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Isolation and Molecular Identification of *Avibacterium Paragallinarum* in Suspected Cases of Poultry

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Abstract

Infectious Coryza (IC) is an infectious upper respiratory disease of chickens and birds, caused by *Avibacterium Paragallinarum* (*Av. Paragallinarum*). It is characterized by facial swelling and nasal discharge. Here, the study aimed to identify the causative agent of infectious coryza from diseased cases using conventional and molecular methods, forty-one different samples (swollen heads and nasal discharges) from layers and broiler chickens were examined bacteriologically for isolation of *Av. Paragallinarum*. The total isolation percentage was 9.75%, and the percentage was 66.7% from total layer samples. The samples underwent examination by polymerase chain reaction (PCR) using HPG-2. Three samples were confirmed by PCR with a percentage of 7.3% (3/41). Multiplex PCR was used for typing the tested strains using HMTp210 gene thus, all strains were of type B. sequence analysis showed a maximum identity percentage (100%) between the tested strains of *Av. Paragallinarum*. In conclusion, typical signs for IC disease were appeared and identified bacteriologically as *Av. Paragallinarum* also three strains were only confirmed by PCR and identified type B strains. PCR technique is a reliable and sensitive test for confirmation.

KEYWORDS
Avibacterium Paragallinarum, HPG-2- phylogenesis, PCR

INTRODUCTION

Infectious Coryza (IC) is described as a disease of upper respiratory system, especially in chickens it caused by *Av. Paragallinarum* (Blackall *et al*., 2005), and infect different types of chickens of different ages (Akter *et al*. 2013). The economic impact of IC is correlated to egg reduction to a percentage about 10-40% leading to a high increase in the numbers of rejected chickens (Tabbu, 2000; Rajurkar *et al*., 2009; Ali *et al*., 2013). Coryza is recognized as a disease with high morbidity and low mortality (Bragg, 2002; Akter *et al*., 2013). The most obvious symptoms of IC are acute to chronic inflammation of the respiratory tract especially the upper part, including sinuses with serous to mucoid nasal discharge, infraorbital sinus swelling, facial edema, conjunctivitis, and lacrimation (Bragg, 2002; Han *et al*., 2016). As the fastidious nature of *Av. Paragallinarum* and slow-growing microorganism that may have difficulties in conventional isolation methods as it needs complex media for growth (Anjaneya *et al*., 2014). However, the use of conventional methods may be unsuccessful in the diagnosis of infectious coryza (Fauziahi *et al*., 2021).

Avibacterium Paragallinarum isolates can be serotyped by two different methods, the first one distinguishes between serovars A, B, and C, types and the second method, which distinguishes three serogroups (A, B, and C) and 9 hemagglutinin serovars (A-1, A-2, A-3, A-4, B-1 and C-1, C-2, C-3, C-4) (Sakamoto *et al*., 2012). The two ways of serotyping schemes use hemagglutination-inhibition testing to type *Av. Paragallinarum*. sensitive and rapid techniques are needed to confirm and diagnose *Av. Paragallinarum*. Using the classical serotyping is laborious and expensive but very essential to get information about the effectiveness of vaccine in the field (Morales-Erasto *et al*., 2014; Han *et al*., 2016). Two different types of PCR techniques conventional PCR (Chen *et al*., 1996) and real-time PCR. that have been used for identification of *Av. Paragallinarum*, (Corney *et al*., 2008).

HPG-2 gene was inspected by conventional PCR to confirm the infectious Coryza. Also, it may be used for direct sinuses squeezing (Chen *et al*., 1996; Badouei *et al*., 2014). PCR used for the identification and confirmation of the bacteria (*Av. Paragallinarum*) from nasal swabs (Anjaneya *et al*., 2014). Molecular typing rises the knowledge about the variation of the strains and gives an overview about the epidemiological situations in a geographical region (Feberwee *et al*., 2019). Molecular serotyping based on multiplex PCR is also being used for molecular serotyping of pathogens (Sakamoto *et al*., 2012). In the present research, we aimed to identify the causative agent for infectious Coryza in Egyptian field samples that showed typical disease pic-

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ture by bacteriological method and identify HPG-2 gene by PCR technique, in addition to typing of the strains by HMTp210, also sequence analysis for HPG-2 gene.

