



Prevalence of multidrug resistant *Avibacterium paragallinarum* in chickens

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ABSTRACT

Chicken represents an essential source of animal-derived protein, essential amino acids, vitamins, and minerals. *Avibacterium paragallinarum* (*A. paragallinarum*) causes an important respiratory disease in chicken known as infectious coryza which is characterized by low feed conversion ratio, drop in egg production and less marketability. This study aimed at isolation, and identification of *A. paragallinarum* from chicken of different ages at different localities in Sharkia Governorate, Egypt using different diagnostic methods including ELISA, bacteriological culture, and PCR. Furthermore, antimicrobial resistance profiling of the recovered *A. paragallinarum* isolates was screened using the disk diffusion method. The obtained results in the present study revealed isolation and identification of *A. paragallinarum* from chicken with coryza-like symptoms from different localities in Sharkia Governorate, Egypt. PCR screening of *A. paragallinarum*-specific 16S ribosomal RNA revealed that 12 (24%) of the coryza-suspected cases were confirmed to be caused by *A. paragallinarum*. Samples from Menia-Alkamd had the highest prevalence rate (6%) of *A. paragallinarum*, while that originated from Belbies city had the lowest prevalence rate at 2%. Conjunctiva had the highest prevalence among the examined tissues. Recovered *A. paragallinarum* isolates showed multidrug resistance profiling with high sensitivity to neomycin and gentamicin. In conclusion, combination of ELISA, bacteriological culture, and PCR is highly recommended for an accurate diagnosis of infectious coryza in chickens. The use of neomycin, and gentamicin is highly suggested in the control of infectious coryza in chickens.

Keywords: *Avibacterium paragallinarum*; Infectious coryza; Chickens; Antimicrobial resistance.

1. Introduction

Chicken represents a major source of animal derived protein that solves the problem of the shortage of red meat in different parts of the world, particularly in Egypt. At the same time, chicken industry is growing all over the world representing a magnificent economical source. However, this industry is challenged by many obstacles including bacterial and viral diseases which might lead to massive economic losses and breakdown of such industry (Eldin et al., 2020).

Infectious coryza is an important respiratory disease affecting chicken and other bird species and caused by *Avibacterium paragallinarum* (*A. paragallinarum*), which was previously known as *Haemophilus paragallinarum* (Blackall et al., 1990). The disease is characterized by acute respiratory manifestations such as sneezing, nasal discharge, and gasping. Facial edema and swelling of the infraorbital sinus are among the most characteristic signs of the disease.

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This disease is affecting chicken of different ages, particularly between two weeks to four months. The disease is reported in many parts of the world as in Africa, Asia, and North and South Americas. The disease is also causing massive economic losses because of the unmarketability of the affected chicken, in addition to the sharp drop in the egg production and feed conversion ratio, and even mortalities reaching to 2-4% (Blackall, 1999; Blackall and Soriano, 2008; Welchman et al., 2010).

Chicken under intensive rearing systems is exposed to a vast array of antimicrobials for the purpose of prevention and control of bacterial diseases. However, in many cases the use of antimicrobials is uncontrolled. This abuse of antimicrobials might lead to the development of drug resistance among different pathogens leading to failure of the treatment protocols and subsequently big economic losses. Bacterial agents had developed several resistance factors which might pass to next bacterial generations (Alsayegh et al., 2021; Darwish et al., 2013).

In sight of the previous factors, this study was conducted to isolate and identify *A. paragallinarum* in chicken of different ages in Sharkia Governorate, Egypt using different diagnostic methods including ELISA, culture methods, and PCR. Furthermore, antimicrobial resistance profiling of the recovered *A. paragallinarum* isolates was conducted using disk diffusion method. To the best of our knowledge, this is the first report investigating the prevalence, and the antibiogram of *A. paragallinarum* in Africa.

2. Materials and Methods

2.1. Ethical approval

All experiments using animals were done according to the guidelines of Zagazig University, Egypt.

