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Isolation and molecular identification of multidrug-resistant *Pseudomonas aeruginosa* **isolated from broiler chickens in Fayoum, Egypt**

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Introduction

P. aeruginosa poses a severe threat to the poultry industry as it influences birds of all ages and causes noteworthy economic losses to the poultry industry. This Gram-negative, motile, non-spore-forming bacillus (1- 3) can contaminate hatcheries, causing severe respiratory symptoms, enteritis, septicemia, keratitis, sinusitis, omphalitis, nephritis, and rapid morbidity and mortality, demonstrating its diverse pathogenic potential within avian populations (4-7).Infection is facilitated by poor hygiene

practices and various routes of contamination, such as skin wounds, tainted vaccinations, egg dipping or inoculation, and contaminated injection needles (8).The emergence of antimicrobial resistance among *P. aeruginosa* strains is an urgent global health concern, especially in developing countries, where indiscriminate antibiotic use is promoting the spread of multidrug-resistant strains (9). *P. aeruginosa* exhibits high adaptability to genetic changes, leading to multidrug resistance, especially when subjected to indiscriminate treatments (10). Notably, antibiotic resistance has been reported, including aminoglycosides,

monobactams, carbapenems, third-generation cephalosporins, and aminopenicillins (11,12). Moreover, *P. aeruginosa* employs both intrinsic and acquired mechanisms to resist antibiotics, including restricted outer membrane permeability, synthesis of antibiotic-inactivating enzymes, e.g., β-lactamases, and efflux systems to expel antibiotics (13). Of particular concern are β-lactamases, particularly class C cephalosporinases (*ampC*-β-lactamases), which confer resistance to cephalosporins, penicillin, and βlactamase inhibitors (14). These mechanisms underscore the challenge of controlling *P. aeruginosa* infections in clinical practices. Recent studies have emphasized the prevalence of multidrug-resistant *P. aeruginosa* strains in poultry, giving rise to an alarming challenge to animal and public health. These strains exhibit resistance to many antibiotics, necessitating the development of alternative strategies for their control (15). Understanding the mechanisms underlying *P. aeruginosa* resistance is essential for effective management and treatment. These mechanisms include changes in genetic structure that render conventional antibiotic therapy ineffective against various antibiotics. To investigate the presence of multidrug-resistant microorganisms of public health concern, PCR screening for the most common antibiotic resistance genesis is needed (16). The *blaOXA* gene of *P. aeruginosa* isolates is one of the major families of ESBLs classified into molecular class D and functional group 2d (oxacillinase-like carbapenemases) and is associated with resistance to oxacillicins, cephalosporins, and carbapenems (17). One of the most distinctive features of antibiotic resistance is the efflux pump system that pumps antibiotics out of cells. For example, the *mexR* gene encodes a transcriptional repressor of the *mex*AB-*opr*M efflux pump that effluxes aztreonam (18). The *ermB* gene encodes a methyltransferase that causes ribosomal methylation, reducing bacterial susceptibility and conferring resistance to macrolides, lincosamides, and streptogramins (19). Moreover, *P. aeruginosa* often develops resistance to antibiotic treatment through biofilm formation. *P. aeruginosa* contains an integron-encoded ribosyl transferase called the aminoglycoside regulatory response (*arr*) gene responsible for biofilm production (20).

This study investigates the resistance profile and molecular mechanisms underlying antibiotic resistance of *P. aeruginosa* isolated from broiler chickens in Fayoum governorate, Egypt. This will contribute to targeted strategies to halt the spread of multidrug-resistant *P. aeruginosa* and protect animal welfare and public health.

Materials and methods

Ethical approval

The animal use protocol in this study was approved by the Ethical Approval Committee of the Faculty of Veterinary Medicine, Benha University, Egypt, under the Ethical approval number (BUFVTM08-03-24).

Sampling

A total of one hundred twenty broiler chickens (n=120) of different ages were obtained from various farms in El-Fayoum Governorates. The samples included 30 one-dayold chicks, 70 birds aged 3-5 weeks, and 20 healthy birds. Four hundred eighty samples were harvested from internal organs (liver, lung, gallbladder, kidney), including 120 samples from each organ. Samples were collected from diseased, freshly dead, and healthy chickens. Samples were transported in ice boxes and then submitted for bacteriological examination.

