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 chicken. Despite worldwide distribution of IC, there is no regular studies on isolation and characterization of *Av. Paragallinarum* in Iran. This investigation was attempted to isolation and antibacterial susceptibility testing (AST) of IC agent from suspected backyard chicken with typical signs of IC in bird’s market places. From 18 collected choanal swab samples, four (22%) isolates of *Av. Paragallinarum* were identified by culture methods based on satellite growth on blood agar which were confirmed by biochemical reaction of Catalase and Oxidase tests and by species-specific PCR (HPG-2). For more characterization, hypervariable region of the hemagglutinin gene of 4 isolates were amplified and obtained sequences deposited at Gene Bank. Meanwhile by PCR (HPG-2) on swab samples, twelve (66%) positive reaction were detected by observing expected 500 bp size bands. Antibiotic susceptibility test (AST) of obtained Isolates were studied via Kirby-Bauer disk diffusion method on horse blood Columbia agar. It was revealed that the isolates are resistant to amoxicillin, oxytetracycline, streptomycin, trimethoprim/ sulfamethoxazole (up to 75%) and sensitive to cefalexin/ ceftaxone, enrofloxacin, florfenicol, gentamycin, lincospectin, neomycin, doxycycline (50%), danofloxacin (75%), flumequine (50%), ofloxacin (75%). Intermediate growth zone inhibition around antibiotic disks have been observed for ampicillin, colestin, erythromycin, penicillin, tiamulin (75%), tylosin (75%). In summary, this is the first report of *Avibacterium paragallinarum* isolation and identification from backyard chicken which may be a source of IC for commercial chicken flocks. Moreover prevalence of resistance to some antibacterial drugs of IC agents may impose the added threat to the poultry rising industry. A more in-depth study is recommended to develop a low cost autogenous IC vaccine for small scale poultry to prevent and management of the disease and antimicrobial resistance development.
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 and growth retardation and reduced egg production (Blackall and Soriano-Vargas, 2013). IC normally characterized with acute onset and spreads rapidly in a flock with the signs of nasal discharge, facial swelling, lacrimation, anorexia, and diarrhea (Blackall and Soriano-Vargas, 2013; Blackall and Soriano-Vargas, 2020).

Unusual IC clinical signs have been reported from Americas which chickens had shown similar clinical signs to swollen-head-like syndrome (Sandoval et al., 1994; Blackall and Soriano-Vargas, 2020). In principal, Prevention of IC mostly rely on practice of sanitation, isolated rising sites and vaccination in poultry farms. Despite these, still sporadic outbreaks of infectious coryza continue to occur and cause significant economic loss especially in developing countries. Despite worldwide distribution of IC and familiarity of the disease to farmers in Iran, there is no regular studies on isolation and characterization of Av. Paragallinarum in country. Bozorgmehri fard in 1980 reported the first study of antibiotic sensitivity of the pathogenic bacteria (Bozorgmeri fard, 1980). Banani, and coworkers has studied truly on isolation and characterization of the pathogenesis of Av. Paragallinarum from infected layer flocks (Banani et al., 2007). In general IC most often has been occurred in backyard chicken in the country. So in order to find out the situation of IC among these kind of chicken, a study was conducted on isolation and identification of Av. Paragallinarum with the aim of helping to IC management and prevention in feature.

Material and Methods
Samples collection
For bacterial isolation and detection, a total of 18 choanal swabs samples from backyard chicken cases suspected to IC infection were obtained and transferred in Brain Heart Infusion (BHI) broth media supplemented with Nicotinamide Adenine Dinucleotide (NAD) (.0025mg/ml). The sampled birds were from retail markets of the Karaj and Tehran cities between November 2018 to May 2019 showing facial swelling, nasal discharge, and respiratory disorder were selected for sampling. For prevention of possible bacterial deactivation, the obtained samples had been tried to culture in laboratory in less than 2 hours.

Bacterial Isolation
The samples were cultured by streaking swabs on 5% sheep blood agar supplemented with 1% sterile-filtered heat-inactivated chicken serum and then followed by cross streaking of Staphylococcus aureus
as NAD provider for media. Bacterial growth were supported by incubation in 37°C and 5% CO2 pressure for 24-48 hours. Bacterial colonies that shows satellite growth were chosen to subculture in 7% horse blood Columbian chocolate agar for further purification and maintenance purpose as recommended (Blackall. and Yamamoto, 1998). Finally in Grams staining negative colonies were screened biochemically for catalase negative and oxidase positive tests.

