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# Isolation and molecular identification of *Avibacterium paragallinarum* in suspected cases of infectious coryza

**Mahdi ASKARI BADOUEI[1,*], Avesta SADRZADEH[2], Nader AZAD[3], Patrick BLACKALL[4], Omid MADADGAR[5], Saeid CHARKHKAR[6]**

[1]Department of Pathobiology, Faculty of Veterinary Medicine, Garmsar Branch, Islamic Azad University, Garmsar, Iran
[2]Department of Poultry Science, Faculty of Veterinary Medicine, Garmsar Branch, Islamic Azad University, Garmsar, Iran
[3]Faculty of Veterinary Medicine, Garmsar Branch, Islamic Azad University, Garmsar, Iran
[4]Queensland Alliance for Agriculture and Food Innovation, University of Queensland, St. Lucia, Queensland, Australia
[5]Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[6]Department of Poultry Science, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran

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**Abstract:** Isolation and identification of *Avibacterium paragallinarum*, the causative agent of infectious coryza, is considered a challenging task in laboratories with limited specialties. In the present study, 14 commercial layer fowls showing the typical symptoms of infectious coryza were subjected to primary isolation followed by polymerase chain reaction confirmation of suspect colonies (culture-PCR). Direct PCR assays on infraorbital sinus swab samples were also carried out. Thirty-five suspected cases of infectious coryza in commercial broiler chickens were also screened using direct PCR on infraorbital sinus swabs. In culture-PCR, only 1 of the 4 suspected isolates was confirmed as *Av. paragallinarum*. In comparison, in direct PCR, 5 layer samples were shown to be positive for *Av. paragallinarum*. All of the broiler samples were negative in the direct PCR assay. Our findings indicate that primary isolation in combination with PCR can be a simple method for diagnosis of infectious coryza, although with a lower sensitivity than direct PCR. While direct PCR is comparably the more rapid and sensitive test, there will be instances in which the bacterial isolate is needed for further use. Hence, the culture-PCR method can be a practical and simple approach, especially in laboratories with limited specialty in identification of this fastidious organism.

**Key words:** Infectious coryza, *Avibacterium paragallinarum*, culture-PCR, direct PCR

## 1. Introduction

Infectious coryza is an acute respiratory disease of chickens caused by *Avibacterium paragallinarum*, once known as *Haemophilus paragallinarum*. The disease is highly contagious and produces an acute disease of the upper respiratory tract of chickens, which can turn into a chronic respiratory disease when complicated by other pathogens (1,2). The disease occurs worldwide and the greatest economic losses associated with infectious coryza are attributed to poor growth performance in growing birds and marked reduction (10%–40%) in egg production in layers and breeding fowls (3,4). While traditionally regarded as a disease of laying birds, there are reports of the impact of the disease in broiler chickens. In developing countries, the presence of other pathogens and poor management can result in outbreaks with greater significance and considerable economic losses (4,5).

The traditional method for identification of coryza in a chicken flock is the isolation and biochemical characterization of *Av. paragallinarum*. Since most of the *Av. paragallinarum* isolates show a requirement for nicotinamide adenine dinucleotide (NAD) for growth, the isolation procedure requires experience and use of special artificial media (3,4). The occurrence of similar but nonpathogenic NAD-dependent species such as *Av. avium*, *Av. volantium*, and *Avibacterium* spp. in some countries makes identification of the agent a more difficult and challenging task (6). A sensitive and specific polymerase chain reaction (PCR) was developed for identification of *Av. paragallinarum* (7). More recently, a sensitive and rapid real-time PCR assay was developed for identification of *Av. paragallinarum* (8).

In the current study, 2 diagnostic approaches were used. The first approach was used on layer bird samples and consisted of culture-PCR in which CLBA medium (Columbia agar base plus 7% equine lysed blood) was used as a simple and inexpensive medium for primary isolation and HPG-2 PCR was used as the confirming test for the

* Correspondence: askari@iau-garmsar.ac.ir

isolates. Direct PCR on clinical samples was the second approach for diagnosis of *Av. paragallinarum* in this study; this method was applied for the layer bird samples as well as the broiler samples.

## 2. Materials and methods

### 2.1. Layer samples

The 14 layer samples consisted of 4 live birds and 10 heads of birds transported to the laboratory inside sterile bags on ice within 5 h of collection. All 14 samples were diagnosed with typical symptoms of coryza including swelling of the head and subcutaneous edema of the face and wattles (sinusitis). The samples were collected from 2 farms, which were designated as Farms A and B. Five head samples were collected from Farm A and 4 live birds and 5 head samples from Farm B.