MATERIALS AND METHODS

Samples collection

Forty-one total suspected cases showed (nasal discharges, lacrimation, and head swelling) were collected from layer and broiler chickens as shown in Fig. 1. The numbers of individual samples were clarified in Table 1. All samples were collected under aseptic conditions and as fresh as possible after slaughtering of chickens.

Isolation and identification of Avibacterium Paragallinarum

Swabs from infraorbital sinus were streaked onto 10% sheep blood agar with *Staphylococcus aureus* (reference culture ATCC 25922) as a feeder (V-factor/ nicotinamide adenine dinucleotide (NAD) in addition to inoculation of swabs onto Buffer peptone water.

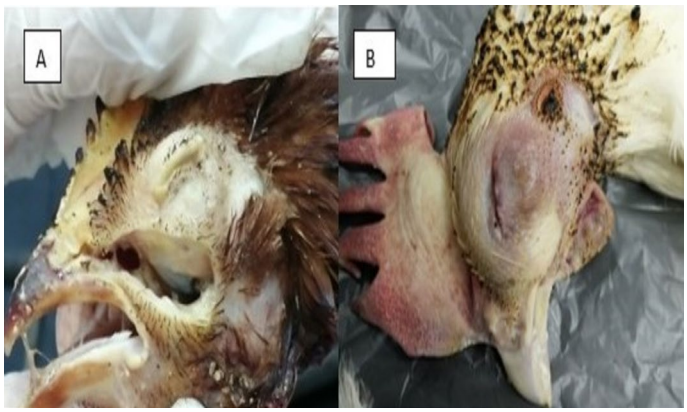

Fig. 1. Typical symptoms of *Av. Paragallinarum*. A) Showed nasal discharge of the case. B) Showed the swollen head and infraorbital sinuses.

Table 1. The examined samples for *A. Paragallinarum*

Poultry species	Common signs	Examined number
Layer chicken	Swollen head	6
	Nasal discharge	1
Total number of layer samples		7
Broiler chicken	Swollen head	12
	Nasal discharge	22
Total number of broiler samples		34
Total number		41

The plates and inoculated swabs were incubated in a candle jar at 37 °C for 18 h (Calderón *et al*., 2010). The bacterial colonies showing satellite growth were taken as culture-positive (Dereja, 2017), or tiny dewdrop colonies were considered positive for *Av. Paragallinarum* (Akter *et al*., 2013). The distinct colonies were picked up for identification of Gram-negative, catalase-negative culture. Fig. 2 showed the satellite shape colonies of the suspected strains.

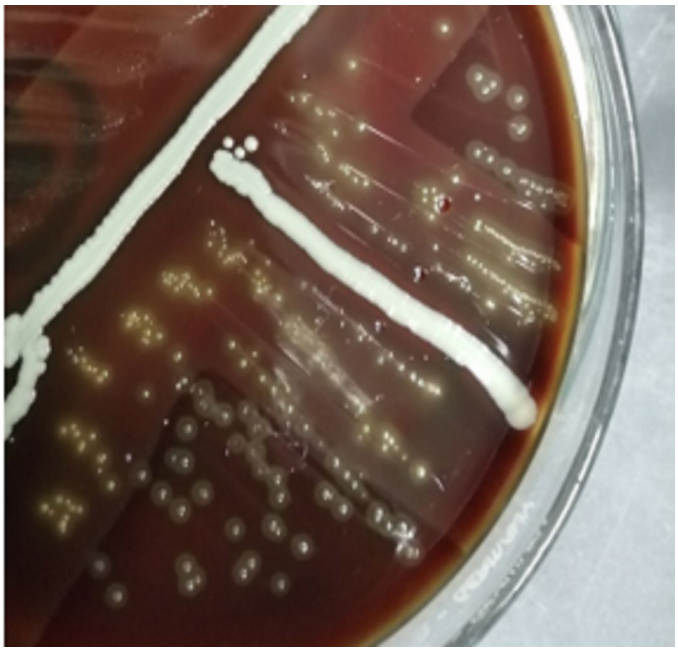

Fig. 2. Suspected colonies on blood agar show satellite shape colonies.