2.2. Collection of samples

Samples for surveillance of *A. paragallinarum* were collected from six localities in Sharkia Governorate, namely, Abo-Hammad, Abo-Kebier, Belbies, Diarb-Negm, Menia-Alkamd and Zagazig during July to October 2021. Samples were collected from suspected coryza cases visiting Educational Veterinary Hospital, Zagazig University, private veterinary clinics, and governmental veterinary hospitals in each locality. Fifty coryza-suspected cases with respiratory symptoms representing 12 chicken flocks were collected (Table 1). The average number of chickens per one flock is 250 ± 50 with an age range of 2-40 weeks of different breeds. Birds were sacrificed, and then sera were separated and stored at -20°C for identification of *A. paragallinarum* using ELISA diagnostic kit. Tissue specimens were collected for postmortem examination, and bacterial isolation.

2.3. Postmortem examination and culturing of *A. paragallinarum*

Sacrificed chickens were exposed to postmortem examination and the detected lesions were recorded. Postmortem findings included respiratory lesions such as conjunctivitis, nasal discharge, tracheal exudate, bronchitis, cloudiness of the air sacs, and pneumonia (Jackwood and Saif, 2003). Specimens from each affected tissue were directly streaked on chocolate agar plates (Thermo Scientific™, USA) and incubated for 24-48 h at 37°C in an incubator supplemented with 5% CO_2 . Chocolate agar was chosen as a culture medium because it supplies V factor which is essential for better growth of *A. paragallinarum*. Colonies of *A. paragallinarum* were small, circular, translucent and look like dewdrops (Akhter et al., 2014). *A. paragallinarum* isolates were then subjected to Gram's staining and showed Gram negative coccobacilli. Then, *A. paragallinarum* isolates were subjected to biochemical tests and were positive for lactose, mannitol, and sorbitol, and negative for catalase, and oxidase (Blackall and Soriano, 2008).

2.4. Identification of *A. paragallinarum* using ELISA kit

The collected serum samples during sacrifice were analyzed by commercial ELISA kit (Zoetis, Parsippany, USA) according to the manufacturer's instructions. Serum samples were diluted to 1/100 and assayed in duplicate. The optical density was measured with an ELISA plate reader (BioTek ELx808) at 405 nm.

2.5. DNA extraction and PCR analysis

Bacterial DNA was extracted from the obtained *A. paragallinarum* isolates using QIAamp DNA kit according to the manufacturer's instructions. Primer sequences for *A. paragallinarum* specific-16S rRNA were designed using the public Primer 3 design program <https://primer3plus.com/cgi-bin/dev/primer3plus.cgi> as following F: 5'-cctgcgatctagctggtc-3', and R: 5'-cccattgcgcaatattcccc-3' (Accession number: NR_042932.1). Uniplex PCR was carried out according to Darwish et al. (2018). The thermal cycle of the reaction was started with a single 1 min cycle at 94°C, followed by 40 cycles of 10 sec denaturation at 94°C, 1 min annealing at 59.7°C and 1 min extension at 72°C, followed by a final cycle of extension for 7 min at 72°C. The amplified PCR products were then electrophoresed on 2% agarose gel and stained with ethidium bromide.

Antibiogram

The resistance profiling of the identified *A. paragallinarum* isolates to the commonly prescribed antimicrobials for purpose of the treatment of infectious coryza in Egypt was examined using the disk diffusion method. The interpretation of the results was done according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2017). The used antimicrobial disks (Oxoid Hampshire, UK) were ampicillin (AMP, 10 µg), ceftiofur (FUR, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), erythromycin (E, 15 µg), gentamicin (GEN, 10 µg), lincomycin (LIN, 15 µg), nalidixic acid (NA, 30 µg), neomycin (N, 30 µg), polymyxin B (PB, 300 IU), oxytetracycline (OXY, 30 µg), penicillin (P, 10 IU) and sulpha-trimethoprim (SXT, 25 µg). Calculation of the multiple antimicrobial resistance (MAR) index for the recovered *A. paragallinarum* isolates was done according to the formula designed by Singh et al. (2010) as follow:

MAR index= No. of resistance (Isolates classified as intermediate were considered sensitive for MAR index) / Total No. of tested antimicrobials

2.6. Statistical analysis

Correlation analysis between the age of the birds and ELISA (antibody) titer was performed using Microsoft Excel available from Office 365.

