Phenotypic and genotypic Characterization of *Avibacterium paragallinarum* isolated from layer chicken Flocks in Egypt yearling 2013-2015

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Abstract

Aim: This study was carried out for isolation of *Av. paragallinarum* from suspected clinical samples and to be identified and serotyped by the molecular technique using Multiplex PCR as an alternative serotyping method.

Material and methods: One hundred and twenty field samples were collected from different poultry farms and bacteriologically examined and morphologically characterized. The suspected isolates which have a phenotypic characters as *Av. Paragallinarum* were examined to the serotype serovare level using PCR through two steps. The first used universal primer to check for *Av. Paragallinarum* with specific PCR primers yielding a 500 bp PCR product. The positive reactors with the primary PCR were proceeded to the second step which is multiplex PCR using primer sets specific for serotypes A, B, and C and the obtained PCR products were 0.8, 1.1 and 1.6 kbp fragments for serovars A, B and C, respectively.

Result: Nine *Av. paragallinarum* isolates were identified, 4 isolates were serovar A, 3 isolates were serovar C and 2 isolates were serovar B.

Conclusion: This study concluded to, the co-circulation of the *Av. paragallinarum* serotypes A, B and C in Egypt. Also the reliability of using multiplex PCR (hypervariable region of *HMTp210)* as a useful tool for fast serotyping of *Av. Paragallinarum* isolates.

Keywords: *Avibacterium paragallinarum*, serotyping, Infectious coryza, polymerase chain reaction, poultry.

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Introduction

Avian infectious coryza (AIC) is a serious respiratory tract infection of chickens caused by *Avibacterium paragallinarum* (*Av. Paragallinarum*) previously known as *Haemophilus paragallinarum*, the disease is highly contagious and cause an acute disease of upper respiratory tract of chickens, which can turn into a chronicity when complicated with other pathogens. The disease is essentially limited to chickens and may occur in both growing and layers chickens. [1,2].

AIC have an economic implication on poultry industry, due to loss of condition in broilers and reduced egg production in laying flocks which ranged between 10 and 40%, the presence of other pathogens and poor management can result in outbreaks with greater significance and considerable economic losses [3].

The disease is characterized clinically by nasal discharge, swelling of the face, lacrimation, anorexia, diarrhea and high morbidity about (20%-50%) and low mortality (5%-20%) in infected chickens. Lesions associated with the disease reflect an acute catarrhal inflammation of the upper respiratory tract, mucoid sinusitis with edema and congestion of sinus mucosa [4,5].

Av. Paragallinarum was classified into three serovars A, B and C using haemagglutination inhibition test (HI) [6]. On the other hand, [7] used the Kume serogrouping based on HI test into nine serovars (A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3,

and C-4). Transmission may occur via direct contact or indirectly through contaminated drinking water with nasal discharge, airborne route but not egg transmission. Apparently, healthy carrier birds serve as the main reservoir of infection. [5].

Up to the past ten years *Av. paragallinarum* infection is diagnosed by isolation and identification based on its culture characters and biochemical properties [8] that have several disadvantages. Meanwhile, serological diagnosis of the disease has not been performed because the progress of this disease is rapid and antibodies are not likely to be induced in chickens infected with *Av. paragallinarum*, particularly with serovar C, even after the disease onset. However, the procedure is complicated, and the sensitivity is insufficient. The replacement of the conventional diagnostic method by some form of DNA-based test would be a major step forward. Nucleic acid molecular tests for diagnosis, identification and serotyping of *Av. Paragallinarum* have been developed and evaluated by some researchers [9]. Hence, the objective of the present study was to attempt isolation and identification of *Av. paragallinarum* from suspected clinical samples and to characterize them by biochemical tests and PCR.

Materials and Methods

Clinical samples processing and bacteriological isolation

One hundred and twenty infraorbital sinus swabs were collected from suspected flocks which had respiratory symptoms seems to be infectious coryza with marked drop in egg production. The swabs were examined bacteriologically by streaking on to brain heart infusion agar media and chocolate agar both supplemented with NADH (V- factor) 25µg/ml. Plates were incubated at 37 ºC for 24 hours in the CO_2 incubator and then sub-

cultured for purification. Suspected colonies were picked up and examined microscopically after staining with Gram's stain for further examination [10].

Biochemical characterization

All isolates with phenotypic characteristic suggestive of *Av. paragallinarum* were characterized biochemically. The isolates were tested for catalase, oxidase, urease, nitrate and indole positivity and ability to ferment sugars that are, glucose, sucrose, maltose, mannitol, sorbitol, galactose and fructose [11].

Molecular characterization

Bacterial DNA extraction: The cultures were grown overnight at 37^{o}C and harvested by centrifugation. The genomic DNA was extracted by using a commercial kit, Qiagen DNA extraction kit according to the manufacturer's instructions under section "bacterial DNA extraction". Extracted DNAs were tested using a species-specific PCR through two steps.