Isolation and biochemical identification of *P.aeruginosa*

Each bird's liver, lung, gallbladder, and kidney samples were individually cultured in nutritional broth (Oxoid) and incubated for 24h at 37°C for primary enrichment. A loopful of broth was spread on Pseudomonas Cetrimideagar (Oxoid) and followed by incubation under aerobic conditions at 37°C for 24h (21). The isolates were presumptively identified as *P. aeruginosa* based on cultural characteristics and biochemical tests. Furthermore, *P. aeruginosa* could be determined by its characteristic production of the blue-green pigment pyocyanin and its characteristic grape-like odor, and its colonies are mostly oxidase-positive (21-23).

Bacterial preservation

Single colonies with characteristic colonial appearance and morphological features of *P. aeruginosa* were selected and inoculated into a 0.5% semisolid agar medium. The agar was then incubated at 37°C for 24 hours and kept at a temperature of 4°C.In addition, a 20% bacterial glycerol stock was prepared and stored at −20°C (21).

In-vitro antibiotic susceptibility testing of *P. aeruginosa* **isolates**

Thirty isolates (n=30) were selected for antimicrobial susceptibility testing using the disk diffusion technique (20 isolates from diseased birds and 10 from apparently healthy birds). Suspensions of isolates were prepared according to McFarland Turbidity Standard Tube No. 0.5 (equivalent to approximately 1.5×10^8 CFU/ml) and inoculated on Mueller-Hinton agar plates (Oxoid). Twelve antibacterial discs (Oxoid) including Amikacin 30µg/disk, Gentamycin 10µg/disk, Apramycin 30µg/disk, Cefotaxime 30µg/disk, Cefepime 30µg/disk, Ceftazidime 30µg/disk, Doxycycline 30µg/disk, Ciprofloxacin 5µg/disk, Levofloxacin 5µg/disk, Ofloxacin 5µg/disk, Amoxicillin 10µg/disk and Piperacillin 100µg/disk were used and then incubated at 37°C for 24h. Zones of inhibition were then measured and interpreted according to Clinical and Laboratory Standard Institute guidelines (24).

Molecular identification of *P. aeruginosa* **antimicrobialresistant genes**

The molecular identification of antimicrobial-resistant genes in *P. aeruginosa* was conducted using polymerase chain reaction (PCR) targeting five resistance genes: *blaOXA*, *ermB*, *arr*, *mexR*, and *ampC*. Ten representative *P. aeruginosa* strains (n=10) were selected for genotypic resistance screening. Genomic DNA from confirmed cultures was extracted using the QIAamp DNA Extraction Miniprep Kit according to the manufacturer's instructions. The primer sequences and sizes of the amplified products are shown in table1. The PCR amplification was done in a 25 µl reaction mixture consisting of 12.5 µl Emerald Amp GT PCR master mix (Takara, Code No. RR310A), 1 µl each of forward and reverse primers, 5.5 µl of Nuclease-free molecular biology grade water, and 5 µl of test DNA. The thermal profile involved a primary denaturation step at 94°C for 5 minutes, followed by 35 cycles of secondary denaturation at 94°C for 30 seconds, annealing at 55°C for 40 seconds (for *arr* and *mexR* genes), 50°C for 40 seconds (for *ampC* and *erm*A genes), or 54°C for 40 seconds (for *blaOXA*-1 gene), and extension at 72°C for 60 seconds. This was followed by a final extension step at 72°C for 10 minutes, and the reaction was then held at 4°C until stopped.