Molecular assessment

All the samples underwent confirmation by PCR with direct colony preparation and direct examination of enriched swabs samples.

Uniplex PCR

All 41 samples underwent confirmation by screening for a common gene using conventional PCR HPG-2 gene by using uniplex PCR. DNA was purified from pre-enriched samples using QIAamp DNA Mini kit (Qiagen, Gmbh, Germany) following manufacturer instructions. Specific PCR primers were provided from Bio-search technologies (Denmark) as listed in Table 2, For HPG2 screening. PCR reactions were performed in a 25-µl master mix reactions of

Table 2. Oligonucleotide primers used in this study for PCR Technique

Target gene	Oligonucleotide sequence (5′- 3′)	Annealing temperature (˚C)	Amplified fragment (bp)	Reference
HPG2	Forward: TGAGGGTAGTCTTGCACGCGAAT	63	500	Zhao *et al*. (2010)
	Reverse: CAAGGTATCGATCGTCTCTCTACT			
HMTp210	ABC: Forward GGCTCACAGCTTTATGCAACGAA	56	800	Sakamoto *et al*. (2012)
	A reverse: CGCGGGATTGTTGATTTTGTT			
	ABC Forward: GGCTCACAGCTTTATGCAACGAA		1100	
	B reverse: GGTGAATTTCACCACACCAC			
	ABC Forward: GGCTCACAGCTTTATGCAACGAA		1600	
	C reverse: AATTTTCTTATTCCCAGCATCAATACCAT			

12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl primer forward and reverse (10 pmol conc.), 3 µl of purified DNA, the reaction volume was completed to 25 µl by 7.5 µl PCR grade water. Briefly the thermocycler condition was done by primary denaturation at 94˚C for 5 min followed by 35 cycles at different temperature denaturation, annealing and extension at 94 ˚C for 45 sec, at 63 ˚C for 45 sec and 72 ˚C for 45 sec respectively, and final extension step at 72 ˚C for 10 min (Zhao *et al*. (2010).

Multiplex PCR for typing of the strains

Reactions were performed in a 50-µl master mix reactions (25µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl primer forward and reverse (10 pmol conc.), 5 µl of purified DNA, and 16 µl PCR grade water). for multiplex PCR for the typing gene HMTp210. Briefly the thermocycler condition was done by primary denaturation at 94˚C for 5 min followed by 35 cycles at different temperature denaturation, annealing and extension at 94 ˚C for 45 sec, at 56˚C for 45 sec and 72˚C for 45 sec respectively, and final extension step at 72˚C for 10 min (Sakamoto *et al*. 2012).

Analysis of amplified product

In Applied Biosystem 2720 thermal cyclers (Thermo-Fisher scientific, Germany) the PCR reactions were cycled. PCR products were electrophoresed in 1% agarose gel (Abgene, Thermo-Fisher scientific, Germany), a Generuler 100 bp ladder (Fermentas, Thermo, Germany), and 100 bp DNA ladder H3 RTU (Genedirex, Taiwan) was used to check the PCR product. Gel images were captured by a gel documentation system (Alpha Innotech, Biometra) using electronic software (Automatic Image Capture, USA).

DNA sequence

The purification for PCR products was done by a QIAquick PCR Product extraction kit (Qiagen, Gmbh, Germany).

