Infection and Drug Resistance

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ORIGINAL RESEARCH

*opr*L Gene Sequencing, Resistance Patterns, Virulence Genes, Quorum Sensing and Antibiotic Resistance Genes of XDR *Pseudomonas aeruginosa* Isolated from Broiler Chickens

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Background: *Pseudomonas aeruginosa* is incriminated in septicemia, significant economic losses in the poultry production sector, and severe respiratory infections in humans. This study aimed to investigate the occurrence, *opr*L sequencing, antimicrobial resistance patterns, virulence-determinant, Quorum sensing, and antibiotic resistance genes of *P. aeruginosa* retrieved from broiler chickens.

Methods: Two hundred samples were collected from 120 broiler chickens from broiler farms at Ismailia Governorate, Egypt. Consequently, the bacteriological examination was conducted and the obtained *P. aeruginosa* strains were tested for *opr*L gene sequencing, antibiogram, and PCR screening of virulence, Quorum sensing, and antibiotic resistance genes.

Results: The overall prevalence of *P. aeruginosa* in the examined birds was 28.3%. The *opr*L gene sequence analysis underlined that the tested strain expressed a notable genetic identity with various *P. aeruginosa* strains isolated from different geographical areas in the USA, India, China, Chile, and Ghana. PCR evidenced that the obtained *P. aeruginosa* strains, carrying virulence-related genes: *opr*L, *tox*A, *apr*A, *phz*M, and *exo*S in a prevalence of 100%, 100%, 42.5%, 33.3%, and 25.9%, respectively. Moreover, the recovered *P. aeruginosa* strains possessed the Quorum sensing genes: *las*I, *las*R, *rhl*I, and *rhl*R in a prevalence of 85.2%, 85.2%, 81.5%, and 81.5%, respectively. Furthermore, 40.7% of the isolated *P. aeruginosa* were XDR to seven antimicrobial classes, possessing *sul*1, bla_{TEM}, *tet*A, bla_{CTX-M}, bla_{OXA-1}, and *aad*A1 genes.

Conclusion: As we can tell, this is the first report emphasizing the evolution of XDR *P. aeruginosa* strains from broiler chicken in Egypt, which is supposed to be a serious threat to public health. The emerging XDR *P. aeruginosa* in poultry frequently harbored the *opr*L, *tox*A, and *apr*A virulence genes, the *las*I, *las*R, *rhl*I, and *rhl*R Quorum sensing genes, and the *sul*1, bla_{TEM}, *tet*A, bla_{CTXM}, bla_{OXA-1}, and *aad*A1 resistance genes.

Keywords: *P. aeruginosa*, MDR, XDR, *opr*L sequence analysis, Quorum sensing, virulence genes, resistance genes

Introduction

Pseudomonas species are ubiquitous microorganisms that are retrieved from various origins, such as poultry, drinking water, domestic and wild animals, human cases, and different food products.[1,2] *Pseudomonas* infections in poultry are of the highest significance as the infection might spread speedily among the poultry flocks resulting in elevated mortalities in various ages.[3] The genus *Pseudomonas* includes several species, but *Pseudomonas aeruginosa* (*P. aeruginosa*) is the

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most common specie incriminated in poultry infection all over the world. *P. aeruginosa* is an opportunistic microorganism able to infect various host tissues.[4,5] *P. aeruginosa* infections in chickens mainly occur via skin wounds, contaminated vaccines, egg dipping, and contamination of needles used for injection. Moreover, the disease could be transmitted from infected flocks to susceptible ones in the same area due to poor hygienic conditions. Even though chickens of any age can be infected with *P. aeruginosa*, young chicks frequently are the most susceptible.[5]

P. aeruginosa are Gram-negative motile rods arranged mainly in a single manner or short chains. It is a strictly aerobic pathogen that rapidly grows on ordinary media and typically produces water-soluble fluorescein or pyocyanin pigments.[6] *P. aeruginosa* is an opportunistic pathogen that is involved in high mortalities of young chicks as a result of yolk sac infections and omphalitis gained through hatchery.[7] Furthermore, *P. aeruginosa* retains both cell-mediated and secreted virulence determinants. The cell-mediated virulence factors, such as lipopolysaccharide (LPS), flagella, and pili, play a vital role in motility, colonization of bacteria in the host tissues, and the invasion of bacterial active proteins into the target cells.[8] Besides, the secreted virulence types enable microbial invasion and propagation, strengthen inflammatory conditions, initiate potent host-tissue damages, and increase the severity of infection. The most common secreted virulence determinants accompanying *P. aeruginosa* are exotoxin A and exotoxin S. Exotoxin A is accountable for the prevention of protein synthesis in the host cell, whereas exotoxin S is an extracellular protein that incriminated in the cell-apoptosis through the initiation of the GTPase and ribosyltransferases actions. Moreover, the pathogen secretes the biologically active phenazine compounds that play a vital role in bacterial virulence. The Quorum-Sensing phenomenon is a cell signaling mechanism present in certain bacterial species, enabling the bacterial cell response to extracellular signals. It is regulated by the *las* and *rhl* genes.[9,10] Molecular techniques are essential for the rapid detection of *P. aeruginosa* by amplification of species-specific primers, especially *opr*L gene sequencing.[11–13]

Recently, multidrug resistance (MDR) has augmented worldwide that is deliberated public health threat. Several recent epidemiological investigations revealed the occurrence of XDR (extensively drug-resistant: resistant to ≥ 1 agent in all tested antimicrobial classes but ≤ 2 classes) and MDR (Multidrug-resistant: resistant to ≥ 1 agent in ≥ 3 antimicrobial classes) bacterial pathogens from different origins.[14–17] *P. aeruginosa* frequently displayed various resistance patterns against several antibiotics. The antibiotic resistance in *P. aeruginosa* is accredited mainly to acquired and intrinsic resistance mechanisms to various antibiotics through low permeability of the outer membrane, antibiotic resistance genes, and active efflux pumps.[18] In *P. aeruginosa*, the outer membrane proteins (*opr*L) play a significant role in antibiotics and antiseptics resistance.[11] Moreover, the Extended β-lactamases (ESBLs), encoded by ESBLs genes, are responsible for the resistance of β-lactam antimicrobials (penicillin and cephalosporins). The $bla_{\text{CTX-M}}$ and bla_{TEM} are the most common ESBLs genes related to *P. aeruginosa*.[19,20] Therefore, the PCR screening of the most common antimicrobial resistance genes has to be accomplished to investigate the occurrence of MDR pathogens of public health importance.[21]

This work was conducted to demonstrate the occurrence, Sequence analysis of *opr*L gene, virulence, Quorum sensing, and resistance genes of XDR *P. aeruginosa* recovered from broiler chickens.