3. Results and Discussion

The obtained results in the present study revealed isolation and identification of *A. paragallinarum* from chicken with coryza-like symptoms including excessive lacrimation, swelling of the infraorbital sinuses, nasal discharge, sneezing, gasping, and pneumonia. The results recorded in Table 1 revealed that ELISA, bacteriological culture, and PCR could be used for diagnosis of *A. paragallinarum* (infectious coryza) in chicken. However, PCR showed the most accurate diagnostic method. As out of 50 coryza-suspected cases from different localities in Egypt, 36 samples (72%) showed positive reactions against *A. paragallinarum* specific ELISA kit, while after bacteriological culture only 24 (48%) showed typical *A. paragallinarum* colonies. Interestingly, just 12 (24%) samples showed positive reaction against *A. paragallinarum*-specific 16S ribosomal RNA gene (Fig. 1). The reason for the high positive values at ELISA examination is the cross-reactivity with other diseases. Similarly, several microorganisms might be able to grow during traditional culture methods. Therefore, it is highly recommended to combine all diagnostic methods for accurate diagnosis of *A. paragallinarum*, particularly PCR. Samples from Menia-Alkamdah had the highest prevalence rate (6%) of *A. paragallinarum*, while that originated from Belbies city had the lowest prevalence rate at 2%. Likely, *A. paragallinarum* was isolated from severe infectious coryza cases in broiler breeders in Panama (Calderón et al., 2010), commercial chickens in Thailand (Chukiatsiri et al., 2012), India (Patil et al., 2017), and Central California, USA (Crispo et al., 2019).

ELISA is among the most sensitive methods for rapid screening of changes in the antibody status in the body of the bird. However, it gives an indication about both the past and present infections in a certain flock (Tsai and Saif, 1991). Therefore, the high prevalence of *A. paragallinarum* in the present study when tested with ELISA indicates the presence of *A. paragallinarum*-specific antibodies in the sera of the tested chickens. This

might reflect both past and present infections with *A. paragallinarum*. While PCR gives an indication about only the recent status of an infectious agent. That was evidenced when a scatter plot was made between the ELISA titer against *A. paragallinarum*, and the age of the examined birds, as a strong significant and positive correlation ($R^2 = 0.8224$, $P < 0.05$) was recorded (Fig. 2). In agreement with the obtained results of the present study, in Turkey, Türkyilmaz et al. (2009) reported presence of antibodies against *Bordetella avium* (*B. avium*), the causative agent of coryza in turkeys, in serum samples of turkeys using ELISA, but all samples were negative to *B. avium* when tested using PCR. In addition, Eldin et al. (2020) reported that PCR is the most accurate method for diagnosis of coryza in turkeys compared with ELISA, and culture methods. In correspondence with the used PCR method in the current investigation, Patil et al. (2017) targeted *A. paragallinarum*-specific 16S rRNA for accurate diagnosis of infectious coryza in chicken. In agreement with the positive correlations between ELISA titer and the age of the birds, Beach et al. (2012) recorded an increase titer of anti-*B. avium* antibodies in the sera of turkeys by the increase of their age. Besides, Eldin et al. (2020) recorded a similar positive correlation between the increases in the titer of anti-*B. avium* antibodies and the age of turkeys in Egypt.