The sequence reaction

The sequence reaction was accomplished by Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer), the sequence identity of *A. Paragallinarum* GenBank published accessions, a BLAST® search (Basic Local Alignment Search Tool) was executed (Altschul *et al*., 1990). The sequence identities were obtained by the MegAlign module of Lasergene DNAStar (Thompson *et al*., 1994). The maximum likeli¬hood, neighbor-joining, and Mega6 were used to construct the phylogenic tree with 1000 bootstraps (Tamura *et al*., 2013). Three strains were analyzed and submitted to GeneBank, then accessions (ON152717-ON152718 and ON152719) for the HPG2 gene were assigned.

RESULTS

Isolation for the agent

After a trial for isolation, there were four suspected strains of *A. Paragallinarum* with a total percentage of 9.75% from layer chickens. It was obvious that the high number of positivity were from the swollen head samples from layer flock with a percentage of 66.7%. And the percentage of isolation was 57.1 % when calculated according to the symptom of swollen heads in layer chickens. As shown in Table 3.

PCR results

The results showed that the only three examined strains incorporated the HPG-2 gene, with a percentage of detection 7.3% (3/41).

Fig. 3, showed the detection of positive strains of *Avibacterium Paragallinarum*.

The three strains were also examined by multiplex PCR technique for typing using HMTp210 gene, the three strains were of type B and gave an amplification product at a specific weight for the gene at 1100bp. As shown in Figure 4.

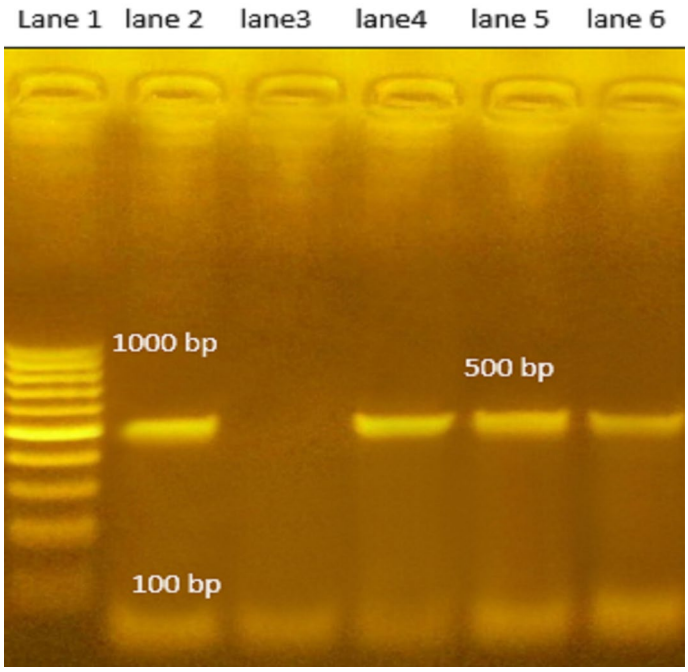

Fig. 3. PCR amplification products with primers of HPG-2 gene-targeted three *Av. Paragallinarum* isolates. Lane 1: represent DNA Generuler 100 bp ladder, Lane 2 the positive control, Lane3: negative control, Lane 4-6 showed positive amplification at 500 bp of *Av. Paragallinarum*.