Table1: Oligonucleotide primers sequences

Primer		Sequence	Product size (bp)	Reference
arr	Forward	AGCGCATCACCCCCAGCAAC	685	(25)
	Reverse	CGCCAAGTGCGAGCCACTGA		
mexR	Forward	GCGCCATGGCCCATATTCAG	637	(26)
	Reverse	GGCATTCGCCAGTAAGCGG		
ampC	Forward	TTCTATCAAMACTGGCARCC	550	(27)
	Reverse	CCYTTTTATGTACCCAYGA		
$blaOXA-I$	Forward	ATATCTCTACTGTTGCATCTCC	619	(28)
	Reverse	AAACCCTTCAAACCATCC		
ermB	Forward	GAAAAAGTACTCAACCAAATA	639	(29)
	Reverse	AATTTAAGTACCGTTACT		

Results

Prevalence of *P. aeruginosa* **isolates in broiler chickens**

The results showed a substantially high isolation rate of *P. aeruginosa* from the 480 internal organ samples collected from broiler chickens (n=120) regardless of age and health status, with percentages ranging from 70.8% to 83.3% (Figure 1a). Analysis of the site of isolation revealed varying prevalence rates of *P. aeruginosa* across different organs, with the highest prevalence observed in the gall bladder 100/120 (83.3%), followed by the lung 97/120 (80.8%), kidneys 87/120 (72.5%) and liver 85/120 (70.8%), (Figure 1b).

Prevalence of *P. aeruginosa* **isolates in broiler chickens of different age groups**

In addition, the isolation rates of *P. aeruginosa* from specific internal organs of broiler chickens of different age groups were studied. The results revealed that for the oneday-old chicks (n=30), the highest prevalence was detected in the gall bladder, with 100% of samples (n=30) yielding Pseudomonas isolates, followed by the lung 27/30 (90%), liver 24/30 (80%), and kidneys 21/30 (70%). Among 3-5 week-old broilers (n=70), the highest isolation rate was observed in the lung 60/70 (85.7%), followed closely by the kidneys 59/70 (84.2%), liver 58/70 (82.8%), and gall bladder 55/70 (78.5%). Notably, in healthy birds $(n=20)$, the gall

bladder exhibited the highest isolation rate 15/20 (75%), followed by the lung 10/20 (50%), kidneys 7/20 (35%), and liver 3/20 (15%) (Figure 1c).

Antimicrobial susceptibility profile of *P. aeruginosa* **isolates**

The antimicrobial susceptibility profile of *P. aeruginosa* isolates was investigated. A total of 30 *P. aeruginosa* isolates, including 20 from diseased birds and 10 from apparently healthy ones, were tested. Strikingly, all tested strains exhibited 100% resistance to amoxicillin and cefotaxime, with similarly high levels of resistance observed towards Piperacillin (96.66%), gentamycin (86.66%), and ofloxacin (80%). Varying degrees of resistance were also recorded against cefepime and ceftazidime (63.33% for each) and levofloxacin and ciprofloxacin (53.3% for each). In contrast, lower resistance levels were observed towards apramycin and doxycycline (36.66% for each). Notably, the isolates displayed the highest sensitivity to amikacin (66.6%). Remarkably, 29 isolates (96.66%) exhibited resistance to more than three antibiotic agents across different antimicrobial classes, indicative of multi-drug resistance (MDR). Additionally, isolates recovered from diseased birds demonstrated notably higher degrees of resistance and lower susceptibility to most antibiotics compared to those recovered from apparently healthy birds, except for amoxicillin and cefotaxime where both *P.*

aeruginosa isolated from diseased and healthy birds showed 100% resistance, in addition to apramycin, ofloxacin and ceftazidime, where *P. aeruginosa* from apparently healthy birds showed higher resistance compared to *P. aeruginosa* isolated from diseased broiler chickens (Figure 2).

Figure1: Prevalence of *P. aeruginosa* in broiler chicken: a. Prevalence of *P. aeruginosa* in one-day-old chicks (n=30), Broilers 3-5 weeks old (n=70) and healthy broilers (n=20) collected from Fayoum Governorate. b. The total prevalence of *P. aeruginosa* in different internal organs harvested from the total broiler chicken examined (n=120). c. Prevalence of *P. aeruginosa* in different internal organs harvested from one-day-old chicks (n=30), Broilers 3-5 weeks old (n=70), and healthy broilers (n=20).