The recorded results in Fig. 3 revealed the prevalence rates of *A. paragallinarum* in the different affected tissues of the PCR-confirmed coryza cases. The results showed that conjunctiva had the highest prevalence rate (100%), followed by both of trachea, and sinuses (83.33% for each), air sacs (66.66%), and lungs (33.33%), respectively. Same tissues were affected by coryza in turkeys sampled from Egypt, where trachea and sinuses had the highest prevalence rates of *B. avium* (Eldin et al., 2020).

Antimicrobials are extensively used in chicken farms in Egypt for the purpose of prevention and control of infectious diseases. In addition, some antimicrobials are used to increase the feed conversion ratio. However, the abuse of such antimicrobials during intensive rearing programs had led to the development of drug resistance phenomenon among several pathogens (Alsayeqh et al., 2021; Darwish et al., 2013). In the present study, *A. paragallinarum* isolates showed multidrug resistance profiling. *A. paragallinarum* isolates were highly resistant to penicillin (91.66%), and nalidixic acid (83.33%). The drug resistance rates for the other tested antimicrobials were as following: lincomycin (66.66%), ceftiofur (66.66%), ampicillin (41.66%), enrofloxacin (41.66%), oxytetracycline (41.66%), ciprofloxacin (33.33%), erythromycin (33.33%), sulpha-trimethoprim (33.33%), polymyxin B (33.33%), chloramphenicol (25%), neomycin (25%), and gentamicin (16.66%), respectively (Fig. 4). Eleven isolated *A. paragallinarum* had multidrug resistance profiles with an average MAR index of 0.440 (Table 2). In agreement with the obtained results in the present study *A. paragallinarum* showed high level of resistance to lincomycin and erythromycin in a study conducted in Thailand (Chukiatsiri et al., 2012). Besides, high resistance of *A. paragallinarum* was recorded to tetracyclines in studies conducted in Netherlands (Heuvelink et al., 2018), and USA (Crispo et al., 2019).

4. Conclusion

The obtained results in the present study revealed that occurrence of *A. paragallinarum*, as causative agent for infectious coryza, in several localities in Egypt. A combination of ELISA, traditional bacteriological culture, and PCR are highly recommended for an accurate diagnosis of *A. paragallinarum* cases. Gentamicin and neomycin are considered reliable antimicrobials for the control of *A. paragallinarum* cases in chicken.

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5. References

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Table 1: Prevalence rates of *A. paragallinarum* in suspected coryza cases at different localities in Sharkia Governorate, Egypt using ELISA, culture, and PCR.

Location	No. of flocks	No. of samples	Positive by ELISA	Positive by Culture	Positive by PCR
Abo-Hammad	3	8	5 (10%)	3 (6%)	2 (4%)
Abo-Kebier	4	8	6 (12%)	4 (8%)	2 (4%)
Belbies	2	6	4 (8%)	3 (6%)	1 (2%)
Diarb-Negm	3	8	6 (12%)	3 (6%)	2 (4%)
Menia-Alkambh	4	10	8 (16%)	6 (12%)	3 (6%)
Zagazig	2	10	7 (14%)	5(10%)	2 (4%)
		50			12
Total	12	(100%)	36 (72%)	24 (48%)	(24%)

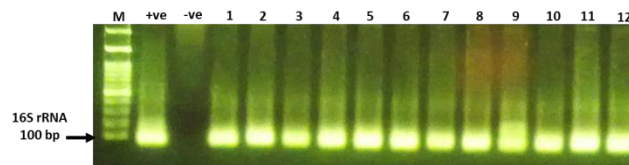


Fig. 1: Agarose gel electrophoresis of *A. paragallinarum*-specific 16S rRNA gene for PCR confirmation of the recovered *A. paragallinarum* isolates from diseased chicken. M refers to a 100 base pairs DNA marker, lane +ve refers to a reference strain for *A. paragallinarum* (positive control), lane -ve refers to no template (negative control) 1-12 refers to the recovered *A. paragallinarum* isolates.