**Detection and identification of *Av. Paragallinarum* by PCR.**

Required DNAs for the test were extracted from both cultured bacterial colonies and swab samples. The DNA extractions from bacterial culture was followed the methods as described by Moor (2002) via boiling of the appropriate amount of clone purified isolates in sterile distilled water for 45-60 minutes (Moore, 2002). The swab samples were prepared to DNA extraction after wrapping the wetted swabs in media around tube vessel, the remained transport media were centrifuged at 13,000 rpm and the obtained precipitate were harvested in 400µl sterile distal water. The DNA extraction was followed by phenol-chloroform method as described previously (Moore, 2002; Nouri et al., 2018). We included in our tests a previously characterized *Av. Paragallinarum* and *Ornithobacterium rhinotracheale* bacteria as positive and negative bacterial genome control respectively.

The extracted DNAs from both swab samples and bacteria were using in test of HPG-2 PCR specific for *Av. Paragallinarum*, as described by (Chen et al., 1996). The primers set, N1 and R1 (Table 1) to generates an amplicon of approximately 500bp in final electrophoretic analysis. Furthermore for deeper identification and verification of isolated bacteria the middle third (hypervariable region) of hemagglutinin gene sequence of isolates were amplified and sequenced using published (Sakamoto, 2012) primer sets of 5-1 forward and 5-1 reverse with the size of approximately 1.6 Kbp. All PCR tests were carried out in 25-ul volumes containing appropriate amount of template DNA (15-150 ng), MgCl2 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 identification of *Av. Paragallinarum*

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence</th>
<th>PCR product Size (Ref.)</th>
</tr>
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<tbody>
<tr>
<td>R1 N1</td>
<td>5'-CAA GGT ATC GAT CGT CTC TCT ACT-3' 5'-TGA GGG TAG TCT TGC ACG CGA AT-3'</td>
<td>500 bp (Chen, 1996)</td>
</tr>
<tr>
<td>5-1 forward 5-1 reverse</td>
<td>5'-GATGGCAACAATTACATTAC-3' 5'-ACCTTGAGTGCTAGATGCTGTTAGTGTC-3'</td>
<td>1.6 kbp (Sakamoto, 2012)</td>
</tr>
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**Antibacterial susceptibility test**
*Av. Paragallinarum* is a slow-growing, fastidious bacterium, most strains requiring V-(nicotinamide adenine dinucleotide, NAD) factor for their growth and there is not recommended standardized medium for susceptibility testing. So Columbia blood agar (CBA) with 7% horse blood was used to carry out antibacterial susceptibility test via Kirby-Bauer disk diffusion method in accordance with clinical and laboratory standard Institute (CLSI. 2018) guideline. A suspension of the fresh culture of isolates (of approximately 1 × 10^8 CFU/mL) was prepared in BHI broth supplemented with NAD which adjusted to 0/5 McFarland standard turbidity, then spread evenly onto CBA in a Petri dish. Then for investigating antibiotic susceptibility, twenty antibiotics were chosen based on those commonly used in farms. All of the antimicrobial discs were from. Discs of antibiotics (Patan-Teb, Iran) were aseptically placed onto the surface of the inoculated agar plate media. After incubation (16–24 h at 37 °C with 5% CO2 pressure) zones of growth inhibition around each of the antibiotic discs was measured to the nearest millimeter. Using reference table of company, the size of zone of growth inhibition related to each antibiotics were 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 (10 μg), cephalexin (30 μg), ceftriaxone (30 μg), doxycycline (30 μg), danofloxacin (5 μg), erythromycin (15 μg), florfenicol (30 μg), flumequine (μg) fosfomycin (200 μg), gentamycin (10 μg), lincomycin (μg), neomycin (30 μg), enrofloxacin (5 μg), ofloxacin (5 μg), penicillin (10 μg), streptomycin (10 μg), trimethoprim-sulfamethoxazol (1.25 + 23.15 μg), oxytetracyclin (30 μg), tiamulin (30 μg) and tylosin (30 μg).

