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Original Article

The impact of quorum sensing and biofilm formation on antimicrobial resistance and virulence of XDR and MDR *Pseudomonas aeruginosa* in laying chickens

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Abstract

Background: *Pseudomonas aeruginosa* plays a major influence on poultry outbreaks. Several factors may contribute to its pathogenicity. **Aims:** This study aimed to investigate the prevalence of *P. aeruginosa* infection among layer chickens with phenotypic and genotypic characterization of the isolates. **Methods:** Samples (n=160) were collected from respiratory distressed layer chickens according to the lesion and bacteriologically examined for isolation of *P. aeruginosa* from Sharkia province, Egypt. The antimicrobial sensitivity was performed against 18 antimicrobial agents. A qualitative assessment of biofilm production was performed using the Tube method. The isolates were genetically examined for confirmation, detection of quorum sensing genes, virulence genes, and biofilm production genes by conventional PCR. **Results:** *P. aeruginosa* was isolated from 25% of the samples. Moreover, 95% of the isolates were extensively drug-resistant (XDR) with multiple antibiotic resistance indices (MARI) of 0.67 to 0.83. A total of 38 isolates were able to produce biofilm with different degrees. PCR of *16S rRNA* (*P. aeruginosa*) and *opr*L genes confirmed the existence of *P. aeruginosa* isolates. For quorum sensing genes, *lasI* and *lasR* were successfully amplified at 100% and 89.5%, respectively. For virulence genes, *tox*A and *exo*U were amplified by a percentage of 78.9%, while the *higBA* gene was in 100% of the isolates. *ppr*A and *ppr*B genes were amplified at 100% and 89.5%, respectively. For biofilm genes, *psl*A, *fli*C, and *pel*A were amplified in 100%, 84.2%, and 10.5%, respectively. **Conclusion:** A strong correlation between quorum sensing genes, biofilm genes, and virulence genes was detected. Further, biofilm production increases the resistance of the isolates to antimicrobial agents.

Key words: Antibiotic resistance, Biofilm, *Pseudomonas aeruginosa*, Virulence, XDR

Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacteria that may affect birds and cause septicemia, respiratory infections, and other disorders which cause high mortality rates in newly hatched chicks and mass embryo deaths at a later stage, in addition to enormous financial losses to the farm (Elsayed *et al*., 2016). After colonization, *P. aeruginosa* can create many extracellular products that induce severe tissue destruction, blood invasion, and dispersion, contributing to its higher mortality rate compared to other Gram-negative infections (Ali *et al*., 2009).

According to Thi *et al*. (2020), *P. aeruginosa* exhibits the capability to form biofilms that serve as a protective barrier against external environmental pressures, hinder phagocytosis, and consequently enable the organism to establish long-term colonization. The dense extracellular matrix of biofilms is a significant contributor to the drug resistance of *P. aeruginosa*. This phenomenon reduces the effectiveness of antibiotics and detergents, thereby underscoring the crucial role played by biofilm in this regard (Yan and Wu, 2019).

The complete eradication of *P. aeruginosa* is challenging due to its antibiotic resistance, as noted by Pang *et al*. (2019). *P. aeruginosa* poses a substantial threat to public health due to its intrinsic resistance to multiple antibiotics and its capacity to immediately

acquire antibiotic resistance determinants, as stated by Roulová *et al*. (2022). These mechanisms include reduced permeability of the outer membrane, the presence of antibiotic resistance genes, and the activity of active efflux pumps (Algammal *et al*., 2023). As per the findings of Langendonk *et al*. (2021), the World Health Organization has classified *P. aeruginosa* as a priority one pathogen, and the emergence of multidrug-resistant (MDR) strains has resulted in a crucial demand for new therapies.