Methods

Animal Ethics

All protocols were conducted according to relevant USDA Animal Welfare guidelines followed for the welfare of the laboratory animals. Scientific Research Ethics Committee, Suez Canal University, Egypt, approved the handling of chickens and all the procedures (Approval no. 2022056).

Sampling

A total of 200 samples were congregated from 120 broiler chickens (2–4 weeks old) (40 apparently healthy birds: tracheal swabs (n=40), 40 Diseased birds: tracheal swabs (n=40), and 40 freshly dead birds: liver, lung, and heart (n=40 for each) from four commercial broilers farms (30 birds from each farm; 10 apparently healthy, 10 diseased birds, and 10 freshly dead birds, were examined) at Ismailia Province, Egypt (from April to June 2021). Diseased birds exhibited

respiratory manifestations. Samples collection was carried out aseptically, and samples were immediately transmitted in the icebox to the laboratory as soon as possible.

Isolation and Identification of *P. aeruginosa*

The collected samples were inoculated in nutrient broth (Oxoid, UK) and incubated aerobically for 24 hrs at 37 °C. A loopful from the inoculated broth was streaked onto cetrimide agar and MacConkey agar (Oxoid, UK) and incubated at 37°C for 24 hrs under aerobic conditions. The recovered isolates were identified according to their morphological characteristics using Gram's stain, culture characters, pigment production (fluorescent pigments), motility, and biochemical reactions using the following tests; oxidase, indole, H_2S production, catalase, urease, methyl red, citrate utilization, gelatin hydrolysis, mannitol fermentation, and Voges-Proskauer tests as previously described by Mac Faddin.[22] Likewise, the identification of *P. aeruginosa* was affirmed genetically using the species-specific set of primers aiming the *opr*L gene according to Xu.[23]

*P. aeruginosa opr*L Gene Sequencing

In this study, all the isolated *P. aeruginosa* strains disclosed coordination in their phenotypic traits (morphological, culture, and biochemical characteristics). Therefore, the PCR products of one randomly chosen *P. aeruginosa* strain were purified using the PureLink purification kit (Life Technologies, Renfrew, UK). Moreover, the attained sequences were stored in the GenBank (Accession no.: MW056321). Furthermore, numerous alignments were carried out on the recovered sequences. The phylogenetic tree was designed consistent with the neighbor-joining approach by the MEGA X software.[24]

Antibiogram of the Retrieved *P. aeruginosa* Isolates

The resistance patterns of the retrieved *P. aeruginosa* isolates were investigated using the disc diffusion test on Muller Hinton agar (Difco, USA). Eleven antimicrobial discs (Oxoid, UK) were implicated; streptomycin (S/10 µg), trimethoprim-sulfamethoxazole (SXT/1.25/23.75µg), ceftriaxone (CTX/30µg), norfloxacin (NOR/10µg), ampicillin (AMP/10µg), erythromycin (E/ 15µg), colistin sulfate (CT/10µg), amikacin (AK/30 µg), cefotaxime (CXT/30µg), tetracycline (TE/30 µg), and amoxicillin-clavulanic acid (AMC/30µg). The interpretation of the test results was accomplished in line with the instructions of CLSI, 2018.[25] The *P. aeruginosa* ATCC 27853 was involved as a test control. Moreover, the tested *P. aeruginosa* strains were grouped into XDR and MDR in compliance with Magiorakos.[26] Besides, the multiple antibiotic-resistance (MAR) indices were estimated according to Krumperman.[27]

Dissemination of Virulence, Quorum Sensing, and Resistance Genes in the Retrieved *P. aeruginosa* Isolates

PCR was used to monitor the virulence-related genes (*opr*L, *tox*A, *apr*A, *exo*S, and *phz*M), Quorum sensing genes (*las*I, *las*R, *rhl*I, and *rhl*R), and resistance genes (bla_{TEM}, bla_{OXA}, bla_{CTX-M}, *sul*1, *aad*A1, and *tet*A) in the isolated *P. aeruginosa*. DNA was extracted using the PureLink DNA Extraction Kit (Life Technologies, Renfrew, UK / Cat. No. K182001). In each reaction, positive controls (Positive *P. aeruginosa* strains provided by the AHRI, Dokki, Egypt), as well as negative controls (DNA-free reaction) were applied. Moreover, the separation of PCR products was carried out with agar gel electrophoresis. Subsequently, the gel was photographed. The used primers (Life Technologies, Renfrew, UK) and PCR conditions are presented in Table 1.

Statistical Analyses

The analyses of the data frequencies were performed by the Chi-square test using SAS software (version 9.4, SAS Institute, Cary, NC, USA) (p-value < 0.05 specifies a significant difference). Furthermore, the correlations between the tested antibiotics and the resistance genes were estimated with the R-software (version 4.0.2; https://www.r-project.org/).

Table 1 Oligonucleotide Sequences and Conditions of PCR Assay

Type of Gene	Target Genes	Primer-Sequences	Amplicon Size (bp)	PCR Conditions (Cycles, *n*=35)			References
				Denaturation	Annealing	Extension	
Virulence genes	*opr*L	ATG GAA ATG CTG AAA TTCGGC CTT CTT CAG CTC GAC GCGACG	504	96°C 1 min.	55°C 1 min.	72°C 1 min.	[23]
	*tox*A	GGAGCGCAACTATCCCACT TGGTAGCCGACGAACACATA	150	95°C 40 sec.	50°C 1 min.	72°C 2 min.	[28]
	*apr*A	GTCGACCAGGCGGCGGAGCAGATA GCCGAGGCCGCCGTAGAGGATGTC	993	95°C 40 sec.	65°C 1 min.	72°C 2 min.	
	*exo*S	GCGAGGTCAGCAGAGTATCG TTCGGCGTCACTGTGGATGC	118	94°C 30 sec.	58°C 30 sec.	68°C 1 min.	[29]
	*phz*M	ATGGAGAGCGGGATCGACAG ATGCGGGTTTCCATCGGCAG	875	94°C 30 sec.	54°C 30 sec.	72°C 1 min.	[30]
Quorum sensing genes	*las*I	CGTGCTCAAGTGTTCAAGG TACAGTCGGAAAAGCCCAG	295	95°C 40 sec.	60°C 1 min.	72°C 2 min.	[28]
	*las*R	AAGTGGAAAATTGGAGTGGAG GTAGTTGCCGACGACGATGAAG	130	95°C 40 sec.	60°C 1 min.	72°C 2 min.	
	*rhl*I	TTCATCCTCCTTTAGTCTTCCC TTCCAGCGATTCAGAGAGC	155	95°C 40 sec.	60°C 1 min.	72°C 2 min.	
	*rhl*R	TGCATTTTATCGATCAGGGC CACTTCCTTTTCCAGGACG	133	95°C 40 sec.	60°C 1 min.	72°C 2 min.	
Beta-lactam resistance	bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94°C 30 sec.	54°C 30 sec.	72°C 1 min.	[31]
	bla_{OXA-1}	ATATCTCTACTGTTGCATCTCC AAACCCTTCAAACCATCC	619	94°C 30 sec.	48°C 30 sec.	72°C 1 min.	
	bla_{CTX-M}	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	94°C 45 sec.	45°C 45 sec.	72°C 45 sec.	[32]
Aminoglycosides resistance	*aad*A1	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	484	94°C 30 sec.	54°C 40 sec.	72°C 45 sec.	[33]
Tetracycline resistance	*tet*A	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576	94°C 30 sec.	56°C 40 sec.	72°C 45 sec.	
Sulfonamides resistance	*sul*1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	94°C 30 sec.	60°C 40 sec.	72°C 45 sec.	[34]