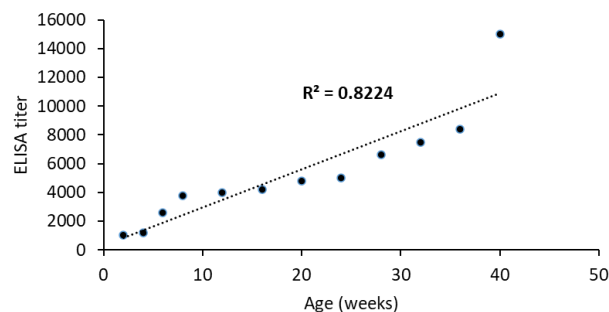


Fig. 2: Scatter plot between ELISA titer of *A. paragallinarum* in the sera of coryza-diseased chicken confirmatory-diagnosed by PCR, and their age (weeks)

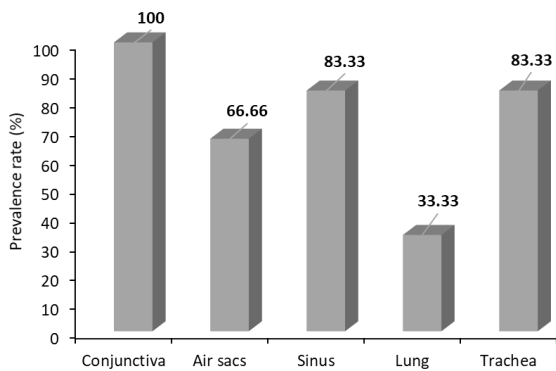


Fig. 3: Prevalence rates of the recovered *A. paragallinarum* isolates in the different tissues of the examined coryza-diseased chicken

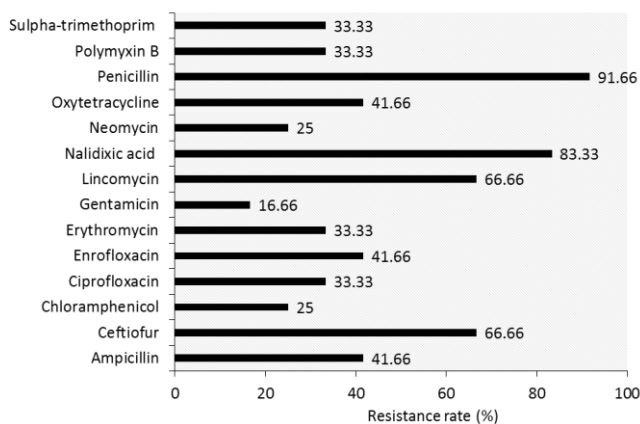


Fig. 4: Antimicrobial resistance rates of the recovered *A. paragallinarum* from diseased chicken

Table 2: Antimicrobial resistance patterns of the recovered *A. paragallinarum* from diseased chicken

Isolate	Resistance pattern	MAR index
1	AMP, FUR, C, CIP, ENR, LIN, NA, N, OXY, P, PB, SXT	0.857
2	AMP, FUR, C, CIP, ENR, LIN, NA, N, OXY, P, SXT	0.786
3	AMP, FUR, C, CIP, ENR, LIN, NA, OXY, P, SXT	0.714
4	AMP, FUR, CIP, ENR, LIN, NA, P, PB, SXT	0.643
5	AMP, FUR, ENR, LIN, NA, P, PB	0.5
6	FUR, GEN, LIN, NA, P, PB	0.429
7	FUR, GEN, LIN, NA, P	0.357
8	FUR, LIN, NA, P	0.286
9	E, NA, N, P	0.286
10	E, NA, P	0.214
11	E, P	0.143
12	E	0.071
Average MAR index		0.440

MAR: multiple antimicrobial resistance; AMP: ampicillin; FUR: ceftiofur; C: chloramphenicol; CIP: ciprofloxacin; ENR: enrofloxacin; E: erythromycin; GEN: gentamicin LIN: lincomycin; NA: nalidixic acid; N: neomycin; PB: polymyxin B; OXY: oxytetracycline; P: penicillin; SXT: sulpha-trimethoprim