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Original Article

Isolation, Identification and Antimicrobial Susceptibility of Avibacterium *Paragallinarum* from Backyard Chicken in Retail Markets of Karaj and Tehran Cities, Iran

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Abstract

Avibacterium (Haemophilus) paragallinarum (*Av. Paragallinarum*) is the causative agent of Infectious Coryza (IC) in chickens. Despite the worldwide distribution of IC**,** no systematic study, to the best of our knowledge, was conducted on isolation and characterization of *Av. Paragallinarum* in Iran. The present study aimed to isolate and perform antibacterial susceptibility testing (AST) of IC agents from suspected backyard chickens with typical symptoms of IC in avian markets. From 18 collected choanal swab samples, four (22%) isolates of *Av. Paragallinarum* were detected by culture methods based on satellite growth on blood agar, which was confirmed by the biochemical reaction of Catalase and Oxidase tests and species-specific PCR (HPG-2). The hypervariable region of the hemagglutinin genes of 4 isolates was amplified and obtained sequences were deposited at a gene bank for more characterization. Meanwhile, 12 (66%) positive reactions were detected by observing expected 500 bpb and using PCR (HPG-2) on swab samples. Antibiotic susceptibility testing (AST) of obtained isolates were analyzed using the Kirby-Bauer disk diffusion method on Columbia agar with horse blood. Isolates were found to be resistant to amoxicillin, oxytetracycline, streptomycin, trimethoprim/sulfamethoxazole (up to 75%) and sensitive to cefalexin, ceftriaxone, enrofloxacin, florfenicol, gentamycin, linco-spectin, neomycin, doxycycline (50%), danofloxacin (75%), flumequine (50%), ofloxacin (75%). An intermediate growth inhibitionzone has been observed around antibiotic discs for ampicillin, colistin, erythromycin, penicillin, tiamulin (75%), tylosin (75%). In summary, to the best of our knowledge, this study is the first report of isolation and identification of *Avibacterium paragallinarum* from backyard chickens which may be a source of IC for commercial chicken flocks. Moreover, the prevalence of resistance to some antibacterial drugs of IC agents may impose an additional threat to the poultry industry. A more in-depth study is recommended to develop a low-cost autogenous IC vaccine for small-scale flocks of poultry to prevent and manage the disease and establish antimicrobial resistance.

Keywords: Infectious Coryza; Avibacterium paragallinarum; PCR; Isolation; Antibiotic susecptibility test

1. Introduction

Avibacterium (Haemophilus) paragallinarum (*Av. Paragallinarum*) is the causative agent of infectious coryza (IC), an important disease of chicken associated with an acute upper respiratory infection, growth retardation, and reduced egg production (1). IC is normally characterized by an acute onset and spreads rapidly in a flock with the symptoms of nasal discharge, facial swelling, lacrimation, anorexia, and diarrhea (1, 2).

Unusual clinical symptoms of IC are similar to those of swollen head syndrome reported among chickens in the United States (2, 3). In principle, the prevention of IC mostly relies on sanitation practices, secluded sites,

and vaccination in poultry farms. Despite these, the sporadic outbreak of infectious coryza is still widespread which causes significant economic losses, especially in developing countries. Despite the worldwide distribution of IC and familiarity of the farmers with the disease, no systematic study, to the best of our knowledge, was conducted on isolation and characterization of *Av. Paragallinarum* in Iran. Bozorgmehri fard (4) conducted the first study of antibiotic sensitivity of the pathogenic bacteria. Banani, Pourbakhsh (5) studied isolation and characterization of the pathogenesis of *Av. Paragallinarum* from infected layer flocks. In general, IC often occurs in backyard chickens in Iran. Therefore, a study should be conducted on isolation and identification of *Av. Paragallinarum* to manage and prevent IC among these chickens in the future.

2. Material and Methods

2.1. Samples Collection

A total of 18 choanal swabs samples were obtained from backyard chicken suspected of IC infection for bacterial isolation and detectionand transferred to Brain Heart Infusion (BHI) broth media supplemented with Nicotinamide Adenine Dinucleotide (NAD) (.0025mg/ml). The birds with facial swelling, nasal discharge, and respiratory disorder were selected for sampling from retail markets of Karaj and Tehran between November 2018 to May 2019. The obtained samples were cultured in a laboratory for less than 2 hoursto prevent possible bacterial deactivation.