Results of occurrence of targeted resistance genes among *P. aeruginosa* **isolates**

The results for PCR amplification of some resistance genes in ten multidrug-resistant *P. aeruginosa* isolates showed that all examined isolates were positive for*mexR* and *ampC* resistance genes with a PCR product size in a percentage of 100% per each gene. The incidence rates for the *blaOXA*, *ermB*, and *arr* genes were 90%, 80%, and 50%, respectively. Furthermore, the results revealed that 3 of the 10 isolates were PCR positive for the 5 resistance genes examined (Figure 3).

Figure 2: a. antimicrobial resistance profile of *P. aeruginosa* isolated from broiler chicken: b. antimicrobial resistance profile of *P. aeruginosa* isolated from diseased broiler chicken vs. *P. aeruginosa* isolated from apparently healthy broiler chicken.

Figure 3: Prevalence of some resistance genes among the examined MDR *P. aeruginosa* isolates Lanes 1-10: tested DNA, L; 100-1000 bp DNA ladder, P: Positive control, N: Negative control: a. Prevalence of *arr* resistance gene among the examined MDR *P. aeruginosa* isolates with PCR amplification product of 685 bp b. Prevalence of *ermB* resistance gene among the examined MDR *P. aeruginosa* isolates with PCR amplification product of 639 bpc. Prevalence of *mexR* resistance gene among the examined MDR *P. aeruginosa* isolates with PCR amplification product of 637 bpd. Prevalence of *blaOXA*-1 resistance gene among the examined MDR *P. aeruginosa* isolates with PCR amplification product of 619 bpe. Prevalence of *ampC* resistance gene among the examined MDR *P. aeruginosa* isolates with PCR amplification product of 550 bp.

Discussion

The presence of *P. aeruginosa* in poultry is of great concern due to the associated economic losses to the poultry industry and its ability to induce severe respiratory infections in humans. *P. aeruginosa* has been consistently isolated from various poultry sources, including chicken meat and oral and cloacal swabs (6,30). Notably, *Pseudomonas* infections have been linked to substantial financial burdens in chicken farms, with confirmed fatalities and sequelae including sinusitis, keratitis, respiratory symptoms, and septicemia (31). Hence, prompt isolation and identification of *P. aeruginosa* is essential for effective control measures. In this study, we conducted bacteriological and molecular studies on samples obtained from the liver, lung, gall bladder, and kidneys (120 each) from broiler chickens of different ages to investigate further the prevalence and antimicrobial resistance profile of *P. aeruginosa* isolates.

Our findings demonstrated high isolation rates of *P. aeruginosa* across various internal organs in broiler chickens regardless of age group and health status, with percentages ranging from 70.8% to 83.3%. The gall bladder exhibited the highest isolation rate among one-day-old chicks and healthy broilers, highlighting the potential reservoir for pathogen dissemination. These findings highlight the widespread prevalence of *P. aeruginosa* in broiler chickens and emphasize the importance of continued surveillance and control measures to minimize the related hazards to animal and human health. Our findings of relatively high prevalence rates of *P. aeruginosa* in broiler chickens contradict those reported by previous studies. For instance, Badr *et al.* (32) identified thirteen isolates of *P. aeruginosa* from diseased chickens, whereas Elsayed *et al.* (2) reported a lower infection rate of 22.9% among broiler chickens.

Similarly, Abd El-Hafeez*et al.* (33) investigated the frequency of *P. aeruginosa* in broiler chicken kidneys and reported an infection rate of 10.4%. Ohore *et al.* (34) reported a prevalence of 28.3% in poultry samples. These discrepancies in isolation rates could be due to changes in pathogenicity, virulence factors, disease severity, the host's immunological status, geographical locations, or environmental factors influencing bacterial colonization.

In recent years, the increasing use of antimicrobials in animal husbandry has significantly contributed to the global burden of antimicrobial resistance (35). The intensification of farming practices, particularly in developing countries, has increased the use of antimicrobials for infection prevention, treatment, and growth promotion (36,37). Consequently, it is essential to determine the susceptibility patterns of pathogenic microorganisms such as *P. aeruginosa* to guide judicious use of antibiotics and reduce the risk of promoting antibiotic resistance (38), and highlighting the importance of exploring other strategies to mitigate bacterial infections in poultry industry as feed additives and immunomodulatory substances (39-51).