Results

Phenotypic Characteristics and the Occurrence of *P. aeruginosa* in the Examined Birds

The bacteriological assay proved that all recovered *P. aeruginosa* isolates (n=54) were Gram-negative, motile rods arranged singly or in short chains. On cetrimide agar, the colonies of retrieved *P. aeruginosa* isolates were large and irregular with a fruity odor and disseminate the characteristic fluorescent pigment (yellowish-green). Moreover, the recovered isolates displayed smooth, pale (non-lactose fermenter), and flat colonies on macConkey agar. Biochemically, the tested *P. aeruginosa* isolates were positive for oxidase, mannitol fermentation, gelatin hydrolysis, catalase, citrate utilization, and nitrate reduction tests. Furthermore, the retrieved *P. aeruginosa* isolates were negative for methyl red, H_2S production, indole, urease, and Voges-Proskauer tests. Besides, all the recovered isolates were positive for the species-specific *opr*L gene.

The occurrence of *P. aeruginosa* in the inspected birds was 28.3% (34/120). Moreover, the percentage of *P. aeruginosa* in the inspected apparently healthy, diseased, and freshly dead chickens was 12.5% (5/40), 42.5% (17/40), and 30% (12/40), respectively (as described in Table 2). Statistically, there is a significant difference ($p < 0.05$) in the occurrence of *P. aeruginosa* in the examined apparently healthy, diseased, and freshly dead birds. With regard to the intensity of *P. aeruginosa* in the examined internal organs of the freshly dead birds, the predominant affected organs were the lung and liver (22.2% for each), followed by the heart (14.8%) (Table 3 and Figure 1).

Sequence Analysis of the *oprL* Gene of *P. aeruginosa*

The *opr*L gene sequence analysis underlined that the tested strain (Accession No. MW056321) exposed high genetic identity (100%) with other *P. aeruginosa* strains isolated from different origins and geographical areas (Figure 2), for example, *P. aeruginosa* strain PA0750 of India (Accession no. CP034908), *P. aeruginosa* strain LIUYANG-C of China (Accession no. CP050053), *P. aeruginosa* PAC1 of USA (CP053706), *P. aeruginosa* strain delta 6_4 of USA (Accession no. CP047063), *P. aeruginosa* strain delta 6_6 of USA (Accession no. CP047065), *P. aeruginosa* strain Cas9_1 of USA (Accession no. CP047067), and *P. aeruginosa* strain PGN4 of USA (Accession no. CP032540). Besides, it proved 99.7% genetic identity with *P. aeruginosa* strain PA-1 of human origin in Chile (Accession no. CP097709) and *P. aeruginosa* strain PA0011 isolated from tissue biopsy in Ghana (Accession no. CP100761).

Antibiogram of the Retrieved *P. aeruginosa* Isolates

The tested *P. aeruginosa* isolates disclosed high resistance to different antimicrobial agents including; trimethoprim-sulfamethoxazole, penicillin, and tetracycline (100% for each), ceftriaxone and cefotaxime (92.6% for each),

Table 2 Prevalence of *P. aeruginosa* Among the Examined Birds

Apparently Healthy Birds (n=40)		**Diseased Birds (n=40)**		**Freshly Dead Birds (n=40)**		**Total (n-120)**	
Positive		**Positive**		**Positive**		**Positive**	
n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
5	12.5	17	42.5	12	30	34	28.3
Chi square		6.4118					
***p* value**		*p*-value = 0.04052					

Table 3 The Dissemination of *P. aeruginosa* Among Different Examined Samples of Chickens

Type of Samples	**Apparently Healthy Birds**		**Diseased Birds**		**Freshly Dead Birds**		***P. aeruginosa* Isolates**	
	n=40		**n =40**		**n =40**			
	Positive Samples		**Positive Samples**		**Positive Samples**		**n**	**%***
	n	**%**	**n**	**%**	**n**	**%**		
Tracheal swabs	5/40	12.5	17/40	42.5	–	–	22	40.7
Liver	–	–	–	–	12/40	30	12	22.2
Lung	–	–	–	–	12/40	30	12	22.2
Heart	–	–	–	–	8/40	20	8	14.8
Total	5/40	12.5	17/40	42.5	32/120	26.6	54	100

Note: %*: The percentage was calculated according to the total number of isolates (n=54).

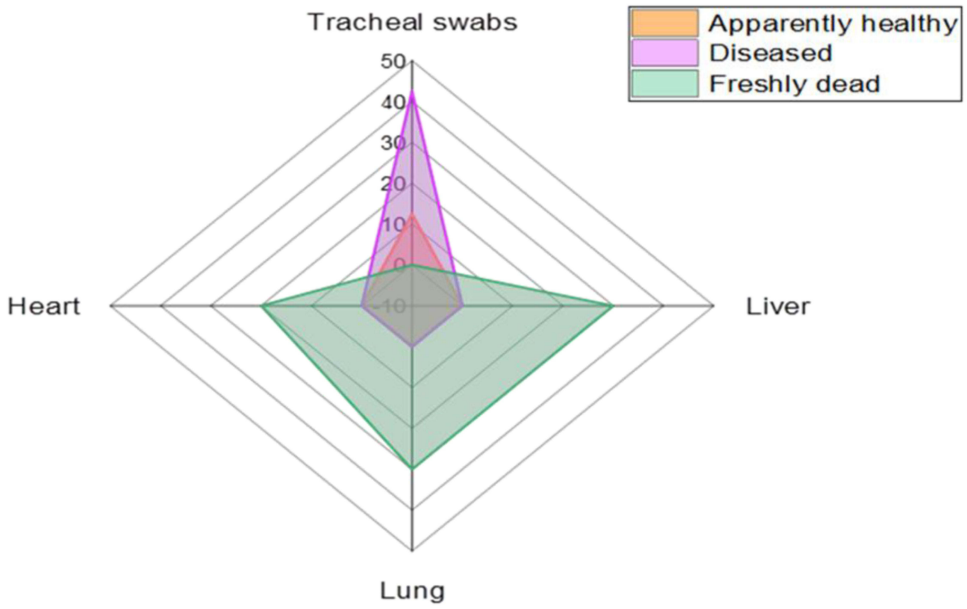

Figure 1 The dissemination of *P. aeruginosa* among various examined samples.