### 2.2. Broiler samples

The broiler samples were collected from 3 farms designated as C, D, and E. Four head samples were collected from Farm C (13-day-old broilers) with the birds suffering from chronic respiratory disease and head swelling. Six samples were from Farm D, a 35-day-old flock with signs of respiratory distress, swelling of the infraorbital sinus, and moderate nasal discharge. From Farm E (43-day-old broilers), 25 head samples were collected from birds showing respiratory signs, including mild head swelling, and complicated symptoms typical for viral diseases like infectious bronchitis and Newcastle disease.

### 2.3. Isolation and molecular identification of *Avibacterium paragallinarum* (culture-PCR)

Samples were taken by squeezing the swollen nasal sinus of the affected birds and collecting the excreted mucus from the nostril using a sterile loop; samples were immediately streaked on 2 plates of CLBA medium with and without a *Staphylococcus epidermidis* cross-streak as described before (9). The plates were incubated in a candle jar for 24 to 48 h and the suspected colonies were examined by Gram staining and catalase test. Pure cultures were made from the suspected gram-negative and catalase-negative isolates for further use.

For total genomic DNA extraction, a loopful of bacteria was removed from the surface of the agar plate and suspended in 300 µL of ultrapure water by vigorous shaking. The suspension was boiled for 10 min and centrifuged at 12,000 × *g* for 2 min (10). The supernatant was used as the template in PCR.

The crude cell lysates were subjected to PCR using the previously described N1 5' TGA GGG TAG TCT TGC ACG CGA AT 3' and R1 5' CAA GGT ATC GAT CGT CTC TCT ACT 3' primers (7). PCR was carried out in a total volume of 50 µL containing 1 µL of prepared DNA, 0.4 µM of each oligonucleotide primer, 0.2 mM dNTP mix, 2 mM $MgCl_2$, 5 µL of 10X PCR buffer, 1.25 U of Taq DNA polymerase (Fermentas, Lithuania; EP0402) and PCR-grade water up to 50 µL. The reaction conditions consisted of 25 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The 500-bp specific products were visualized after electrophoresis in 1.2% agarose gels and staining with ethidium bromide. A genetically confirmed isolate of *A. paragallinarum* in this study was used as the positive control and sterile water as the negative control in all of the PCR assays.

### 2.4. Direct PCR on clinical samples

Samples were collected using sterile swabs from nostril secretions after squeezing the swollen sinus or after dissecting the affected sinus. The swabs were soaked in 400 µL of phosphate-buffered saline for 1 h at room temperature and processed using a modification of the method described previously (7,11). In brief, the samples were centrifuged at 2000 rpm for 5 min to settle the blood. The supernatant was then transferred to a new tube and centrifuged at 13,000 rpm for 15 min. The supernatant was discarded, and the pellet was suspended in lysis buffer containing 10–20 µL of 1X PCR buffer containing 0.5% Nonidet P-40 (Applichem, Germany; A1694), 0.5% Tween 20 (Applichem; A4974), and 200 µg $mL^{-1}$ proteinase K (Fermentas; EO0491) and incubated at 56 °C for 1 h. Next, the sample was heated at 98 °C for 10 min to inactivate the proteinase K. The processed samples were subjected to PCR as described above.

## 3. Results

Among the 14 layer samples, 4 samples yielded suspect isolates (gram-negative, catalase-negative) in culture but only 1 sample (from Farm B) was confirmed as *Av. paragallinarum* after DNA extraction and PCR testing. This positive PCR product was sequenced and, after BLAST confirmation, the sequence was deposited in GenBank (Accession No. HQ397677). Direct PCR testing on the 14 infraorbital sinus swabs was able to identify 5 positive cases of infectious coryza (including the bird identified by culture-PCR) (Table). Thirty-five broiler head samples were screened using direct PCR on infraorbital sinus secretions but none of the samples showed positive results in PCR. The direct PCR assay on some layer samples is presented in the Figure, showing 2 of the positive samples.

## 4. Discussion

The fastidious nature of *Av. paragallinarum* and the subsequent requirement for special media has made the isolation and identification of this organism a costly and laborious task (3,11). Furthermore, *Av. paragallinarum* is a relatively slow-growing organism that can be easily overgrown by other contaminating bacteria that commonly inhabit the nasal and upper respiratory

**Table**. Comparison of primary culture, PCR confirmation of the isolates, and direct PCR.

| Layer samples | Culture[a] | PCR-positive isolates | Direct PCR[b] |
|---|---|---|---|
| 1 | - | - | - |
| 2 | - | - | - |
| 3 | - | - | - |
| 4 | - | - | - |
| 5 | - | - | - |
| 6 | + | - | + |
| 7 | + | - | + |
| 8 | - | - | + |
| 9 | + | - | + |
| 10 | - | - | - |
| 11 | + | + | + |
| 12 | - | - | - |
| 13 | - | - | - |
| 14 | - | - | - |

[a]: Suspected isolates of *Av. paragallinarum* (gram-negative and catalase-negative).
[b]**:** Direct PCR on clinical samples (infraorbital sinuses).