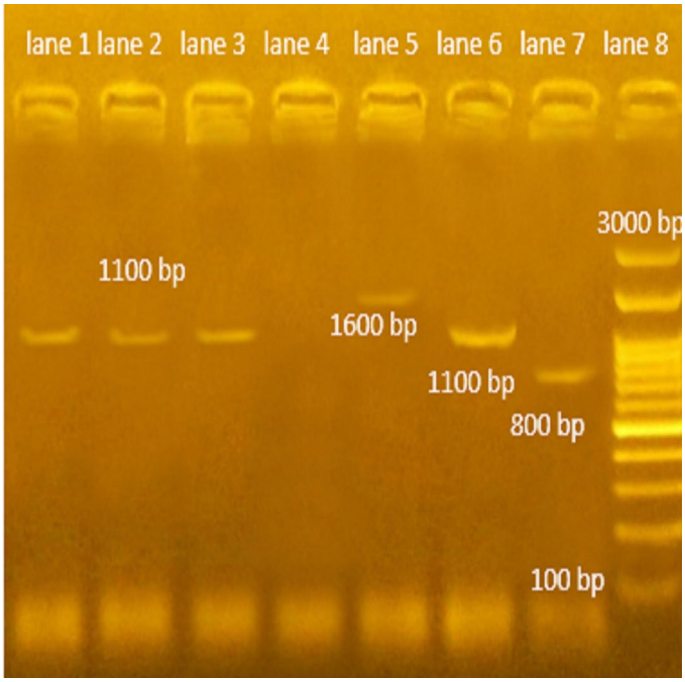

Fig. 4. Agarose gel electrophoresis for multiplex PCR for genetic serotyping of *A. Paragallinarum* using HMTp210 gene. Lanes 1 to lane 3: the three strains showed positive amplification for type B at a specific weight of 1100bp. Lane 4: represent the negative control. Lane 5,6, and 7: represent positive control for each type (C, B, and A) respectively. Lane 8: represents 100 bp plus ladder H3 RTU (marker- 100 bp to 3000 bp).

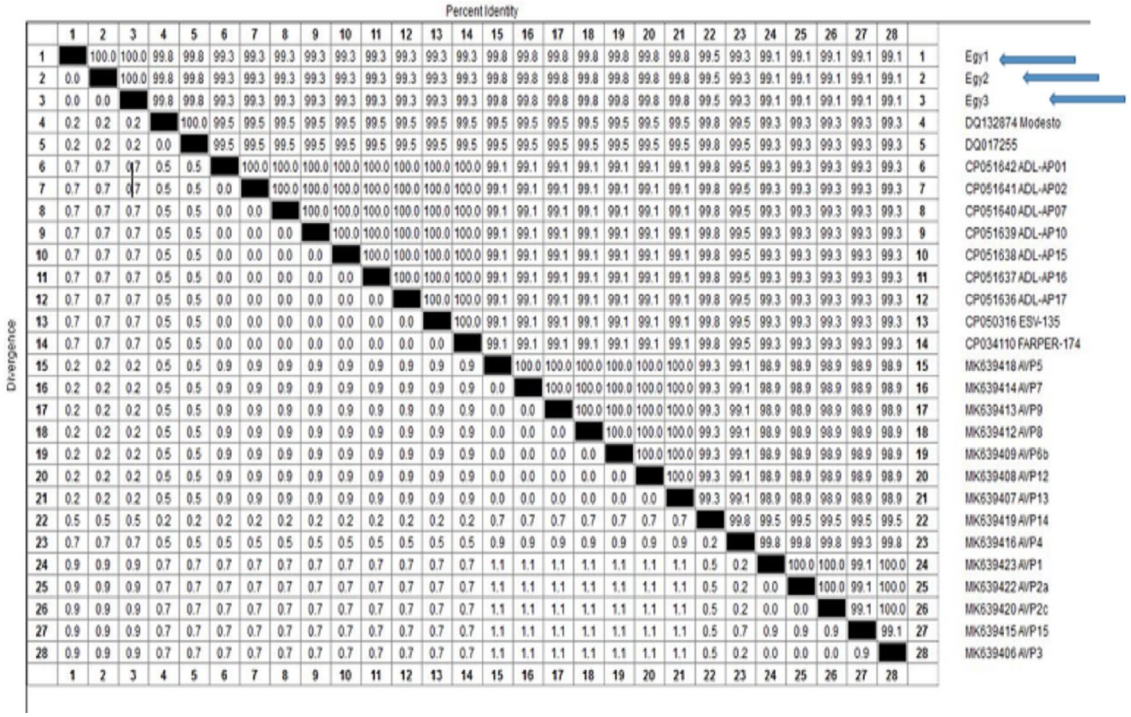

Fig. 5. The Genetic Distance between the three A *Paragallinarum* strains carrying the HPG-2 gene and randomly selected strains from GenBank.