2.2. Bacterial Isolation

The samples were cultured witha swab streaked on 5% sheep blood agar supplemented with 1% heat-inactivated, sterile-filtered chicken serum and then followed by cross-streaking of *Staphylococcus aureus* as NAD provider for media. Bacterial growth was supported by incubation at37° C and 5% CO_2 pressure for 24-48 hours. Bacterial colonies whichindicated satellite growth were selected to subculture in 7% horse blood Columbian chocolate agar for further purification and maintenanceas recommended (6). Finally, gram staining negative colonies were biochemically screened for catalase-negative and oxidase-positive tests.

2.3. Detection and Identification of *Av. Paragallinarum* by PCR

DNAs required for the test were extracted from both cultured bacterial colonies and swab samples. The DNA extractions from bacterial culture were performed following the methods as described by Moore (7) via boiling an appropriate amount of clone purified isolates in sterile distilled water for 45-60 minutes. The swab samples were prepared for DNA extraction after wrapping the wets wabs in media around the tube vessel, the remained transport media were centrifuged at 13,000 rpm and the obtained precipitate was harvested in 400µl of sterile distal water. The DNA extraction was followed by the phenol-chloroform method as previously described (7, 8). In performed experiments, a previously characterized *Av. Paragallinarum* and *Ornithobacte riumrhinotracheale* were includedas positive and negative bacterial genome control, respectively.

The extracted DNAs from both swab and bacterial samples were used in the test of HPG-2 PCR specific for *Av. Paragallinarum*, as described by Chen, Miflin (9). The primers, N1 and R1 (Table 1) set to generate an amplicon of approximately 500bp in the final electrophoretic analysis. Furthermore, the middle third (hypervariable region) of the hemagglutinin gene sequence of isolates were amplified and sequenced for deeper identification and verification of isolated bacteriausing published primer sets of 5-1 forward and 5-1 reverse with the size of approximately 1.6 Kbp. All PCR tests were performedin 25-ul volumes containing an appropriate amount of template DNA (15-150 ng), $MgCl_2$, and primer concentrations using Eppendorf® Mastercycler® thermal cycler. PCR products were visualized by UV light after electrophoresis in 1% agarose gel for 25 min at 80V.

Table 1. primers sequence used to identify *Av. Paragallinarum*.

Oligonucleotide	Sequence	PCR product size
R1 N1	5'-CAA GGT ATC GAT CGT CTC TCT ACT-3' 5'-TGA GGG TAG TCT TGC ACG CGA AT-3'	500 bp (9)
5-1 forward 5-1 reverse	5'-GATGGCACAATTACATTTACA-3' 5'-ACCTTGAGTGCTAGATGCTGTAGGTGC-3'	1.6 kbp

2.4. Antibacterial Susceptibility Test

Av. Paragallinarum is a slow-growing and fastidious bacterium. Most of its strains require V-(nicotinamide adenine dinucleotide, NAD) factor for their growth and there is no recommended standardized medium for susceptibility testing. Therefore, Columbia blood agar (CBA) with 7% horse blood was used to perform antibacterial susceptibility test by Kirby-Bauerdisk diffusion method following the guidelines of Clinical and Laboratory Standard Institute. A suspension of the fresh culture of isolates (of approximately 1×10^8 CFU/mL) was prepared in BHI broth supplemented with NAD which was adjusted to 0/5 McFarland turbidity standard, then evenly spread onto CBA in a Petri dish. Then, 20 commonly used antibiotics in farms were selected for investigating antibiotic susceptibility. All antimicrobial discs were from antibiotic discs of Patan-Teb, Iran which were aseptically placed onto the surface of inoculated agar plate media. Zones of growth inhibition around each of the antibiotic discs were measured to the nearest millimeter after incubation (16–24 h at 37 °C with 5% CO_2 pressure). Using the reference table of the company, the size of the zone of growth inhibition related to each antibiotic was recorded as: "the isolate is susceptible (S), intermediately susceptible (I), or resistant (R)". Antibiotics and their potencies were as follow: ampicillin (10 µg), amoxicillin (25 µg), colestin (10µg), cephalexin (30µg), ceftriaxone (30 µg), doxycycline (30µg), danofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), flumequine (30 µg) fosfomycin (200 µg), gentamycin (10 µg), lincospectin (20µg), neomycin (30 µg), enrofloxacin (5 µg), ofloxacin (5 µg), penicillin (10 µg), streptomycin (10 µg), trimethoprim-sulfamethoxazol (1.25 + 23.15 µg), oxytetracycline (30 µg), tiamulin (30 µg) and tylosin (30 µg).