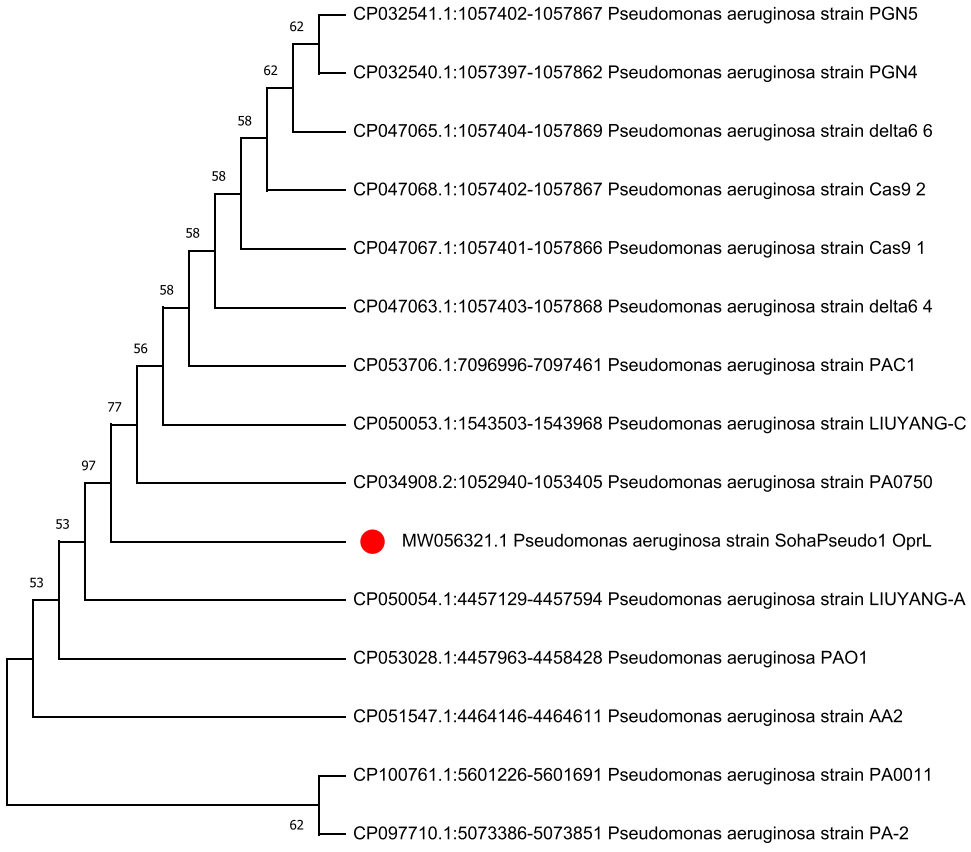

Figure 2 The phylogenetic analyses of the *opr*L gene sequencing: the tree elucidates the genetic similarity of the recovered *P. aeruginosa* strain and other strains placed in the GenBank database. The *P. aeruginosa* strain in the current work is noticeable with a red circle.

streptomycin and amikacin (90.7% for each), amoxicillin-clavulanic acid (88.8%), and erythromycin (77.7%). Furthermore, the tested isolates displayed a striking sensitivity to norfloxacin (81.5%) and colistin sulfate (74.07%) (Table 4 and Figure 3). Statistically, there is a significant difference ($p < 0.05$) in the sensitivity of *P. aeruginosa* strains to

Table 4 Antimicrobial Resistance Patterns of the Retrieved *P. aeruginosa*

Classes	Antimicrobial Agents	*P. aeruginosa* (n=54)					
		Sensitive (S)		Intermediate (I)		Resistant (R)	
		n	%	n	%	n	%
Penicillin	Ampicillin	0	0	0	0	54	100
β-Lactam-β-lactamase-inhibitor combination	Amoxacillin-clavulanic acid	0	0	6	11.2	48	88.8
Cephalosporin	Ceftriaxone	2	3.7	2	3.7	50	92.6
	Cefotaxime	1	1.85	3	5.5	50	92.6
Fluroquinolone	Norfloxacin	44	81.5	0	0	10	18.5
Tetracycline	Tetracycline	0	0	0	0	54	100
Polymyxin	Colistin sulfate	40	74.07	4	7.4	10	18.5
Sulfonamides	Trimethoprim-sulfamethoxazole	0	0	0	0	54	100
Macrolides	Erythromycin	2	3.7	10	18.5	42	77.7
Aminoglycosides	Streptomycin	0	0	5	9.3	49	90.7
	Amikacin	1	1.9	4	7.4	49	90.7
Chi square *P* value		343.4 $p<0.0001$		32.647 0.0003122		64.038 $p<0.0001$	

different antimicrobial agents. Likewise, notable positive correlations were verified between TE and AMP (r = 1); SXT, TE, and AMP (r = 1); AK, CXT, CTX, SXT, TE, and AMP (r = 1); E, AMC, and S (r = 1); S, AK, CXT, CTX, SXT, TE, and AMP (r = 1); AMC, S, AK, and CXT (r = 1); NOR and CT (r = 1) (Figure 4).

The Occurrence of Virulence, Quorum Sensing, and Resistance Genes in the Isolated *P. aeruginosa* Isolates

Using PCR revealed that the tested *P. aeruginosa* strains possessing the virulence-related genes: *opr*L, *tox*A, *apr*A, *phz*M, and *exo*S with a prevalence of 100%, 100%, 42.5%, 33.3%, and 25.9%, respectively. Moreover, the recovered *P. aeruginosa* strains possessed the Quorum sensing genes: *las*I, *las*R, *rhl*I, and *rhl*R in a prevalence of 85.2%, 85.2%,

