystems Ltd., London, UK), 400 nM of each primer (MB-HA8 and MB-HA11), and 4.00 µL of extracted DNA in a total volume of 50.00 µL. The thermocycling profile consisted of an initial denaturation at 95.00 ˚C for 1 min, followed by 35 cycles of 95.00 ˚C for 15 sec, 60.00 ˚C for 15 sec, and 72.00 ˚C for 30 sec. Following amplification, PCR products were excised from a 1.00% agarose gel and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific). The purified PCR products were ligated into the pJET1.2/blunt vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). The ligation mixture was used to transform competent *Escherichia coli* TOP10 cells via heat shock at 42.00 ˚C for 1 min. The transformants were streaked onto Luria-Bertani agar (Merck) plates supplemented with 50.00 µg mL^{-1} ampicillin. Colony PCR was performed to confirm the presence of the correct insert using the same primers used in the full-gene amplification. Plasmids were extracted from overnight bacterial cultures using the High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH). Sanger sequencing was performed using the pJET1.2 universal forward and reverse primers and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Waltham, USA) on an Applied Biosystems 3,500 Genetic Analyzer according to the manufacturer's instructions. Sequence data were assembled and analyzed using BioEdit (version 7.0; Ibis Therapeutics, Carlsbad, USA).[13]

Phylogenetic analysis. Nucleotide and deduced amino acid sequences were aligned using ClustalW algorithm in MEGA Software (version 11.0; Biodesign Institute, Tempe, USA).[14] The BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted to identify homologous sequences in the GenBank® database. Phylogenetic analyses were carried out using the maximum likelihood method under the general time reversible model. Bootstrap analysis was performed with 1,000 replicates to assess the reliability of the tree topology in MEGA Software.[14]

Nucleotide sequence accession numbers. All sequences were deposited in GenBank under the accession numbers PZ618029-PZ618051.

Table 1. Summary of *Avibacterium paragallinarum* isolates used in this study

Isolates	Host	Location of isolation	Date of isolation
AP-1/layer/Mashhad/2018	Commercial layer	Razavi Khorasan-Mashhad	2018-10
AP-2/backyard poultry/Karaj/2018	Backyard poultry	Alborz-Karaj	2018-11
AP-3/backyard poultry/Tehran/2019	Backyard poultry	Tehran-Tehran	2019-11
AP-4/backyard poultry/Tehran/2019	Backyard poultry	Tehran-Tehran	2019-11
AP-5/backyard poultry/Tehran/2019	Backyard poultry	Tehran-Tehran	2019-11
AP-6/layer/Varamin/2019	Commercial layer	Tehran-Varamin	2019-11
AP-7/layer/Varamin/2019	Commercial layer	Tehran-Varamin	2019-11
AP-8/layer/Nazarabad/2023*	Commercial layer	Alborz-Nazarabad	2023-01
AP-9/layer/Shahriar/2023	Commercial layer	Tehran-Shahriar	2023-04
AP-10/backyard poultry/Tehran/2023	Backyard poultry	Tehran-Tehran	2023-04
AP-11/backyard poultry/Tehran/2023	Backyard poultry	Tehran-Tehran	2023-04
AP-12/backyard poultry/Tehran/2023	Backyard poultry	Tehran-Tehran	2023-04
AP-13/backyard poultry/Tehran/2023	Backyard poultry	Tehran-Tehran	2023-05
AP-14/backyard poultry/Tehran/2023	Backyard poultry	Tehran-Tehran	2023-10
AP-15/backyard poultry/Tehran/2023	Backyard poultry	Tehran-Tehran	2023-11
AP-16/layer/Karaj/2024	Commercial Layer	Alborz-Karaj	2024-01
AP-17/backyard poultry/Kermanshah/2024	Backyard poultry	Kermanshah-Kermanshah	2024-01
AP-18/backyard poultry/Tehran/2024	Backyard poultry	Tehran-Tehran	2024-01
AP-19/backyard poultry/Marand/2024	Backyard poultry	East Azerbaijan-Marand	2024-02
AP-20/backyard poultry/Marand/2024	Backyard poultry	East Azerbaijan-Marand	2024-02
AP-21/backyard poultry/Tehran/2024	Backyard poultry	Tehran-Tehran	2024-03
AP-22/layer/Tehran/2024	Commercial layer	Tehran-Tehran	2024-05
AP-23/backyard poultry/Marand/2024	Backyard poultry	East Azerbaijan-Marand	2024-05

*From this isolate onward, all subsequent isolates were collected in this study.