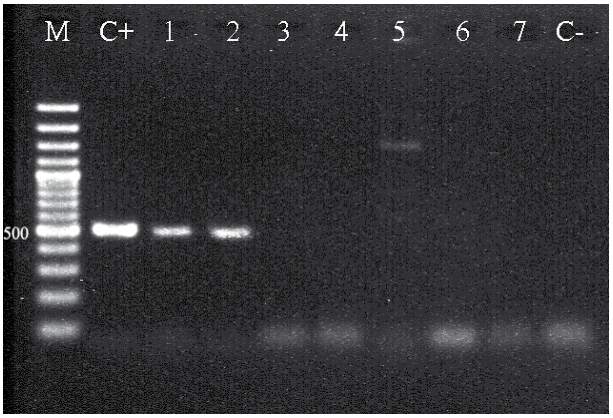


**Figure.** Molecular identification of *Av. paragallinarum* in layer samples by direct PCR. M, marker, 100 bp-plus (Fermentas, Lithuania; SM0321); C+, positive control (500 bp); 1, 2: 2 of the positive layer samples; 3–7: negative samples; C-, negative control.

passages. Additionally, nonpathogenic haemophili, formerly known as *Av. avium* and *Av. volantium*, can also be present in chickens, which makes the isolation and identification process problematic (11). The use of PCR tests after initial isolation instead of biochemical identification can reduce the complexity of the diagnostic task (11). Another advantage of this method is its speed, because the results are obtainable within 24–48 h. In the present study, the culture-PCR approach was used for the diagnosis of coryza in 14 layer cases. Among 4 suspect isolates obtained from culture, only 1 produced the specific band for *Av. paragallinarum*. The culture medium used in this work (CLBA) does not show satellitism as it contains all required nutrients for the growth of *Av. paragallinarum*. While *Av. paragallinarum* is indeed gram-negative and catalase-negative, there is a range of other bacteria that have these same 2 properties, including *Ornithobacterium rhinotracheale*. In the future, the additional screening of the suspect property of satellitism may reduce the number of incorrect suspect isolates selected for PCR examination.

Another approach for diagnosis of infectious coryza is direct PCR on clinical samples. HPG-2 PCR was developed for this purpose with very promising results, and in Australia, this method was found equivalent to culture (7). Direct PCR examination of sinus swabs outperformed traditional culture in routine diagnostic submissions when practiced in China (11). In developing countries problems like improper sampling, delayed transport, and poor quality of media could result in higher failure rates in isolation (3). In contrast to culture, direct PCR can also be performed on swab samples that were stored at 4 °C or –20 °C for up to 180 days (12). In South Africa the diagnosis of infectious coryza is complicated by the presence of atypical forms of *Av. paragallinarum* (NAD-independent) and *Ornithobacterium rhinotracheale* as well as the traditional form of *Av. paragallinarum* (NAD-dependent); therefore, HPG-2 PCR has also proven to be very useful in these situations (13). Our study on layer samples also showed that direct-PCR is a more sensitive approach than culture-PCR, since 5 samples were positive in the direct method as compared to 1 positive sample in culture-PCR.

Infectious coryza can have a significant impact on broiler chickens and the importance of infectious coryza in broiler flocks and related economic losses have been addressed in previous studies (14–16). Banani et al. studied 14 broiler flocks in Iran by culture method but *Av. paragallinarum* was not isolated (17). Usage of antibiotics, especially sulfonamides, can increase the failure rate of bacterial isolation. In the present study direct PCR was applied to all clinical samples to overcome the stated issue. The broiler head samples were highly contaminated; therefore, we were not able to apply the culture method to them. To our knowledge, infectious coryza has not yet been reported in broiler chickens in Iran, and in agreement with the culture-based study of Banani et al., all broiler samples were negative in the direct PCR assay in this study (17).

The present study indicates that direct PCR is a very sensitive approach in the diagnosis of infectious coryza. Similar findings were observed when direct PCR was compared to PCR on isolates in China (11). It should be kept in mind that expertise and laboratory resources can be the critical factors when comparing culture and PCR for this fastidious, fragile, and relatively slow-growing organism. There would be instances where the bacterial

isolates are required for serotyping or antibacterial susceptibility testing, and the culture-PCR method can be used as a simple diagnostic option in laboratories with limited facilities for identification of *Av. paragallinarum*. Further researches with larger sample sizes and sophisticated sampling methods in different seasons and geographical areas are required to rule out the presence of *Av. paragallinarum* in broiler chickens in Iran.

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