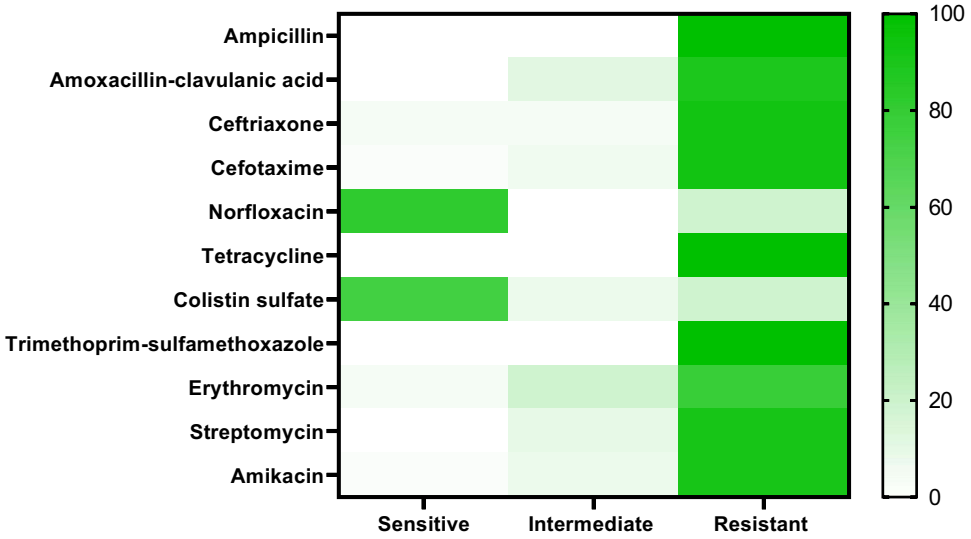

Figure 3 The heat-map explicates the antibiogram of the retrieved *P. aeruginosa* strains from broiler chickens.

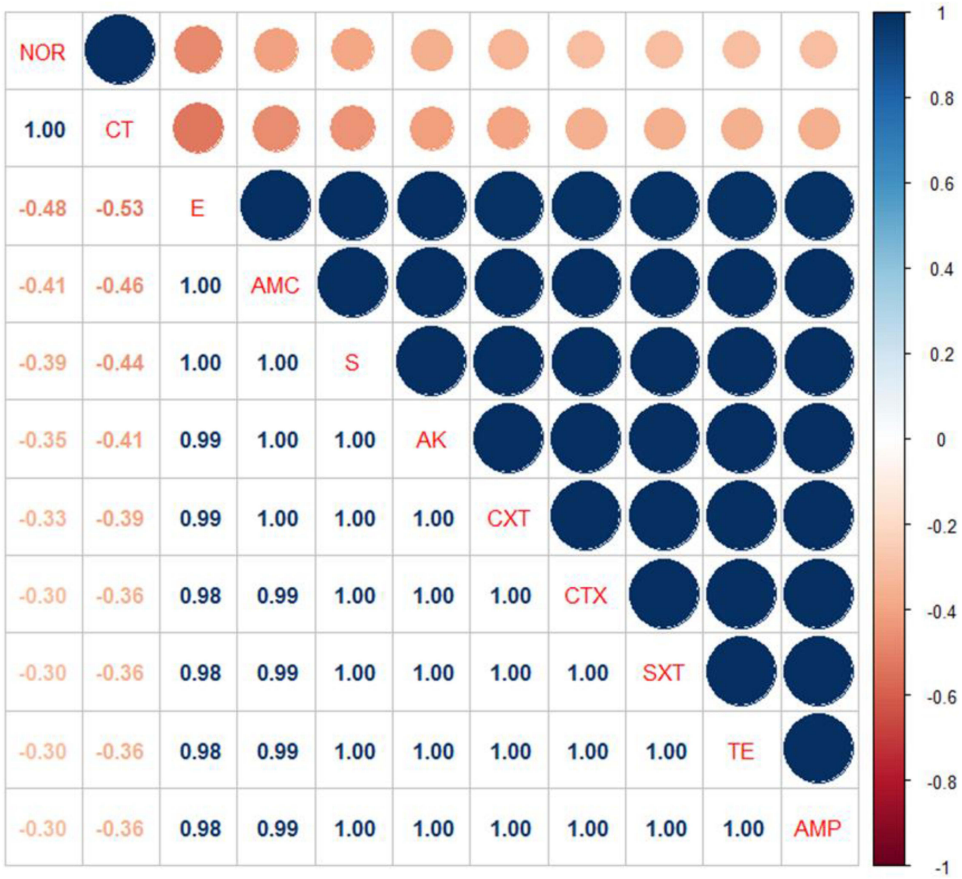

Figure 4 The heat-map simplifies the correlation coefficient (r) between the tested antimicrobial agents in this study.

81.5%, and 81.5%, respectively. With reference to the dissemination of the resistance genes, all the isolated *P. aeruginosa* strains (100%) held the bla_{TEM}, *sul*1, and *tet*A resistance genes. Besides, recovered *P. aeruginosa* isolates harbored the antimicrobial-resistance genes; *aad*A1, $bla_{CTX\text{-}M}$, and bl_{aOXA} in a prevalence of 90.7%, 88.8%, and 81.5%, respectively. The statistical analysis emphasized a significant difference ($p < 0.05$) in the dissemination of virulence genes in the obtained *P. aeruginosa*. On the contrary, a non-significant difference ($p > 0.05$) was recorded in the occurrence of Quorum sensing and resistance genes in the tested *P. aeruginosa* (Table 5 and Figure 5).

Resistance Patterns and the Distribution of Antimicrobial Resistance Genes Between the Isolated *P. aeruginosa* Strains from Birds

It was noticed that 40.7% (22/54) of the obtained *P. aeruginosa* isolates were XDR to 9 antimicrobial agents in seven antimicrobial classes and carrying *sul*1, bla_{TEM}, *tet*A, $bla_{CTX\text{-}M}$, $bla_{OXA\text{-}1}$, and *aad*A1 genes. Moreover, 18.5% (10/54) of the tested *P. aeruginosa* strains revealed extensive-drug resistance to 9 antimicrobial agents in seven antimicrobial classes and carried *sul*1, bla_{TEM}, *tet*A, bla_{CTXM}, and *aad*A1 genes. Furthermore, 16.7% (9/54) of the obtained *P. aeruginosa* isolates were XDR to 10 antimicrobial agents in eight classes, possessing *sul*1, bla_{TEM}, *tet*A, $bla_{OXA\text{-}1}$, $bla_{CTX\text{-}M}$, and *aad*A1 genes. Besides, 9.3% (5/54) of the obtained *P. aeruginosa* isolates were MDR to 6 antimicrobial agents in five classes, carrying *sul*1, bla_{TEM}, *tet*A, $bla_{OXA\text{-}1}$, and bla_{CTXM} genes. Also, 7.4% (4/54) of the tested *P. aeruginosa* isolates were MDR 7 antimicrobial agents in six classes and possessing *sul*1, bla_{TEM}, *tet*A, $bla_{OXA\text{-}1}$, and *aad*A1 genes as illustrated in Table 6 and Figure 6. Likewise, our results emphasized that the MAR index values were > 0.2, suggesting that the *P. aeruginosa* strains recovered from birds derived from high-risk contamination. Additionally, our findings reported notable positive correlations between the bla_{TEM} gene and AMP (r=1); bla_{TEM}, CTX, and CXT (r=1); *sul*1 and