Table 2. The sequence of primers used in this study for detection and sequencing

Target genes	Primers	Sequences	Product size (bp)	Application	References
hagA	MB-HA8	5′-AAGCTTTTATTTTAGATTTATTG-3′	1321 - 1324	Amplification of *hagA* gene	11
	MB-HA11	5′-CGCACGGCATTGATATTGTG-3′			
pyrG and *yihA*	MB-ICN1	5′-TGAGGGTAGTCTTGCACGCGAAT-3′	510 - 511	Detection of *Av. paragallinarum*	12
	MB-ICR1	5′-CAAGGTATCGATCGTCTCTCTACT-3′			

Results

Homology and phylogenetic analysis. The identity matrix of the studied isolates revealed nucleotide sequence identities ranging from 94.10 to 99.90%, while protein sequence identities varied between 92.10 and 100% (data not shown). These findings indicate a relatively high degree of genetic conservation among the isolates, with only minor variations observed at both the nucleotide and protein levels. A BLAST search was performed on 23 *Av. paragallinarum* isolates to determine their genetic relatedness to previously reported strains. The analysis revealed that Iranian isolates share a close evolutionary relationship with strains from China, Germany, South Korea, and USA, with the highest sequence similarity observed among Chinese isolates (Table 3). Isolates AP-12, AP-15, and AP-17 exhibited distinct genetic characteristics compared with the other isolates in this study. The BLAST analysis revealed a high degree of similarity between these isolates and strain AG21-0333, identified as a non-typeable variant.[15] Further examination of *hagA* gene coding sequences revealed that all isolates encode proteins consisting of 344 - 345 amino acids. However, eight isolates (AP-8, AP-12, AP-14, AP-15, AP-16, AP-17, AP-20, and AP-21) were found to produce a truncated HagA protein.

Phylogenetic analysis. To further explore the evolutionary relationships among the studied isolates, a phylogenetic tree was constructed using representative *Av. paragallinarum* sequences retrieved from GenBank® (Fig. 1). The resulting phylogeny revealed that Iranian isolates were segregated into five distinct clusters, designated as Iran-1, Iran-2, Iran-3, Iran-4 and Iran-5, indicating the presence of multiple genetic lineages within the country (Fig. 1). Among these clusters, Iran-1 was the largest, comprising 15 isolates that formed a strongly supported clade with strains from China and India. The only isolate that formed a distinct cluster was AP-20, supported by a high bootstrap value, and was positioned near the Iran-4 group. Isolate AP-21 appeared as an outgroup, separate from both Japanese and German strains, suggesting a possible introduction of this genetic lineage from geographically distant regions. In contrast, isolates AP-12, AP-15, and AP-17 clustered with strains from South Korea and USA, consistent with previous BLAST results that indicated atypical genetic profiles.[15] The remaining isolates, AP-8, AP-14, and AP-16, formed a phylogenetic cluster primarily associated with South Korean and Indian strains.

Discussion

Infectious coryza remains a significant respiratory disease impacting poultry worldwide, with confirmed outbreaks reported across many countries.[16] While comprehensive national surveillance data may be limited in Iran, targeted investigations and field observations clearly indicate the ongoing presence of IC. In support of this, *Av. paragallinarum* has been isolated from both commercial layer and backyard flocks showing clinical signs of IC in several Iranian provinces, including Tehran, Alborz, Isfahan, and Qazvin.[4,5,17] To date, no genotyping

Table 3. Closest similarity of *hagA* genes from *Avibacterium paragallinarum* isolates from Iran according to BLAST search

Isolates	Most similar to:
AP-1/layer/Mashhad/2018	C-AP1/fowl/China/2021 (99.33%)*
AP-2/backyard poultry/Karaj/2018	C-AP1/fowl/China/2021 (99.52%)
AP-3/backyard poultry/Tehran/2019	C-AP1/fowl/China/2021 (99.90%)
AP-4/backyard poultry/Tehran/2019	C-AP1/fowl/China/2021 (99.42%)
AP-5/backyard poultry/Tehran/2019	C-AP1/fowl/China/2021 (99.23%)
AP-6/layer/Varamin/2019	C-AP1/fowl/China/2021 (99.71%)
AP-7/layer/Varamin/2019	C-AP1/fowl/China/2021 (99.33%)
AP-8/layer/Nazarabad/2023	16AD002/chicken/South Korea/2016 (99.13%)
AP-9/layer/Shahriar/2023	C-AP1/fowl/China/2021 (99.52%)
AP-10/backyard poultry/Tehran/2023	C-AP1/fowl/China/2021 (99.81%)
AP-11/backyard poultry/Tehran/2023	C-AP1/fowl/China/2021 (99.33%)
AP-12/backyard poultry/Tehran/2023	AG21-0333/chicken/USA/2021 (97.97%)
AP-13/backyard poultry/Tehran/2023	C-AP1/fowl/China/2021 (99.62%)
AP-14/backyard poultry/Tehran/2023	16AD002/chicken/South Korea/2016 (99.71%)
AP-15/backyard poultry/Tehran/2023	AG21-0333/chicken/USA/2021 (98.26%)
AP-16/layer/Karaj/2024	16AD002/chicken/South Korea/2016 (99.71%)
AP-17/backyard poultry/Kermanshah/2024	AG21-0333/chicken/USA/2021 (97.97%)
AP-18/backyard poultry/Tehran/2024	C-AP1/fowl/China/2021 (99.33%)
AP-19/backyard poultry/Marand/2024	C-AP1/fowl/China/2021 (99.81%)
AP-20/backyard poultry/Marand/2024	16AD002/chicken/South Korea/2016 (98.16%)
AP-21/backyard poultry/Tehran/2024	2671/chicken/Germany/unknown (99.61%)
AP-22/layer/Tehran/2024	C-AP1/fowl/China/2021 (99.81%)
AP-23/backyard poultry/Marand/2024	C-AP1/fowl/China/2021 (100%)