3. Results

Av. Paragallinarum bacteria were isolated from 4 samples of 18 choanal swab samples by showing typical morphological and biochemical characteristics of satellite growth, Grams negative polymorphic short rod bacteria, oxidase activity, and non-catalase reaction. Cultivation of positive samples generally appeared in form of tiny dewdrops and non-hemolytic colonies even in the first round of cultivations (Figure 1). Meanwhile, these isolates had also no growth in culture media of MacConkey agar and at normal air incubation.

Molecular detection and identification of these isolates were confirmed by observing the expected 500bp band in electrophoresis of their species-specific PCR products and sequencing partial hemagglutinin protein gene. After proper alignment of obtained partial nucleotide sequences of related hemagglutinin gene of isolates, they were deposited at the Genbank genetic sequence database (http://www.ncbi.nlm.nih.gov/genbank) by accession number (MN928965-8). In Molecular tests by *Av. paragallinarum* species-specific PCR, 12of 18 (66%) of sampled birds were infected with *Av. Paragallinarum*.

Also, antimicrobial susceptibility tests revealed that all four *Av. Paragallinarum* isolates were resistant to amoxicillin, oxytetracycline, streptomycin, trimethoprim/

sulfamethoxazole (up to 75%) and sensitive to cefalexin, ceftriaxone, enrofloxacin, florfenicol, gentamycin, linco-spectin, neomycin, doxycycline (50%), danofloxacin (75%), flumequine (50%), ofloxacin (75%). An intermediate growth inhibition zone was observed around antibiotic discs for ampicillin, colistin, erythromycin, penicillin, tiamulin (75%), tylosin (75%) according to the reference table of the company (Patan-Teb, Iran).

Figure 1. Satellite growth of *Avibacterium paragallinarum* around strike culture of *Staphylococcus aureus* on sheep blood agar plate.

4. Discussion

Infectious Coryza is almost universally distributed and to the best of our knowledge, no information exists on the exact prevalence of the disease among commercial breeding herds and/or backyard chicken in Iran. It is believed that the persistence of IC agents in poultry farms could be based on the multi-age poultry production system and partial immunity due to serovar content of commercial vaccine against regional prevalent serovars of *Av. Paragallinarum* and also the role of backyard chicken as a source of IC infection for nearby chicken flocks (1, 2).

Despite some reports of IC from Pakistan, no molecular monitoring data exists to describe the epidemiology of IC in the neighboring countries of Iran. The occurrence of IC in commercial poultry flocks of Iran has not been officially reported by the Iran Veterinary Organization, which could beat some point a good outcome of vaccination protocols performed by the imported vaccines against serogroups A, B, and C during the last decades. However, in recent years, the clinical picture of IC has been unofficially reported in some layer farms as IC was bacteriologically confirmed by isolation of causative bacteria by authors in one case (not published yet). Generally, IC appears to be controlled in poultry farms due to the vaccination used in most sensitive poultry flocks particularly, layer and breeder chicken. However, the disease in chicken of rural regions and live bird marketing venues frequently has been a challenging clinical issue for veterinarians. *Av. Paragallinarum* has been isolated from different organs of diseased birds such as the infraorbital sinus, choanal cleft, trachea, nasal secretion, and internal organs of the liver, air sacs (1, 10). In this study, chickens presented in the retail live market were selected with typical clinical symptoms of conjunctivitis, unilateral or bilateral facial swelling, and infra-orbital sinus swelling and nasal secretion. Sample birds were notably around 6-8 months old that had been separately taken to market from different parts of the country in groups of 20 to 50 birds. The results of bacterial culture from 18 groups of swab samples obtained from each suspected group of birds resulted in the isolation of four *Av.*

Paragallinarum (22 %). All isolates were V-factor dependent which presented typical 'satellite' growth on solid plate cultures. The result of the culture method (22%) compared to PCR detection (66%) reflects the low efficiency of accurate detection of IC only based on the culture method of the choanal cleft in naturally infected chicken. This result is comparable to the report of Chen et al. (1996) as the PCR test in the normal condition was positive in15/39 birds and 6/8 commercial farms while the positive result of culture was observed in 8/39 birds and 4/8 farms. Another experimental challenge study concluded that *Av. Paragallinarum* could be detected in 50% or less of chickens as positive at 21 and 24 days post-challenge in sinus swab samples by both PCR and culture method and PCR detection and culture methods indicated similar results up to 18 days post-challenge (11). The sensitivity of the PCR method in detecting *Av. paragallinarum* appears to depend on the time of sampling and nature of the infection. Recently, Clothier, to rain (12) reported the frequency of *Av. paragallinarum* infection using real-time PCR in 294 samples from small poultry flocks submitted to the California Animal Health and Food Safety Laboratory System (Davis, CA) among which 86 (30%) samples were PCR-positive for *Av. paragallinarum*. Therefore, it seems that molecular genomic detection in field conditions by PCR or culture methods is less sensitive than in the case of experimental conditions. Selection of the main respiratory organs (infra-orbital sinus, trachea) of birds for *Av. Paragallinarum* isolation is critical. Meanwhile, considerable attention should be paid to avoid the deletion in sampling times in respect to the onset of the disease in birds and rolling out the other disease conditions with similar clinical picture to IC to reduce the discrepancies in efficiency of diagnostic methods.