Table 5 Distribution of Virulence, Quorum Sensing, and Antimicrobial Resistance Genes

Types	Genes	*P. aeruginosa* n= 54	Chi Square	*P* value
Virulence genes	*oprL*	54 (100%)	48.074	$p<0.0001$
	toxA	54 (100%)		
	aprA	23 (42.5%)		
	phzM	18 (33.3%)		
	exoS	14 (25.9%)		
Quorum-sensing genes	*lasI*	46 (85.2%)	0.088889	0.9931^{NS}
	lasR	46 (85.2%)		
	rhlI	44 (81.5%)		
	rhlR	44 (81.5%)		
Antimicrobial resistance genes	bla_{TEM}	54 (100%)	1.7177	0.7875^{NS}
	bla_{CTX-M}	48 (88.8%)		
	bla_{OXA-1}	43 (81.5%)		
	tetA	54 (100%)		
	sulI	54 (100%)		
	aadA1	49 (90.7%)		

Abbreviation: NS, non-significant.

SXT (r=1); *tetA* and TE (r=1); *aadA1*, AK, and S (r=1); bla_{CTX-M} and CTX (r=1); bla_{CTX-M} and AMP (r=1); bla_{CTX-M} and CXT (r=0.99); bla_{TEM} and AMC (r=0.99); bla_{CTX-M} and AMC (r=0.98); bla_{OXA-1}, AMP, and CTX (r=0.97); bla_{OXA-1} and CXT (r=0.96); bla_{OXA-1} and AMC (r=0.93) (Figure 7).

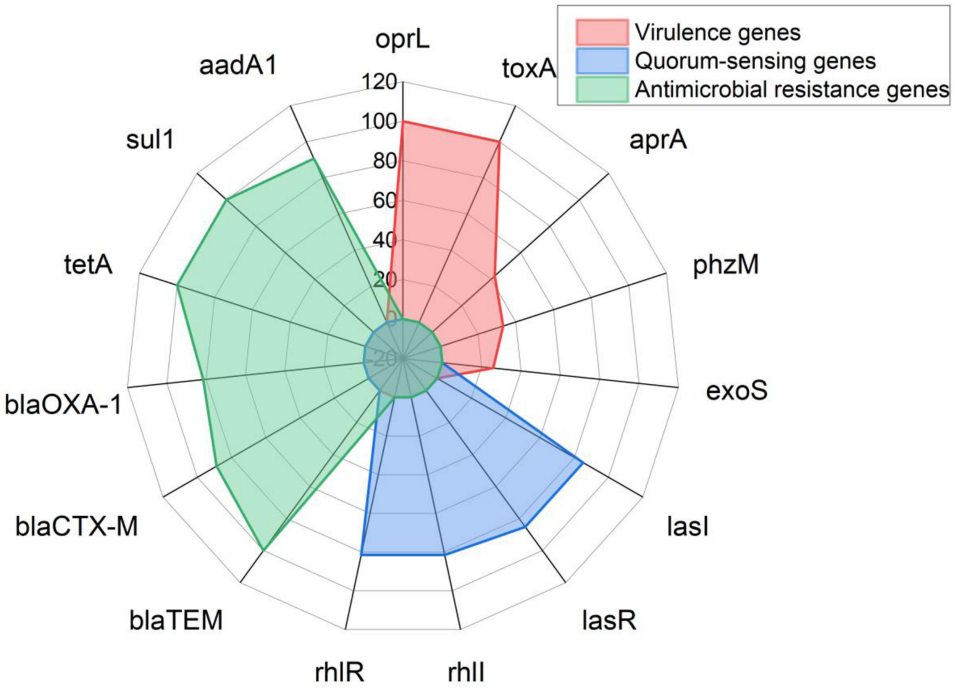

Figure 5 The distribution of virulence, quorum sensing, and resistance genes in the recovered *P. aeruginosa* isolates.

Table 6 Resistance Patterns and Distribution of Resistance Genes Between *P. aeruginosa* Strains Isolated from Birds

No. of strains	%	Resistance patterns	Phenotypic resistance	Resistance genes	MARI
22	40.7	**XDR**	**9 Antimicrobial agents/Seven classes** SXT, AMP, TE, AMC, CTX, CAZ, E, S, and AK	*sul*1, bla_{TEM}, *tet*A, bla_{CTX-M}, bla_{OXA-1}, *aad*A1	0.82
10	18.5	**XDR**	**9 Antimicrobial agents/Seven classes** SXT, AMP, TE, AMC, CTX, CAZ, E, S, and AK	*sul*1, bla_{TEM}, *tet*A, bla_{CTX-M}, *aad*A1	0.82
9	16.7	**XDR**	**10 Antimicrobial agents /Eight classes** SXT, AMP, TE, AMC, CTX, CAZ, NOR, E, S, and AK	*sul*1, bla_{TEM}, *tet*A, bla_{OXA-1}, bla_{CTX-M}, *aad*A1	0.91
5	9.3	**MDR**	**6 Antimicrobial agents/Five classes** SXT, AMP, TE, CTX, CAZ, and CT	*sul*1, bla_{TEM}, *tet*A, bla_{OXA-1}, bla_{CTX-M}	0.54
4	7.4	**MDR**	**7 Antimicrobial agents/Six classes** SX, AMP, TE, AMC, S, AK, and CT	*sul*1, bla_{TEM}, *tet*A, bla_{OXA-1}, *aad*A1	0.64
3	5.5	**MDR**	**8 Antimicrobial agents/Six classes** SXT, AMP, TE, AMC, CTX, CAZ, S, and AK	*sul*1, bla_{TEM}, *tet*A, bla_{OXA-1}, bla_{CTX-M}, *aad*A1	0.82
1	1.8	**XDR**	**10 Antimicrobial agents /Nine classes** SXT, AMP, TE, NOR, E, CTX, CAZ, CT, S, and AK	*sul*1, bla_{TEM}, *tet*A, bla_{CTX-M}, *aad*A1	0.91

Abbreviations: AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CTX, ceftriaxone; NOR, norfloxacin; CXT, Cefotaxime; E, erythromycin; S, streptomycin; SXT, trimethoprim-sulfamethoxazole; CT, colistin sulfate; TE, tetracycline; AK, amikacin; MARI, the number of antimicrobial agents to which the isolates are resistant/ the total number of tested antimicrobial agents.