* Similarity is based on coding sequence of *hagA* gene (1,035 - 1,038 bp).

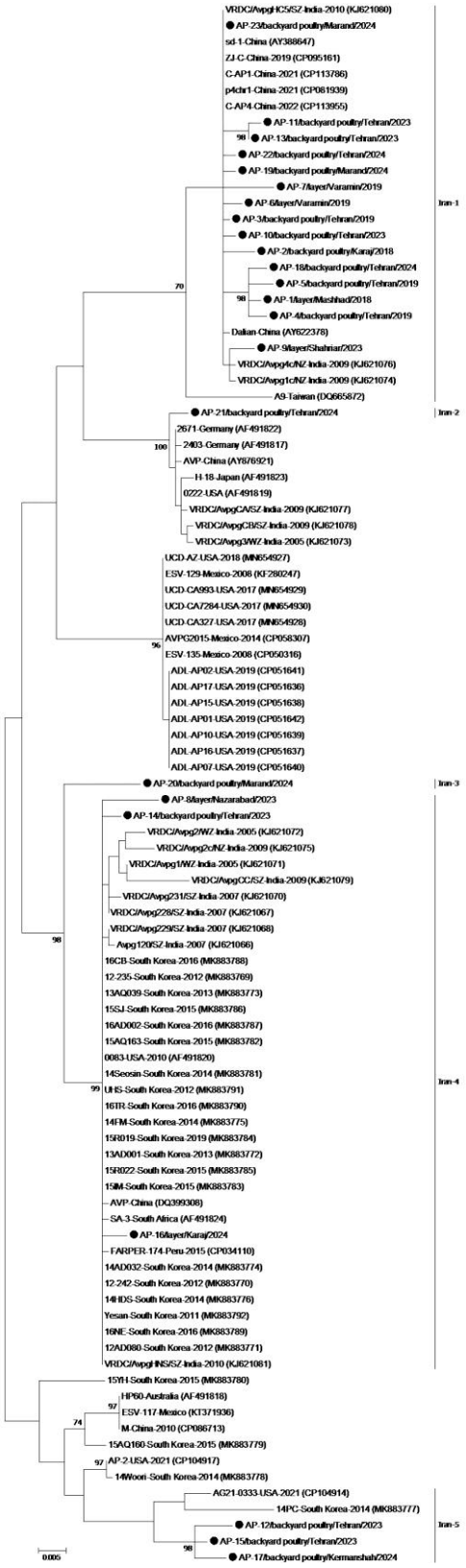

Fig. 1. Phylogenetic analysis of Iranian *Avibacterium paragallinarum* isolates along with other representatives from different geographical regions worldwide. The isolates included in this study are marked with filled circles. Bootstrap support values greater than 70.00% are shown near the nodes. The scale bar indicates nucleotide substitution/site.

studies of *Av. paragallinarum* have been conducted in Iran. Therefore, this study aimed to genotype 23 Iranian isolates using the *hagA* gene sequences, offering novel insights into the genetic diversity of circulating strains.

Over the past few decades, molecular methods for detecting and characterizing *Av. paragallinarum* have evolved significantly. One key advancement was the development of PCR-based assays targeting species-specific genetic markers. The HPG-2 PCR assay, first introduced by Chen *et al.*, became widely adopted due to its high specificity and sensitivity.[12,18] Traditional culture-based detection of *Av. paragallinarum* is often hindered by the bacterium's fastidious nature and complex growth requirements, making successful isolation difficult. In contrast, PCR methods, particularly the HPG-2 assay, have demonstrated higher sensitivity and robustness, especially in samples with low bacterial loads or reduced viability.[12]

More recent molecular investigations have provided deeper insights into the genetic landscape of *Av. paragallinarum*. For example, Putra *et al.*, using partial *pyrG* sequence analysis, observed clustering of all clinical samples with known reference sequences, indicating genetic conservation within their dataset.[19] Our findings are consistent with such observations. We found high sequence identity among Iranian isolates, ranging from 94.10 - 99.90% at the nucleotide level and 92.10 to 100% at the protein level, suggesting a relatively conserved genome with limited variation among strains.