**Results**

*Av. Paragallinarum* bacteria with showing typical morphological and biochemical characteristic of satellite growth, Grams negative polymorphic short rod bacteria, oxidase activity and non-catalase reaction was isolated from 4 out of 18 of choanal swab samples. Culture of positive samples generally looked as a dominated in form of tiny dewdrop, non-hemolytic colonies even in first round of cultivations (Picture 1). Meanwhile these isolates had also no growth in co-cultures of MacConkey agar media and at normal air incubation condition.
Molecular detection and identification of these isolates were confirmed by both observation of expected 500bp band in electrophoresis of their species-specific PCR products and partial hemagglutinin protein gene sequencing. After proper alignment of obtained partial nucleotide sequences of related hemagglutinin gene of isolates, they were deposited at the Genbank genetic sequence data base (http://www.ncbi.nlm.nih.gov/genbank) by accession numbers of MN928965-8. In Molecular tests by Av. paragallinarum species-specific PCR, 12 out of 18 (66%) of sampled bird had involved with Av. Paragallinarum infection.

Also antimicrobial susceptibility tests showed that all of 4 Av. Paragallinarum isolates were resistant to amoxicillin, oxytetracycline, streptomycin, trimethoprim/ sulfamethoxazole (up to 75%) and sensitive to cefalexin, ceftriaxone, enrofloxacin, florfenicol, gentamycin, lincospectin, neomycin, doxycycline (50%), danofloxacin (75%), flumequine (50%), ofloxacin (75%). Intermediate growth zone inhibition around antibiotic disks were observed for ampicillin, colestin, erythromycin, penicillin, tiamulin (75%), tylosin (75%) according to reference table of company (Patan-Teb. Iran).
Discussion

Infectious Coryza has almost global distribution and to the best of our knowledge there is no information about the exact prevalence of the disease among commercial breeder flocks and/or backyard chicken in Iran. There is a common believe that persistence of IC agent among chicken farms could be as result of multi-age production system of poultry and partial immunity offered by of serovar content of commercial vaccine against regional prevalent serovars of *Av. Paragallinarum* and also reservoirs role of backyard chicken as a sources of IC infection for nearby chicken flocks (Blackall and Soriano-Vargas, 2013; Blackall and Soriano-Vargas, 2020).

Despite some report of IC from Pakistan, there has been no even molecular surveillance data to describe the epidemiology of IC in our neighboring countries. Occurrence of IC in commercial poultry flocks of Iran officially have not been reported by national veterinary organization so far, which could be in some point of view a good outcome of vaccination protocols that performed by imported vaccine against serogroups A, B, and C throughout the last decades. But in recent years, clinical picture of IC unofficially was reported in some layer farms as in one case IC was bacteriologically confirmed by isolation of causative bacteria by authors (not published yet). In overall, IC in chicken farms appears to be under controls due to the vaccination used in most sensitive poultry flocks particularly layer and breeder chicken. But meanwhile the clinically the disease in chicken of rural region and live bird marketing places frequently has been a challenging matter to the veterinary practitioners. *Av. Paragallinarum* has been isolated from different organs of diseased birds such as infera-orbital sinus, choanal cleft, trachea, nasal secretion, and internal organs of liver, air sacs (Blackall and Soriano-Vargas, 2013; Clothier et al., 2019a), in this study, chicken presented in retail live market with typical clinical sign of conjunctivitis, unilateral or bilateral facial swelling and infera-orbital sinus swelling and nasal secretion were selected. Selected birds for sampling were notably around 6-8 months old that separately had been taken to market from different part of country in groups of consisted about 20 to 50 birds by sellers. The results of bacterial culture from 18 groups of swab specimen obtained from each suspected groups of birds, Resulted into isolation of 4 *Av. Paragallinarum* (22 %). All isolates were V-factor dependent, showing typical ‘satellite’ growth on solid plat cultures. The result of culture method (22%) in comparison to PCR detection (66%) reflects the low efficiency of exact detection of IC solely based on culture method from choanal cleft in naturally infected chicken. This result is comparable with report of Chen, et al (1996) as, PCR test in natural condition yielded 15/39 birds and 6/8 commercial farms positive as compared with 8/39 birds and 4/8 farms positive by culture. In another study by chen, et al (1998) in an experimental challenge studies, He found out that by both
PCR and culture method, *Av. Paragallinarum* could be detected in 50% or less of chickens as positive at 21 and 24 days post challenge in sinus swab samples and also up to 18 day post-challenge, PCR detection and culture methods gave similar results (Chen et al., 1998). It seems that sensitivity of PCR method in *Av. paragallinarum* detection is dependent on the time of sampling and nature of infection. Recently, Clothier (2019), 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), 86 (30%) samples were PCR-positive for *Av. paragallinarum* (Clothier et al., 2019b). Hence it seems molecular genomic detection in field condition by PCR or culture methods have lower sensitivity than in case of experimental condition. It is critical to choosing the main respiratory organs (infra-orbital sinus, trachea) of bird for *Av. Paragallinarum* isolation. Meanwhile to lowering the discrepancies in efficiency of detection methods, considerable attention must be paid to avoiding 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.