Discussion

P. aeruginosa is one of the primary causes of septicemia in broiler chickens, triggering notable economic losses in the poultry production sector all over the world.[7] Regarding the phenotypic features of *P. aeruginosa*, the bacteriological investigation emphasized that all the obtained isolates from birds disclosed the distinctive morphological, cultural, and biochemical features of *P. aeruginosa*. These outcomes are consistent with the findings verified by Abdelmoez.[35]

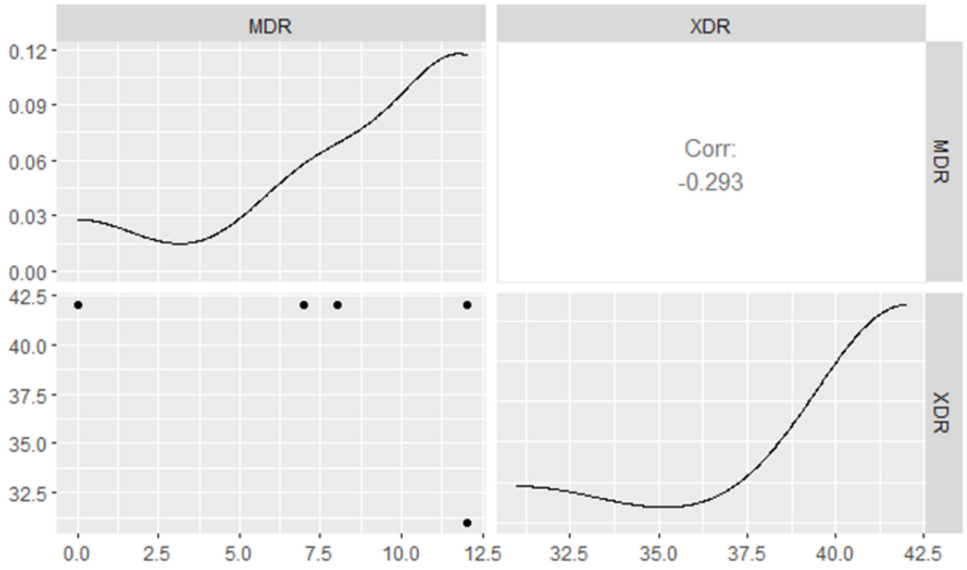

Figure 6 Illustrates the occurrence and distribution of XDR and MDR resistance patterns among the isolated *P. aeruginosa* strains from the examined broiler chickens.

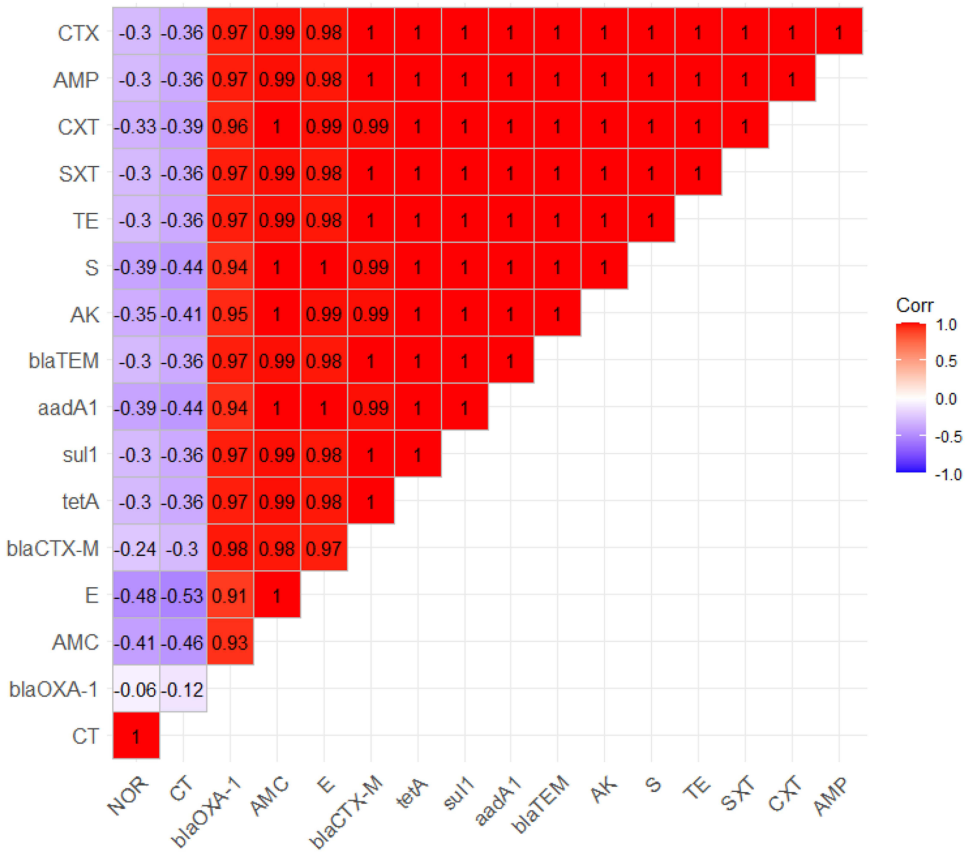

Figure 7 The heat-map elucidates the correlation coefficient (r) among the resistance genes of *P. aeruginosa* strains and different tested antimicrobial agents.

In this work, the overall prevalence of *P. aeruginosa* in the inspected birds was 28.3%. The highest prevalence was noted in the diseased chickens, followed by the freshly dead ones. Higher prevalence was recorded by Abd El-Tawab,[36] who stated that the percentage of *P. aeruginosa* in the inspected broiler chickens was 34%. Moreover, a lower prevalence (20%) was reported by Shahat.[4] *P. aeruginosa* represents a serious pathogen in poultry, causing severe respiratory infections and high mortality rates in broiler chicken flocks.[37]

Disparities in prevalence may be attributed to the hygienic conditions, the time of sampling, geographical variation, management practices, environmental stresses, and bird age and immunity.[7] A high standard of hygienic measures is essential to prevent the spreading of *P. aeruginosa* infection between birds in poultry farms. Regular cleaning and disinfection of poultry farms frequently lead to better results for infection control. Moreover, limitations of antibiotic use in the poultry industry should be carried out.[5,35]

The molecular-based identification of *P. aeruginosa* is essential to overcome the limitations of conventional assays. Moreover, the amplification of species-specific primers, such as the *opr*L gene is beneficial to obtain rapid, reliable, and accurate identification of *P. aeruginosa*.[38] In the present study, all retrieved isolates of *P. aeruginosa* from birds tested positive for the *opr*L gene. Likewise, the *opr*L sequence analyses emphasized that the tested *P. aeruginosa* strain displayed a notable genetic matching (100%) with several *P. aeruginosa* strains originated from various sources and areas. For example, *P. aeruginosa* strain LIUYANG-C of China (Accession no. CP050053), *P. aeruginosa* PAC1 of USA (CP053706), *P. aeruginosa* strain delta 6_4 of USA (Accession no. CP047063), *P. aeruginosa* strain delta 6_6 of USA (Accession no. CP047065), and *P. aeruginosa* strain PA0750 of India (Accession no. CP034908).[39] These outcomes accentuate the epidemiological map and highlight the public health impact of *P. aeruginosa*.