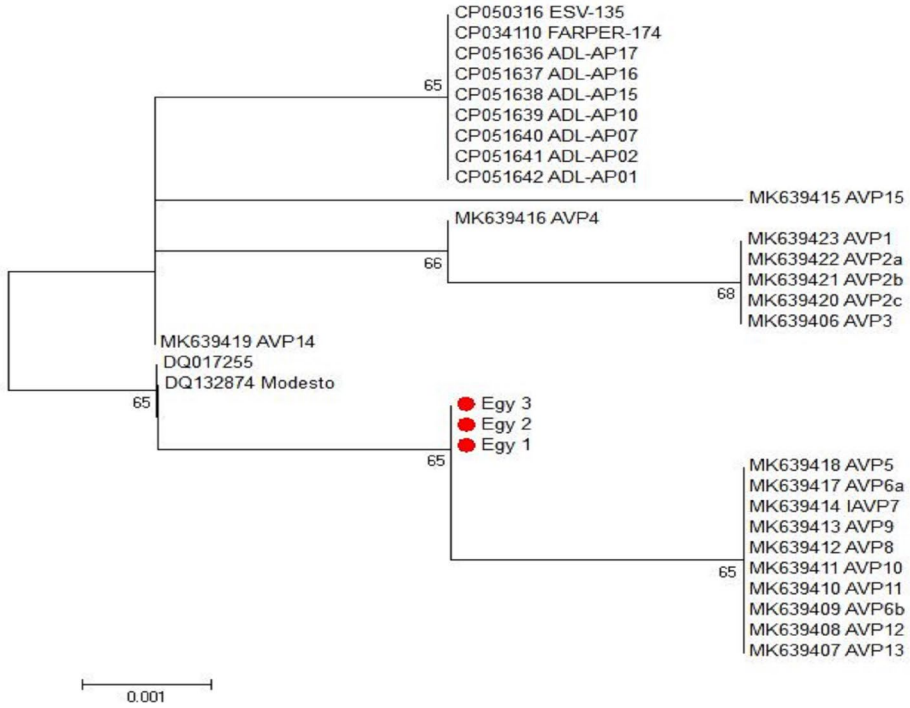

Fig. 6. The phylogenetic tree between the three Egyptian *A. Paragallinarum* strains carrying the HPG-2 gene and other randomly selected strains from GenBank.

Nucleotide sequencing results

DNA sequencing for the 500 bp of HPG-2 gene for the three strains was generated. Sequences were assigned GenBank accessions (ON152717, ON152718 and ON152719). The sequence distances tool of the Lasergen DNAStar software revealed a high percentage of identity (100%) between the three strains (Eg1, Eg2, and Eg 3) as shown in Fig. 5.

Although, the phylogenetic analysis showed clear clustering of the 3 Egyptian strains as shown in Fig. 6.

DISCUSSION

Infectious Coryza could have a significant impact on broiler chickens and economic losses have been addressed in many studies (Droual *et al.*, 1990; Sandoval *et al.* 1994). The disease is reported as an important disease in poultry farming, especially in a high-temperature environment (Feberwee *et al.* 2019). *Avibacterium Paragallinarum* is a fastidious and slow-growing bacteria (Blackall 1999). The fastidious character of *Av. Paragallinarum* makes its isolation very difficult as it needs multiple requirements and a special technique for isolation (Badouki *et al.*, 2014). The organism needs special media for its identification and biochemical

Table 3. The numbers and percentage for isolation of *A. Paragallinarum*

Poultry species	Common signs	Examined number	Number of positive (%)
Layer chicken	Swollen head	6	4 (66.7%)
	Nasal discharge	1	0
Total number of layer samples		7	4 (57.1%)
Broiler chicken	Swollen head	12	0
	Nasal discharge	22	0
Total number of broiler samples		34	0
Total number		41	4 (9.75%)

Each percent was calculated to the total examined samples of each species.

tests, so it is considered an expensive technique (Morales-Erasto *et al*. 2014).