In our study, we performed in vitro susceptibility testing to 12 antimicrobials and found a surprising resistance pattern among *P. aeruginosa* isolates. Remarkably, all isolates tested showed complete resistance to amoxicillin and cefotaxime. In addition, the results showed high levels of resistance to piperacillin96.66%, gentamicin86.66%, and ofloxacin80%. Different resistance levels were seen for cefepime and ceftazidime63.33% and levofloxacin and ciprofloxacin53.3%. In contrast, apramycin and doxycycline showed relatively low resistance rates36.66%, and amikacin had the highest sensitivity at 66.6%. Notably, most isolates96.66% resisted three or more antibiotics, indicative of multi-drug resistance. Furthermore, Isolates obtained from diseased birds showed significantly higher levels of resistance and lower susceptibility to most antibiotics than those obtained from apparently healthy birds. The exceptions were amoxicillin and cefotaxime, both isolates from diseased and healthy birds, which showed a resistance rate of 100%. Additionally, *P. aeruginosa* from apparently healthy birds are more resistant to apramycin, ofloxacin, and ceftazidime than *P. aeruginosa* isolated from diseased broiler chickens. This increased resistance poses significant risks, including treatment failure, economic losses, and public health concerns, as it may facilitate the transfer of resistance genes from animals to humans. Our findings on the resistance patterns of *P. aeruginosa* isolates are comparable with prior research. Badr *et al.* (32) found that while *P. aeruginosa* isolates were resistant to numerous antibiotics, they were responsive to levofloxacin. Elsayed *et al.* (2) found high levels of resistance to Amoxicillin and E-Moxclav among *P. aeruginosa* isolates and Jawher and Hassan (50) who reported 100% resistance to Amoxicillin among *P. aeruginosa* isolates. Elbehiri*et al.* (38) also assessed the antimicrobial resistance profiles of Pseudomonas isolates, finding resistance rates of 81.16% for nitrofurantoin, 71% for ampicillin and ampicillin/sulbactam, 65.22% for cefuroxime and ceftriaxone, and 55% for aztreonam, and found a resistance rate of 49.28% for ciprofloxacin. Furthermore, Orady*et al.* (39) found a significant prevalence of resistance among *P. aeruginosa* isolates, with 90% resistant to ampicillin.

The findings suggest that *P. aeruginosa* demonstrates phenotypic multidrug resistance, probably controlled by genotypic factors such as antimicrobial resistance genes. Plasmids are particularly important in enabling the transfer of genes across different bacterial species. They can stimulate the emergence of new genetic variations and facilitate the exchange of major features, enhancing diversity in microbial communities (44). The transfer of antimicrobial genes across plasmids, known as Inter-plasmid antimicrobial gene transfer, is a significant mechanism that allows plasmids to acquire different antimicrobial resistance genes. This process contributes to understanding how multidrugresistant microbes develop and emerge (45). To further confirm the resistance profile of *P. aeruginosa* isolates in our study, we utilized PCR to examine the presence of five specific antimicrobial resistance genes (*mexR*, *arr*, *blaOXA*, *ampC*, and *ermB*) in 10 multidrug-resistant *P. aeruginosa* isolates. Our findings revealed that the *mexR* and *ampC* resistance genes were present in all isolates, while the *blaOXA*, *ermB*, and *arr* genes had incidence rates of 90%, 80%, and 50%, respectively. The results confirm the resistance profiles of the MDR *P. aeruginosa* obtained in this study, which aligns with previous research that has identified various antibiotic resistance genes in *P. aeruginosa* isolates as Hassan *et al.* (40) reported numerous antibiotic resistance genes, including *bla*_{CTX}, *fox*, and *mexR* in 100%, 80%, and 100% of the isolates, respectively.