In our investigation, Given to the microbiota of buccal cavity of birds, as expected, along with of *Av. Paragallinarum* isolation, other bacteria such as hemolytic Gram-positive (*Staphylococcus* spp) and Gram-negative colonies of coliform-like bacteria beside *Ornithobacterium rhinotracheale* has also been identified. Apart from reported avian viral pathogens that could have participation in pathogenesis of IC (Blackall and Soriano-Vargas, 2013; Clothier et al., 2019b; Blackall and Soriano-Vargas, 2020), *mycoplasma gallisepticum* (Kato, 1965; Blackall and Soriano-Vargas, 2013), *Ornithobacterium rhinotracheale* (Morales-Erasto et al., 2016), Gallibacterium anatis(Paudel et al., 2017) Salmonellosis(Sandoval et al., 1994), Pasteurellosis(Sandoval et al., 1994), *Staphylococcus aureus* (Kishida et al., 2004; Blackall and Soriano-Vargas, 2013) were reported that may worsen the health condition of the affected chicken by *Av. Paragallinarum*(Sandoval et al., 1994; Blackall and Soriano-Vargas, 2013).

The presence of other bacterial pathogens contributed to the severity and complexity of disease (Blackall and Soriano-Vargas, 2013; Clothier et al., 2019b). Siddique and co-worker(2012) in an investigation of viral and bacterial infection in respiratory distress cases of poultry flocks scattered in some districts of Punjab, Pakistan, which were sero-positive and sero-negative for *mycoplasma gallisepticum* using PCR test, reported 5.7% and 3.8% detection rate of *Av. Paragallinarum* respectively(Sandoval et al., 1994). In a study by Dereja (2017) in Jimma, Ethiopia the prevalence of 22.4% of IC in backyard chicken of five rural area by PCR, from November 2011 to April 2012 was reported (Dereja and Hailemichael, 2017). Intensity of chicken population and climate situation of
villages seems have profound effect on prevalence of infectious coryza outbreak (Blackall and Soriano-Vargas, 2013; Dereja and Hailemichael, 2017). Epidemiologically IC in neighboring countries of Iran such as Pakistan (Hasan S et al., 2002; Siddique et al., 2012), Turkey (Findik and Yardimci, 2010), Iraq (Rashid and Pociecha, 1984), and regional country of India (Patil et al., 2017a; Patil et al., 2017b), Bangladesh (Khatun et al., 2016) have been reported in layer and breeder farms in addition to backyard chicken. In our country further studies is needed to find out the prevalence of IC among different type of chicken and it probable role in complex respiratory syndrome.

The variation in antimicrobial sensitivity patterns between isolates from some countries emphasizes the importance of active, ongoing monitoring of Av. Paragallinarum isolates (Luna-Galaz et al., 2016; Nhung et al., 2017). There is very little information on isolation and antibiotic susceptibility of Av. Paragallinarum prevalence in Iran and is limited to the report of Banani and his coworker in 2007. In contrast to his report, this study have shown variation in AST results related to development of complete antibacterial resistance against oxytetracycline and Lincomycin among Av. Paragallinarum isolates. In spite of expected low usage of antibacterial drug in those chicken.

Widespread antibacterial drug usage to combat secondary bacterial infection following viral diseases particularly immunosuppressive viral infection in commercial flocks has overwhelming pressure to emerging multi-antimicrobial resistant bacteria including Av. Paragallinarum (Nhung et al., 2017). In this exploration in spite of careful examination of culture plates, NAD independent strain of Av. Paragallinarum have not been isolated.

Moreover antibacterial drug resistance of IC agents may impose a threat to 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 occurrences. This study is the first report of IC agent isolation and genetically identification by partial hemagglutinin gene sequencing (MN928965-8) from backyard chicken in county. Further studies is needed to investigate NAD independent IC strains and unusual growth requirements variants (Blackall et al., 2011) of Av. Paragallinarum.

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