Despite the accuracy of traditional microbiological procedures, identifying *P. aeruginosa* from clinical and environmental samples might take several days and extensive hands-on work by technicians. PCR allows accurate, specific, sensitive, rapid, and complete identification and characterization of *P.aeruginosa* (Khattab *et al*., 2015; Gholami *et al*., 2016; Abd El-Ghany, 2021). *P. aeruginosa* produces many cell-associated and extracellular virulence agents to aid infection. Quorum sensing (QS) regulates several virulence factors. QS allows bacteria to communicate by producing small diffusible chemicals that neighboring species can detect (Smith and Iglewski, 2003). *P.aeruginosa* has two QS systems, *las* and *Rhl*. *Las*I/R regulates *rhl*I-*rhl*R transcription. *las*I controls the production of N-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL), which impasses, triggers, and activates *Las*R (Latifi *et al*., 1996). QS systems control exotoxin A, alkaline protease, elastase, and pyocyanin synthesis in *P. aeruginosa*. QS regulates biofilm formation, reinforcing the bacteria for host defenses, and promoting bacterial pathogenicity (Steindler *et al*., 2009). Toxin/antitoxin (TA) systems significantly affect persister formation within bacterial organisms (Zhou *et al*., 2021). The TA system is comprised of a stable toxin and a labile antitoxin. The toxin hinders crucial bacterial physiological processes, including DNA replication, transcription, protein synthesis, cell wall synthesis, cell division, or membrane potential reduction, thereby suppressing bacterial growth (Zhou *et al*., 2021). TA systems induce virulence and biofilm development (Li *et al*., 2023). Multiple Two-component systems (TCSs) and regulatory genes make up the *P. aeruginosa* genome (Rodrigue *et al*., 2000). Due to its huge genome and core-essential genes, *P. aeruginosa* is resistant and virulent (Poulsen *et al*., 2019).

P. aeruginosa's outer membrane proteins OprI and OprL influence its interaction with the surroundings and antibiotic resistance. Efflux transport mechanisms impact cell permeability via these outer membrane proteins. These proteins can quickly identify *P. aeruginosa* in clinical specimens due to their limited abundance (Noomi, 2018). *P. aeruginosa* expresses several virulence factors that contribute to its pathogenicity and toxicity, including exotoxin A (t*oxA* gene), exoenzyme S (*exo*S gene), and exoU (*exo*U gene) (Amirmozafari, 2016; Fadhil *et al*., 2016). Exotoxin A (*tox*A) inhibits protein production (Michalska and Wolf, 2015). *Pseudomonas aeruginosa* secretes elastase (*Las*B) and alkaline protease (*Apr*A) to avoid the host immunological response (Jing *et al*., 2021).

This study aimed to investigate the prevalence of *P. aeruginosa* infection among layer chickens and identify antimicrobial sensitivity and biofilm formation of the isolated bacteria. Molecular characterization was performed to detect some quorum sensing genes, virulence genes, and biofilm production genes of the isolates by conventional PCR.

Materials and Methods

Animal ethics

The Animal Ethics Review Committee of Suez Canal University (AERC-SCU 2023055) in Egypt governed every procedure, including chicken handling and experimental methods performed by well-trained scientists.

Sampling

A total of 160 specimens, including liver, heart, lung, and spleen were collected from 40-layer chickens of different ages in Sharkia province, Egypt. The birds suffered from respiratory distress. Samples were collected aseptically in sterile plastic separate bags, marked, and transferred in an ice box to the reference laboratory for veterinary quality control on poultry production in Sharkia province as quickly as possible for bacteriological examination.

Isolation and biochemical identification of *P. aeruginosa*

Isolation and identification of *P. aeruginosa* were performed according to Quinn *et al*. (2002). One gram of each sample was inoculated in a tube containing 9 ml buffered peptone water (BPW) (Oxoid, CM0509) and incubated at 37°C for 24 h. Then, a loopful BPW was streaked on 5% sheep Tryptose Blood Agar (Difco), MacConkey agar (Oxoid, CM0007), and Pseudomonas agar (HIMEDIA, M085) plates and incubated at 37°C for 24-48 h. Suspected colonies from different media were picked up and subjected to morphological and biochemical identification according to Koneman *et al*. (1997) and Quinn *et al*. (2002). The identified isolates were stored in brain heart infusion broth (BHI) with 20% glycerol (LAB M-LAB 49) at -80°C for further investigations. All recovered isolates were confirmed genetically using *Pseudomonas* spp. *16S rRNA*. *P. aeruginosa* confirmation was conducted genetically using a species-specific primer set targeting the *16S rRNA* and *opr*L gene (Table 1).