Concerning the antimicrobial susceptibility testing, the retrieved *P. aeruginosa* strains were highly resistant to several antimicrobial classes including aminoglycosides, tetracycline, sulfonamides, penicillin, cephalosporins, macrolides, and β-Lactam-β-lactamase-inhibitor combination. These findings nearly agreed with those recorded by Kousar[40] and

Mohamed.[41] On the other hand, norfloxacin and colistin-sulfate exhibited optimistic antimicrobial activity toward the tested *P. aeruginosa* strains recovered from broiler chicken. The sensitivity of *P. aeruginosa* to norfloxacin and colistin sulfate was informed previously by Sans-Serramitjana[42] and Rafique.[43] The remarkable resistance of *P. aeruginosa* to various antimicrobials is deliberated public health threat. The widespread use of antibiotics in the poultry production sector and the proficiency of *P. aeruginosa* to attain resistance genes from other superbugs are the fundamental reasons endorsing the occurrence of MDR and XDR strains. Moreover, the antimicrobial resistance in *P. aeruginosa* is attributed mainly to acquired and intrinsic resistance mechanisms through low permeability of the outer membrane as well as harboring resistance genes. Consequently, reliable application of susceptibility testing and investigation of the occurrence of XDR and MDR pathogens are indispensable for choosing the most effective antibiotics.[44,45]

Concerning the distribution of virulence genes, using PCR proved that the retrieved *P. aeruginosa* strains commonly have the *opr*L and *tox*A genes, followed by *apr*A, *phz*M, and *exo*S genes. Our findings are nearly in accordance with those recorded by Al-Dahmoshi,[46] Bakheet and Torra,[47] and Qian.[48] The demonstration of virulence-related genes is crucial for the assessment of the potential pathogenicity of *P. aeruginosa*. The outer membrane lipoprotein (regulated by the *opr*L gene) is accountable for the intrinsic resistance of *P. aeruginosa* to antiseptics and antimicrobial agents.[49,50] Moreover, Exotoxin A (regulated by the *tox*A gene) is a cytotoxic compound that is considered a key virulence determinant of *P. aeruginosa*. It is responsible for hindering protein biosynthesis in the host. Besides, exotoxin S is an extracellular protein that incriminated cell apoptosis through the initiation of the GTPase and ribosyltransferase actions. Likewise, the pathogen secretes the biologically active phenazine compounds (encoded by the *phz*M gene) that play a vital role in bacterial virulence. Alkaline protease (encoded by *apr*A gene) is a metalloprotease enzyme that splits different immune proteins such as TNF-α, IL-6, IFN-γ, and laminin, resulting in reduced immune response.[51,52]

In this study, the majority of the recovered *P. aeruginosa* strains had the *las*I, *las*R, *rhl*I, and *rhl*R Quorum sensing genes. Our results are consent with those stated by Sabharwal.[28] Quorum-Sensing plays a substantial role in the expression of virulence-related genes, antimicrobial resistance, and biofilm formation in *P. aeruginosa*. Quorum-Sensing molecules are regulated by the *las* and *rhl* genes.[53–55]

Regarding the occurrence of resistance patterns in the recovered *P. aeruginosa*, the majorities of the tested *P. aeruginosa* were XDR to seven or eight antimicrobial classes, carrying the *sul*1, bla_{TEM}, *tet*A, bla_{CTXM}, $bla_{\text{OXA-1}}$, and *aad*A1 genes. Besides, a high proportion of the retrieved *P. aeruginosa* disclosed MDR to five or six classes and harbored *sul*1, bla_{TEM}, *tet*A, $bla_{\text{OXA-1}}$, and bla_{CTXM} genes. The tremendous increase in antimicrobial resistance is believed to be a major public health threat globally. The misapplication of antibiotics in poultry farms and health facilities and the transportation of resistance genes between bacteria are the primary predisposing determinants of multi-drug resistance.[56,57] The Extended β-lactamases (ESBLs), encoded by ESBLs genes, are responsible for the resistance of β-lactam antimicrobials (penicillin and cephalosporins). The $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{OXA} are the most common ESBLs genes in *P. aeruginosa*. The bla_{TEM} gene is responsible for penicillin resistance. Moreover, the resistance to cephalosporins is ascribed to the $bla_{\text{CTX-M}}$ gene. Furthermore, the resistance to the β-Lactam-β-lactamase-inhibitor-combinations is endorsed by the synergism between $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-M}}$ resistance genes. Besides, *P. aeruginosa* is usually resistant to aminoglycosides, sulfonamides, and tetracycline due to the occurrence of *aad*A1, *sul*1, and *tet*A resistance genes, respectively.[58–60] In the present study, there is a positive relationship between the occurrence of antimicrobial resistance and the distribution of antimicrobial resistance genes and virulence genes among the recovered *P. aeruginosa* strains from broiler chickens. On the other hand, Gajdács[61] reported no correlation between virulence determinants, antimicrobial resistance, and biofilm production in the in-vitro-tested *P. aeruginosa* strains.

Conclusion

In brief, for all we know, this is the first report that underscored the evolution of XDR *P. aeruginosa* strains from broiler chicken in Egypt. The retrieved XDR *P. aeruginosa* strains commonly harbored the *opr*L, *tox*A, *apr*A, *phz*M, and *exo*S virulence-determinant genes and the *las*I, *las*R, *rhl*I, and *rhl*R Quorum sensing genes. The re-emerging *P. aeruginosa* strains in broiler chickens were XDR to many antimicrobial classes (cephalosporins, tetracycline, aminoglycosides, sulfonamides, penicillin, macrolides, and β-Lactam-β-lactamase-inhibitor combination) and usually carrying *sul*1, bla_{TEM}, *tet*A, $bla_{\text{CTX-M}}$, $bla_{\text{OXA-1}}$, and *aad*A1 genes. Norfloxacin and colistin-sulfate displayed a potent in-

vitro antimicrobial activity toward the emerging XDR *P. aeruginosa* strains. The synergistic application of traditional and molecular diagnostic assays is a precise epidemiological tool for the investigation of *P. aeruginosa*. Worryingly, the existence of XDR *P. aeruginosa* strains is reflected as a public health threat. The evolution of XDR *P. aeruginosa* strains consequently recommends the reliable conducting of sensitivity tests and the restricted use of antibiotics in poultry farms and the health sector.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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