Conventional & Multiplex PCR: Using universal *Av. paragallinarum* Primers following the procedure adopted by [12]. Set of primer was used from the conserved DNA region specific to *Av.paragallinarum*. For multiplex PCR, another set of primer specific for HTM gene correspond to serotypes A, B and C were used for serotyping are shown in table 1 [9].

The PCR reactions were performed in a total volume of 25 µl contained 12.5 µl Taq green PCR master mix (2x) (Thermoscientific), 2µl of DNA sample, 1 µl of each forward and reverse primer and 8.5 Nuclease free water. The PCR condition were initial denaturation at 95ºC for 10 minutes, followed by 35 cycles of denaturation at 95ºC for 1 min, primer annealing at 55 °C for 1 min, primer extension at 72 ºC for 1 min. the final extension was set at 72 ºC for 7 min. The PCR products were subjected to agarose gel electrophoresis using 2% agarose, visualized, and photographed under ultraviolet light

Table (1) Sets of Primers used

Primer	Sequence	Fragment	Ref.
Universal	F: 5'-TGA GGG TAG TCT TGC ACG CGA AT 3' R: 5'-CAA GGT ATC GAT CGT CTC TCT ACT 3'	**500 bp**	**[12]**
Common	F: 5'GGCTCACAGCTTTATGCAACGAA-3'		**[9]**
A	R: 5'-CGCGGGATTGTTGATTTTGTT-3'	**0.8 kbp**	
B	R: 5'-GGTGAATTTCACCACACCAC-3'	**1.1 kbp**	
C	R:5'TAATTTTCTTATTCCCAGCATCAATACCAT-3'	**1.6 kbp**	

Results

Morphological characterization

Isolation of *Av.paragallinarum* was attempted from 120 field samples from different poultry farms. Small dew drop like colonies were detected on brain heart infusion agar and Chocolate agar after 24 hours of incubation at 37°C in 5-10% CO_2 incubator and no growth could be observed on MacConkey agar. All isolates need V- factor (NADH) in their growth. Isolates showed Gram-negative, nonmotile coccobacilli when stained with the Gram stain.

Biochemical characterization

All isolates that have morphological character were subjected to the biochemical test. The isolates were found to be negative for catalase, H_2S, urease, and indole, oxidase positive, reduced nitrate and ferment glucose, sucrose, fructose, maltose, sorbitol, and mannitol but not galactose.

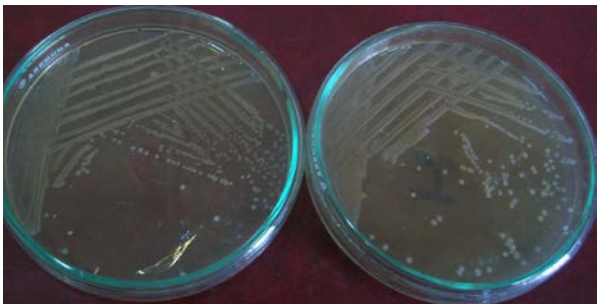

Figure 1. Av. Paragallinarum colonial morphology on brain heart infusion agar: tiny dew drop colony are seen after 24 hours incubation.

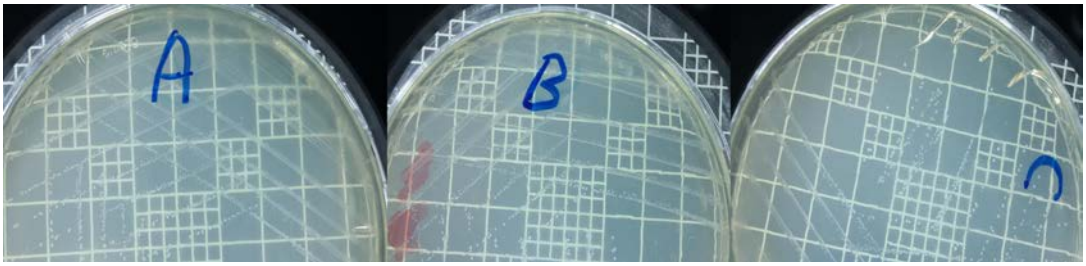

Figure 2 Tiny dew drops like colonies of Av. paragallinarum serotypes (A, B and C).

Molecular identification and serotyping

The isolates which have morphological and biochemical character as *Av.paragallinarum* were primarily confirmed when universal primer were used where PCR specific fragments correlating to ≈ 500 bp were observed as expected size the same as the positive control sample.