In the present study, as expected, according to the microbiota of the buccal cavity of birds, along with *Av. Paragallinarum* isolation, other bacteria such as hemolytic Gram-positive (*staphylococcus*. *spp*) and Gram-negative colonies of coliform-like bacteria has also been identified beside *Ornithobacterium rhinotracheale*. Apart from reported avian viral pathogens that may contribute to the pathogenesis of IC (1, 2, 12), *mycoplasma gallisepticum* (1, 13), *Ornithobacterium rhinotracheale* (14), *Gallibacteriumanatis (15),* Salmonellosis (3), Pasteurellosis (3), *Staphylococcus aureus* (1, 16) have been reported to worsen the health condition of the affected chicken by *Av. Paragallinarum* (1, 3).

The presence of other bacterial pathogens contributed to the severity and complexity of the disease (1, 12). Siddique, SU (17) in the study of viral and bacterial infection in respiratory distress cases of poultry flocks scattered in some districts of Punjab, Pakistan, which were seropositive and seronegative for *mycoplasma gallisepticum* using PCR test, reported 5.7% and 3.8% detection rate of *Av. Paragallinarum*, respectively (3). In a study conducted in Jimma, Ethiopia the prevalence of 22.4% of IC was reported in backyard chicken of five rural areas by PCR from November 2011 to April 2012 (18). The intensity of the chicken population and climate of villages seems to have a profound effect on the prevalence of infectious coryza (1, 18). IC in neighboring countries of Iran such as Pakistan (17, 19), Turkey (20), Iraq (21), and regional country of India (22, 23), and Bangladesh (24) have also been epidemiologically reported in layer and breeder farms in addition to backyard chicken. Further studies should be conducted to investigate the prevalence of IC among different types of chicken and its possible role in complex respiratory syndrome in Iran.

The variation in antimicrobial sensitivity patterns between isolates from some countries emphasizes the importance of active and continuous monitoring of *Av. Paragallinarum* isolates (25, 26). Limited information exists on isolation and antibiotic susceptibility of *Av. Paragallinarum* prevalence in Iran, which is limited to the report of Banani, Pourbakhsh (5) Contrary to his report, the present study alters AST results related to the development of complete antibacterial resistance

against oxytetracycline and Lincomycin among *Av. Paragallinarum* isolates, despite the expected low usage of the antibacterial medications in those chickens.

Extensive use of antibacterial drugs to control secondary bacterial infection following viral diseases particularly immunosuppressive viral infection in commercial flocks imposes great pressure on emerging multi-antimicrobial resistant bacteria including *Av. Paragallinarum* (26). In the present study, NAD independent strain of *Av. Paragallinarum* has not been isolated despite careful examination of culture plates.

Moreover, the antibacterial drug resistance of IC agents may threaten poultry and public health. A more in-depth study is recommended to develop a low-cost autogenous vaccine for backyard poultry to prevent disease and antimicrobial resistance. The present study, to the best of our knowledge, is the first report of IC agent isolation and genetic identification by determining partial hemagglutinin gene sequencing (MN928965-8) from backyard chicken in Iran. Further studies are needed to investigate NAD independent IC strains and unusual growth requirements variants (27) of *Av. Paragallinarum.*

Authors' Contribution

Study concept and design: A. N.
Acquisition of data: M. B.
Analysis and interpretation of data: S. Gh. M.
Drafting of the manuscript: A. Sh.
Critical revision of the manuscript for important intellectual content: M. B.
Statistical analysis: A. N.
Administrative, technical, and material support: M. B.

Ethics

Ethical principles of working with laboratory animals in the present study were approved by Education and Research Deputy of the Jihad-Agriculture Ministry, Tehran, Iran under the project number: (NO: 12-18-18-9452-94003).

Conflict of Interest

The authors declare that they have no conflict of interest.

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