Furthermore, Orady*et al.* (39) discovered resistance genes in *Pseudomonas* species isolates, including sul1, bla_{TEM} , *tetA*, bla_{CTX} - M , $blaOXA-1$, and $aadAI$, indicating the possibility of multidrug resistance. Similarly, Mohamed *et al.* (30) demonstrated antibiotic resistance in Pseudomonas isolates from chickens, notably within the β-lactamase family, and biofilm formation. Antibiotic resistance genes may explain phenotypic resistance to cephalosporin, βlactam, and other tested antimicrobials, raising the possibility of multidrug-resistant bacteria. In addition, these results highlight the effectiveness of PCR as a tool for detecting and validating antimicrobial resistance profiles in different microorganisms.

Although this study offered valuable insights about the antibiogram of *P. aeruginosa* in Fayoum Governorate in Egypt, it also displayed some limitations. Initially, the sample size was relatively small and restricted to broiler chickens from various farms within El-Fayoum Governorate, which may limit the applicability of the results to other regions or poultry populations. Furthermore, the study focused on a limited set of internal organs (liver, lung, gallbladder, kidney), which may not provide a comprehensive representation of the occurrence and dissemination of *P. aeruginosa* in other chicken tissues or systems (52).

Conclusion

In conclusion, our findings highlight a significant presence of *P. aeruginosa* in broiler chickens, accompanied by high levels of antimicrobial resistance and multiple resistance genes. These findings emphasize the urgent need for monitoring and controlling antimicrobial usage in poultry farms to mitigate the dissemination of multidrug-resistant*. aeruginosa* strains. Additionally, our findings suggest amikacin as a possibly effective treatment for *P. aeruginosa* infections.

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Conflict of interests

The author has no conflict of interest

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العزل والتوصيف الجزيئي للزائفة الزنجارية المقاومة لألدوية المتعددة المعزولة من الدجاج الالحم في الفيوم بمصر

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الخالصة

في صناعة الدواجن، تكتسب بكتريا الزائفة الزنجارية سالبة الجرام أهمية كممرض انتهازي ناشئ له آثار سريرية ملحوظة. هذه البكتريا الممرضة سالبة الجرام لديها القدرة على تلويث المفرخات، مما يؤدي إلى مجموعة من الأعراض التنفسية الشديدة، والتهاب الأمعاء، وتسمم الدم، والتهاب القرنية، والتهاب الجيوب الأنفية، والتهاب السمف، والكلية، واإلصابات والوفيات السريعة، مما يشير إلى اإلمكانات المسببة لألمراض المتنوعة لهذه البكتريا الممرضة الطيور. من خالل هذه الدراسة، تم جمع ما مجموعه 480 عينة)120 لكل منها(من الكبد والرئة والمرارة والكلى من دجاج التسمين من مختلف الأعمار والفحص البكتيري. تراوحت معدالت العزل اإلجمالية ل للزائفة الزنجارية من ٪70.8 إلى .٪83.3 ظاهريا، كشف اختبار مقاومة المضادات الحيوية للعزلات المختارة (العدد = ٣٠) أن (٩٦,٦٦٪ كانت مقاومة لثلاثة مضادات حيوية أو أكثر من مجموعات مختلفة من مضادات الميكروبات، مما يشير إلى مقاومة األدوية المتعددة، والتي كانت أعلى مقاومة لها لألموكسيسيلين ،٪100 والبيبيراسيلين ،٪96.66 والجنتاميسين ٪86.66 أوفلوكساسين ،٪80 والسيفيبيم ،٪63.33 والسيفتازيديم ،٪63.33 والليفوفلوكساسين ٪53.3 وسيبروفلوكساسين ٪53.3 يليه أبرامايسينودوكسيسيكلين ٪36.66 لكل منهما بينما كانت ٪66.6 من العزالت حساسة لألميكاسين. وراثيا، تم استخدام تفاعل البلمرة المتسلسل لتحديد خمس جينات مقاومة في عشرة عزالت مختارة من للزائفة الزنجارية متعددة المقاومة للمضادات الحيوية، وكشفت النتيجة أن ٪100 من العزالت المختبرة تحتوي على جينات مقاومة *mexR* و *ampC*، عالوة على ذلك، كان انتشار جينات *blaOXA* و *ermB* و*arr*90 و 80 و ٪50 على التوالي.

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