Antimicrobial sensitivity test

The antibiogram of the isolates was analyzed by the Kirby-Bauer disk diffusion test according to CLSI (2020), as illustrated in Supplementary Table 1 (ST1) with a total of eighteen antimicrobials of ten classes. The eighteen antimicrobial discs (Oxoid, UK) were; ampicillin (AM/10 µg), amoxicillin (AX/10 µg),

Table 1: The sequences of oligonucleotide primers used for confirmation of the isolates, biofilm production, and virulence genes

Gene type	Genes	Oligonucleotides sequences	Amplified product (bp)	PCR conditions (35 cycles)			References
				Denaturation	Annealing	Extension	
Confirmatory gene	16S rRNA (*Ps.* spp.)	F:GACGGGTGAGTAATGCCTA R:CACTGGTGTTCCTTCCTATA	618	94°C 5 min	50°C 40 s	72°C 45 s	Spilker *et al*. (2004)
	16S rRNA (*P. aeurginosa*)	F:GGGGGATCTTCGGACCTCA R:TCCTTAGAGTGCCCACCCG	956	94°C 5 min	52°C 40 s	72°C 50 s	Spilker *et al*. (2004)
Virulence genes	*oprL*	F:ATGGAAATGCTGAAATTCGGC R:CTTCTTCAGCTCGACGCGACG	504	96°C 1 min	55°C 1 min	72°C 1 min	Xu *et al*. (2004)
	toxA	F:GACAACGCCCTCAGCATCACCAGC R:GACAACGCCCTCAGCATCACCAGC	396	94°C 5 min	55°C 40 s	72°C 40 s	Matar *et al*. (2002)
	lasB	F:ACAGGTAGAACGCACGGTTG R:ACAGGTAGAACGCACGGTTG	1220	94°C 5 min	57°C 40 s	72°C 1 min	Finnan *et al*. (2004)
	*exo*U	F:ATGGAAATGCTGAAATTCGGC R:CTTCTTCAGCTCGACGCGACG	134	94°C 5 min	55°C 40 s	72°C 30 s	Winstanley *et al*. (2005)
Quorum sensing genes	*lasI*	F:CGTGCTCAAGTGTTCAAGG R:TACAGTCGGAAAAGCCCAG	291	95°C 40 s	60°C 1 min	72°C 2 min	Bratu *et al*. (2006)
	lasR	F:AAGTGGAAAATTGGAGTGGAG R:GTAGTTGCCGACGACGATGAAG	130	95°C 40 s	60°C 1 min	72°C 2 min	Bratu *et al*. (2006)
Toxin/antitoxin system	*higBA*	F:CTCATGTTCGATCTGCTTGC R:CAATGCTTCATGCGGCTAC	469	94°C 60 s	49°C 1 min	72°C 90 s	Williams *et al*. (2011)
Two-component regulatory system	*ppr*A	F:TGCTGTTGGGCATGGACATCT R:AGACGCGAAAGGATCAGCTT	80-200	95°C 15 s	68°C 30 s	72°C 30 s	Skindersoe *et al*. (2008)
	*ppr*B	F:TCAAGTACGAGGTACACGGCAACA R:TATCTGGTAGTTGGTCAGGCCCTT		95°C 15 s	71°C 30 s	72°C 30 s	
Biofilm genes	*psl*A	F:TCCCTACCTCAGCAGCAAGC R:TGTTGTAGCCGTAGCGTTTCTG	656	94°C 5 min	60°C 40 s	72°C 45 s	Ghadaksaz *et al*. (2015)
	*pel*A	F:CATACCTTCAGCCATCCGTTCTTC R:CGCATTCGCCGCACTCAG	786	94°C 5 min	60°C 40 s	72°C 45 s	
	fliC	F:TGAACGTGGCTACCAAGAACG R:TCTGCAGTTGCTTCACTTCGC	180	94°C 5 min	56.2°C 30 s	72°C 30 s	

