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-Alkamh 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.

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 (Alsayeq 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-Alkamh 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% CO2. Chocolate agar was chosen as a culture medium because is 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 cocobacilli. 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).

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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 EL680) 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'-cctgcattcaggctggtg-3’, and R: 5'-ccctgcattcaggtctcc-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 were as follows: Ampicillin (AMP, 10 µg), ceftriaxone (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 isolated A. paragallinarum isolates was done according to the formula designed by Singh et al. (2010) as follow:

\[
\text{MAR index} = \frac{\text{No. of resistance (Isolates classified as intermediate were considered sensitive for MAR index)}}{\text{Total No. of tested antimicrobials}}
\]

2.6. Statistical analysis

Correlation analysis between the age of the birds and ELISA (antibody) titers 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 chickens. 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-Alkham had the highest prevalence rate (6%) of A. paragallinarum, while that originated from Belbies city had the lowest 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 has 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%), ceftriaxone (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 (Chukiatrics 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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Conflict of interest: None

5. References

Akhter, S., Saha, S., Khan, K.A., Amin, M.M., Haque, M.E. 2014. Isolation and identification of Avibacterium paragallinarum from layer chickens in Gazipur, Bangladesh. Microbes Health, 3, 9-11.

Alsayeqh, A.F., Baz, A.H.A., Darwish, W.S. 2021. Antimicrobial-resistant foodborne pathogens in the Middle East: a systematic review. Environ. Sci. Pollut. Res. Int., 28(48), 68111-68133.

Beach, N.M., Thompson, S., Mutnick, R., Brown, L., Kettig, G., Puffenberger, R., Stockwell, S.
B., Miyamoto, D., Temple, L. 2012. Bordetella avium antibiotic resistance, novel enrichment culture, and antigenic characterization. Vet. Microbiol., 160, 189-196.

Blackall, P.J. 1999. Infectious coryza: overview of the disease and new diagnostic options. Clin. Microbiol. Rev., 12, 627-632.

Blackall, P.J., Morrow, C.J., McIntosh, A., Eaves, L.E., Rogers, D.G. 1990. Epidemiologic studies on infectious coryza outbreaks in northern New South Wales, Australia, using serotyping, biotyping, and chromosomal DNA Restriction Endonuclease Analysis. Avian Dis., 34, 267-276.

Blackall, P.J., Soriano, E.V. 2008. Infectious coryza and related bacterial. In: Disease of Poultry. 12th ed. Blackwell Publishing, Oxford. p.789-803.

Blackall, P.J., Christensen, H., Beekenham, T., Blackall, L.L., Bisgaard, M. 2005. Reclassification of Pasteurellia gallinarum, Haemophilus paragallinarum, Pasteurella avium and Pasteurella multocida as Avibacterium gallinarum gen. nov., Avibacterium paragallinarum comb. nov., Avibacterium avium comb. nov. and Avibacterium multocida comb. nov. Int. J. Syst. Evol. Microbiol., 55, 353-362.

Calderón, E.N., Thomas, K., Morales-Erasto, V., Salgado-Miranda, C., Soriano-Vargas, E. 2010. Identification of Avibacterium paragallinarum serovar B-1 from severe infectious coryza outbreaks in Panama. Avian Dis., 54(3), 1095-7.

Chukiatsiri, K., Sasipreeyajan, J., Blackall, P.J., Yuwatanichsampan, S., Chansiripornchai, N. 2012. Serovar identification, antimicrobial sensitivity, and virulence of Avibacterium paragallinarum isolated from chickens in Thailand. Avian Dis., 56(2), 359-64.

Clinical and Laboratory Standards Institute (CLSI). 2017. Performance standards for antimicrobial susceptibility testing. CLSI Approved Standard M100. http://file.qums.ac.ir/repository/mmrc/clsi 2017.pdf

Crispo, M., Blackall, P., Khan, A., Shivaprasad, H.L., McClure, K., Senties-Cué, C.G., Cooper, G., Blakey, J., Pitesky, M., Mountainspring, G., Cutler, G., Bickford, A., Stoute, S. 2019. Characterization of an outbreak of Infectious Coryza (Avibacterium paragallinarum) in commercial chickens in Central California. Avian Dis., 63(3), 486-494.

Darwish, W.S., Atia, A.S., Reda, L.M., Elhelaly, A.E., Thompson, L.A., Saad Eldin, W.F. 2018. Chicken giblets and wastewater samples as possible sources of mexitilin-resistant Staphylococcus aureus: Prevalence, enterotoxin production, and antibiotic susceptibility. J. Food Safety, 38(4), 12478.

Darwish, W.S., Eldaly, E.A., El-Abassy, M.T., Ikenaka, Y., Nakayama, S., Ishizuka, M. 2013. Antibiotic residues in food: the African scenario. Jpn. J. Vet. Res., 61 Suppl, S13-22.

Eldin, W.F.S., Abd-El Samie, L.K., Darwish, W.S., Elewa, Y.H.A. 2020. Prevalence, virulence attributes, and antibiogram of Bordetella avium isolated from turkeys in Egypt. Trop. Anim. Health Prod., 52(1), 397-405.

Heuvelink, A., Wiegel, J., Kehrenberg, C., Dijkman, R., Soriano, E.V. 2008. Infectious coryza and related bacterial. In: Disease of Poultry. 12th ed. Blackwell Publishing, Oxford. pp.789-803.

John, J., Soriano, E.V. 2009. Infectious coryza and related bacterial. In: Disease of Poultry. 12th ed. Blackwell Publishing, Oxford. pp.789-803.

Kebier, Abo, 2021. Detection of Antibodies against Avibacterium Paragallinarum Specifically using ELISA in the Serum of a Healthy Chicken. Damanhour Journal of Veterinary Sciences, 6(2), (2021) 28-31

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-Alkamh | 4             | 10             | 8 (16%)               | 6 (12%)                 | 3 (6%)              |
| Zagazig      | 2             | 10             | 7 (14%)               | 5 (10%)                 | 2 (4%)              |
| Total        | 12            | 50             |                       |                         |                     |

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 a (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

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: sulphathiazole; SXT: sulphamethoxazole-prim