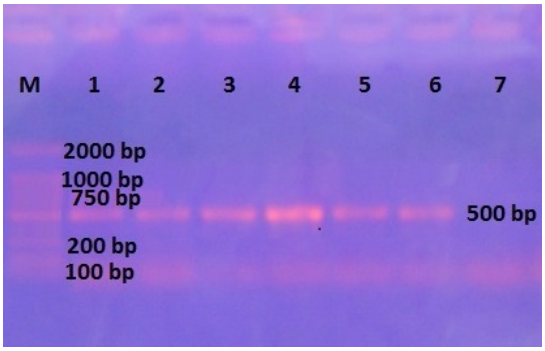

Figure 3 Polymerase chain reaction (PCR) amplification fragments of Av. paragellinarum field isolates using universal primer. M is the 100 bp DNA ladder. Lane 1 positive Av. Paragallinarum, Lane 2.3.4.5.6 field isolates of Av. Paragallinarum and lane 7 is negative control.

Results of multiplex PCR using primer sets specific for HTM gene was used for serotyping. Expected amplicons size were 800, 1100, and 1600 bp which correspond to page's serotype A, B, and C respectively.

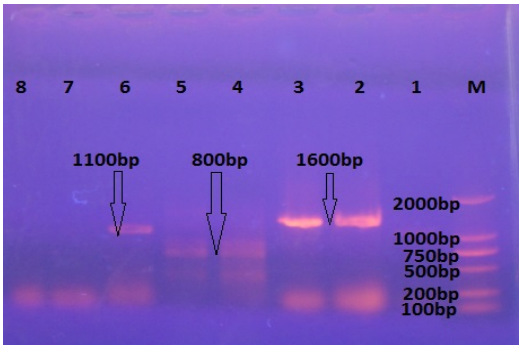

Figure 4 Multiplex PCR of Av.Paragallinarum isolates using specific primers.

Discussion

Respiratory tract infections are of great importance in poultry industry, causing heavy economic losses, *Av. Paragallinarum* is the cause of Infectious Coryza [IC] with worldwide distribution especially in farms keeping multi-aged birds and it is influenced

by environmental factors [13,2]. Economic losses in the poultry industry mainly due to increased culling rates in broiler chickens as well as reduction in egg production [14].

In developing countries, the presence of other complicating factors including other pathogens and poor management can result in outbreaks with greater significance and considerable economic losses, this markedly different from those in the uncomplicated infections typically seen in developed countries, where the disease can have less greater economic impact in well management farms [3].

In the present study, a total of 120 infra orbital sinus swabs were collected from chicken which had respiratory signs and marked drop in egg production. The *Av. Paragallinarum* was isolated and identified via phenotypic character and molecular identification. The typical culture characteristics revealed that colonies of *Av. paragallinarum* including the need of NADH and microaerobic or anaerobic (5-10% CO_2) for growth; small dew drop like colonies on brain heart infusion agar and Chocolate agar; and Gram negative, non-motile coccobacilli were observed after 24 hours of incubation. Also, the biochemical identification of obtained isolates identified them as *Av. Paragallinarum* [2,5,15,16].

Recent literatures showed that serological diagnosis of AIC is not a reliable and/or confidential tool for identification of *Av. paragallinarum* infection, because the progress of this disease is rapid and antibodies are not likely to be induced in chickens infected with *Av. paragallinarum*. Also, the procedure of HI tests, used for identification of *Av. paragallinarum*, is complicated and the sensitivity is insufficient. Hence, the replacement of the conventional serotyping by some forms of DNA based tool would be a major step forward, allowing many more laboratories to perform serotyping.

In the present study the molecular serotyping technique of *Av.paragallinarum* by conventional and Multiplex PCR was used as an alternative serotyping method. The isolates which had phenotypic characters of *Av. paragallinarum* were confirmed using the PCR specific test. These result are correlated to that reported by [17]. Multiplex PCR of *Av. paragallinarum* isolates using primer sets around the hypervariable region amplified 0.8, 1.1 and 1.6 kbp fragments for serovars A, B and C, respectively. Multiplex PCR test was introduced by [9] used for serotyping, targeted the hypervariable region of HMTp210 sequence, which encodes the HA antigen of *Av. paragallinarum*. This test also used successfully by [16] in studies of AIC cases.

In this study 9 *Av. paragallinarum* isolates were identified, 4 isolates were serovar A, 3 isolates were serovar C and 2 isolates were serovar B. The *Av.paragallinarum* serovar A, B, and C were isolated previously in Egypt and 26 isolates were characterized isolates recovered from 36 outbreaks of infectious coryza in layer chicken flocks [18]. In another study carried out in 2004 in Upper Egypt a total of 22 morphologically selected isolates were subjected to biochemical and serological investigations that further indicated the presence of *Av. Paragallinarum* serotypes A, B and C [19].

In conclusion, the results of the current study further confirmed the co-circulation of the *Av. paragallinarum* serotypes A, B and C in Egypt. Moreover, the study further confirmed the reliability of using both conventional and multiplex PCR as a useful tool for fast serotyping of *Av. Paragallinarum* isolates rather than laborious bacteriological isolation and identification techniques.

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