apramycin (APRA/15 µg), ciprofloxacin (CIP/5 µg), neomycin (N/10 µg), gentamycin (CN/30 µg), trimethoprim-sulfamethoxazole (SXT/25 µg), ceftriaxone (CTX/30 µg), cephradine (CE/30 µg), norfloxacin (NOR/10 µg), erythromycin (E/15 µg), streptomycin (S/10 µg), spiramycin (SP/100 µg), colistin sulfate (CT/10 µg), fosfomycin (FF/50 µg), florfenicol (FFC/10 µg), tetracycline (TE/30 µg), and doxycyclin (DO/30 µg). The test results were interpreted according to CLSI (2020). Control was *P. aeruginosa* ATCC 27853. Extensively drug-resistant (XDR) or multidrug-resistant (MDR) was grouped according to Magiorakos *et al*. (2012).

Phenotypic characterization of biofilm production

All isolates were assessed qualitatively for their abilities to produce biofilm using the Tube method (TM) (Christensen *et al*., 1982).

Molecular characterization of isolated *P. aeruginosa*

Nineteen representative isolates were subjected to molecular examinations for monitoring of QS (*lasI*, and *las*R) genes, virulence genes (*oprL*, *toxA*, *exoU*, and *lasB*), toxin/antitoxin system (*hig* BA), two-component regulatory system (*ppr*A, and *ppr*B) and biofilm production genes (*pslA*, *pela*, and *fliC*) by specific primers (Table 1). DNA extraction was performed by QIAamp DNA mini kit instructions (Life Technologies, Renfrew, UK/ at. No. K182001). The positive control was supplied by the Animal Health Research Institution (AHRI), Doki, Egypt, and negative control (DNA-free reaction) was applied for each reaction. PCR products were separated by gel electrophoresis and photographed with a gel documentation system.

Statistical analysis

Using R-software (version 4.0.3; https://www.r-project.org/), a Chi-square test was performed on the frequency distributions of the data ($P<0.05$ indicates statistical significance). Also, it was used to evaluate relationships between the tested antimicrobials and the resistance genes.

Results

Prevalence of *Pseudomonas* among the examined samples

A total of 40 *Pseudomonas* isolates were recovered from 160 examined samples (25%). The heart and lung samples showed the highest number of isolates, while liver samples were the least (Table 2). Suspected *Pseudomonas* isolates were identified phenotypically by Gram stain, motility test, and biochemically. The prevalence among examined organs did not show a significant difference. Species-specific 16 srRNA and *opr*L genes were successfully amplified in all isolates.

Table 2: Distribution of *Pseudomonas* isolates in examined chicken organs

Sample type	Sample No.	No. of +ve samples	%*
Heart	40	13	32.5
Lung	40	13	32.5
Spleen	40	12	30
Liver	40	2	5
Chi-square		8.6	
P-value		0.03511^{NS}	

* Percentage according to the total number of each source of the sample. NS= Non significant, and +ve: Positive

Antibiogram test of *Pseudomonas* isolates

The most powerful antimicrobial agent was colistin sulfate with a percentage of 95%, while complete resistance (100%) was detected against 7 antimicrobial agents with variation in sensitivity to other tested antimicrobial agents (Table 3). There was a significant difference in the antimicrobial sensitivity patterns of various examined antimicrobials.

Multiple antibiotic resistance patterns of *P. aeurginosa*

Of note, 95% (38/40) of the tested *P. aeurginosa* isolates were XDR that showed resistance against 12-15 antimicrobials with multiple antibiotic resistance indices (MARI) indices of 0.67 to 0.83, respectively. Moreover, 5% (2/40) of *P. aeurginosa* isolates were resistant to 7 antimicrobials, with a MAR index over 0.4 (Table 4).