In this study microbiological examinations of different samples were performed. The samples were obtained from diseased layer and broiler chickens, with only four samples suspected to be *A. Paragallinarum*. The positive four samples were from layer chickens having swollen heads. It was obvious that 4/6 layer samples were positive with a percentage of 66.7%, while the total percentage from the total examined samples were 4/41=9.75% as shown in Table 3. The present study was contrary to that done in Iran by Banani *et al*. (2006), who studied fourteen broiler flocks by cultural method but *A. Paragallinarum* has not been isolated. While the results of the present research were compatible with that reported by Xu *et al*. (2019) that was able to isolate and define *Av. Paragallinarum* in many countries around the world. Many factors may cause failure in culturing of the microorganism as the quality and type of samples shipping, and poor quality media, the latter leads to a higher rate of culture failure, and makes PCR a reliable diagnostic choice (Blackall and Soriano-Vargas 2013).

PCR has been extensively used for the detection of many types of samples (Chen *et al*. 1996). The application of PCR tests after trials for isolation instead of multiple biochemical identifications can reduce the problem of the isolation methods (Chen *et al*., 1998). Using HPG gene-based PCR assay targeting of *Av. Paragallinarum* was established in 2008 (Corney *et al*., 2008) and this technique was approved in 2019 (Clothier *et al*., 2019). The present study detected only three strains of *Av. Paragallinarum* by using HPG-2 gene PCR technique which produced a 500 bp amplicon of the HPG-2 gene of the expected size (Fig. 3). Similar findings were observed when direct PCR was compared to PCR on isolates in China for identification of *Av. Paragallinarum* (Chen *et al*.,1998). HPG2-PCR is proved to be a sensitive method for species identification of *Av. Paragallinarum* (Muhammad and Sreedevi 2015). The gained PCR results for *Av. Paragallinarum* are consistent with the findings of a previous study (Chen *et al*. 1996; Anjaneya *et al*. 2014; Patil *et al*. 2017). The present study showed that all the three strains were of type B using multiplex PCR and that finding was compatible with that of Zhang *et al*. (2003) who found that the incidence of infection with serovar B has increased significantly in China. Also, molecular typing increases information about the variations of the strains and the epidemiological situation in a geographical area (Feberwee *et al*., 2019). Sequencing for HPG-2 gene for the tested strains was generated. Sequences were assigned GeneBank accessions ON152717, ON152718, and ON152719). However, all the assigned strains showed 100% identity between each other and showed 99.3% identity with different randomly selected strains isolated from the chicken farm in Pennsylvania, USA. (CP051636 to CP051642). The current research results were well-matched with that of Byukusenge *et al*. (2020) who concluded that the percent of the identity between strains from avian sources closer to 99.9%. Also, the current Egyptian strains showed an identity percentage from 99.1 to 99.8 % with strains isolated from commercial layer flocks in the Netherlands during an outbreak with accession numbers from MK639406 to MK639423. HPG-2 sequence analysis as a tool for the detection of mutation and genotyping needs a large number of isolates to be tested (Feberwee *et al*., 2019). In this study, we found that PCR was a sensitive, rapid, and reliable technique for the identification of organisms especially with difficulty in isolation methods, in addition to the finding of the phylogenesis similarity between the different Egyptian strains that indicated the circulation of the same strain in the country.

CONCLUSION

Av. Paragallinarum is considered fastidious growing organisms that depend on the experience in isolation and laboratory resources are very important factors for confirmation of the presence of the microorganism. PCR can be used as a rapid and sensitive technique for the identification of the circulating strains of *Av. Paragallinarum*. Also, sequence analysis gives an indication of the type that may help for vaccine preparation for the bacteria.

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CONFLICT OF INTEREST

The authors declares that they have no conflicts of interest.

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