Our BLAST analysis confirmed strong genetic similarity between Iranian isolates and international strains from countries such as China, South Korea, Germany, and USA. Notably, one of the closest matches was with the Chinese strain C-AP1/fowl/China/2021. This supports the hypothesis that East Asia may represent a potential source of genetic introduction for *Av. paragallinarum* into the region. In a related study from Egypt, Badr *et al.*, used HPG-2 PCR to identify *Av. paragallinarum* strains and reported similarly high levels of homology, not only among local strains but also with isolates from the USA and the Netherlands.[20] This genetic relatedness across countries aligns with our own findings and suggests possible inter-regional transmission or shared evolutionary origins.

A previous study by Peighambari *et al.*, based on *HMTp210* sequencing had identified only two genotypes in Iran, corresponding to Page serovars B and C.[4] That study targeted only the hypervariable region of *HMTp210*, whereas our broader approach using *hagA* sequencing revealed five distinct phylogenetic clusters, indicating a higher level of genetic diversity than previously reported.

Further evidence comes from Deresse *et al.*, who conducted a comprehensive molecular survey in Ethiopia between 2022 and 2024. Their study confirmed the presence of *Av. paragallinarum* using HPG-2 PCR, with six isolates from northern and eastern Ethiopia showing complete sequence identity to Iranian strains.[21] This suggests transboundary dissemination and points to possible shared evolutionary lineages between regions separated by vast geographic distances.

In a study conducted in South Korea, phylogenetic analysis of 24 *Av. paragallinarum* isolates based on the *hagA* gene divided the strains into four distinct clusters (I - IV).[11] Of these, 20 Korean isolates were grouped into a single cluster (Cluster I). One variant, 15YH, was placed alone in Cluster II. Two other Korean variants, 14Woori and 15AQ160, were grouped into Cluster III, which also contained reference strains for Kume serovars C-2, A-4, and C-4. The fourth Korean variant isolate clustered in Group IV, which comprised isolates from North and South America.[11] In the present study, *Av. paragallinarum* isolates from Iran were grouped into five distinct phylogenetic clusters. The largest cluster, Iran-1, showed strong affiliation with isolates from China and India, suggesting possible common ancestry or past transmission events. Intriguingly, isolate AP-21 formed a distinct cluster positioned near the group of strains from Germany and Japan, suggesting the introduction of a genetically divergent variant. Other isolates, AP-8, AP-14, and AP-16, were closely related to strains from South Korea and India, further indicating the genetic diversity and complex origins of circulating strains in Iran. Iran-1, Iran-2, and Iran-3 formed independent clusters, each genetically distinct from the Korean isolates. Iran-4 contained isolates that clustered with South Korean strains from Cluster I, and included only three Iranian isolates alongside strains from China, India, South Africa, and USA. Notably, isolate 14PC from South Korea, which belonged to Cluster IV in the Korean study, clustered in the Iran-5 group along with Iranian isolates AP-12, AP-15, and AP-17. This group appears to include non-typeable variants, as it also contains an isolate from USA that has been previously classified as a non-typeable strain. Xu *et al.*, similarly observed that most contemporary Chinese isolates clustered closely, yet differed from older local strains, highlighting the dynamic and evolving nature of this pathogen.[22]

Similarly, Saravanan *et al.*, employed *hagA* sequencing for Indian isolates and reported 94.00 - 100% similarity with strains from China, Australia, Mexico, and India.[23] These findings reinforce the interconnectedness of *Av. paragallinarum* strains across global poultry populations, likely facilitated by trade or migratory birds.

This study provides a comprehensive molecular characterization revealing significant genetic heterogeneity within *Av. paragallinarum* circulating in Iranian poultry, evidenced by the segregation of isolates into five distinct phylogenetic clusters. Strong phylogenetic affiliations were observed with diverse international strains, notably from East Asia (China, South Korea), but also involving distinct links to strains from USA, Germany, and Japan. These widespread genetic connections suggest

that international poultry trade has played an important role in the epidemiology and genetic diversity of the pathogen across continents. Although *hagA*-based genotyping does not correlate with traditional serotyping systems such as Page or Kume schemes, our findings underscore the necessity of ongoing molecular surveillance. Such efforts are essential not only for improved disease control but also to guide future vaccine development efforts that better reflect the circulating genotypes.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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