Qualitative assessment of biofilm production

Out of 40 isolates tested for biofilm production by Tube method, 18 (45%) were strongly positive, 14 (35%) were moderately positive, 6 (15%) were weak and 2 (5%) did not show any biofilm formation.

Molecular characterization of *P. aeruginosa* isolates

For QS genes, *las*I was successfully amplified in all isolates (19/100%), while *las*R was detected in 17/89.5% of the isolates. For virulence genes, only 15 strains were positive for *tox*A and *exo*U by a percentage of 78.9%. *hig*BA gene was successfully amplified in all isolates (19/100%). *ppr*A and *ppr*B were amplified with a percentage of 89.5% and 100%, respectively. For biofilm genes, all 19 isolates (100%) were positive for the *psl*A gene and 16 (84.2%) isolates were positive for *fli*C and only two isolates (10.5%) were positive for *pel*A (Table 5), (Figs. 1a and b, ST1). There was a significant difference among recovered isolates in virulence and biofilm genes. QS gene (*las*R) significantly affected *psl*A (biofilm gene), *las*B, *hig*BA, *ppr*A, and *ppr*B by simple regression (P<0.05).

Association between biofilm formation, biofilm genes, and phenotypic antibiotic resistance

The biofilm formation and XDR capacity were analyzed using the Tube method which showed that there was a significant relationship between biofilm formation and XDR properties (X^2=5.8947) (Table 6). Considering the biofilm genes and phenotypic resistance, there was a strong relationship between *psl*A and *fli*C genes and XDR properties, while there was no relationship between *pel*A gene and XDR (Fig. 2). There was a significant affected (P<0.05) of biofilm formation on AX, AM, SP, CE, S, K, FF, SET, TE, DO, CTX by simple regression (rs=1).

Table 3: The results of the antimicrobial sensitivity test of the recovered isolates

Antimicrobial classes	Antimicrobial agents (Conc.)	Resistance		Intermediate		Sensitive	
		No.	%	No.	%	No.	%
Penicillin	AX (10 µg)	40	100	0	0	0	0
	AM (10 µg)	40	100	0	0	0	0
Aminoglycoside	APRA (15 µg)	22	55	18	45	0	0
	CN (30 µg)	38	95	2	5	0	0
	N (10 µg)	28	70	10	25	2	5
	S (10 µg)	40	100	0	0	0	0
Cephalosporin	CTX (30 µg)	38	95	2	5	0	0
	CE (30 µg)	40	100	0	0	0	0
phosphonic acids	FF (50 µg)	40	100	0	0	0	0
Fluoroquinolone	CIP (5 µg)	6	15	10	25	24	60
	NOR (10 µg)	8	20	4	10	28	70
Polymixin	CT (10 µg)	2	5	0	0	38	95
Macrolide	E (15 µg)	36	90	4	10	0	0
	SP (100 µg)	40	100	0	0	0	0
Amphenicols	FFC (10 µg)	30	75	4	10	6	15
Sulphonamide	STX (25 µg)	40	100	0	0	0	0
Tetracycline	TE (30 µg)	38	95	0	0	2	5
	DO (30 µg)	38	95	0	0	2	5
Chi-square		91.66		139.33		401.29	
P-value		P>0.0001		P>0.0001		P>0.0001	

Conc.: Concentration, No.: Number, AM: Ampicillin, AX: Amoxicillin, APRA: Apramycin, CIP: Ciprofloxacin, N: Neomycin, CN: Gentamycin, SXT: Trimethoprim-Sulfamethoxazole, CTX: Ceftriaxone, CE: Cephradine, NOR: Norfloxacin, E: Erythromycin, S: Streptomycin, SP: Spiramycin, and CT: Colistin sulfate

Table 4: The *Pseudomonas* isolates' phenotypic antimicrobial-resistance patterns

No. of strains	(%)	Pattern of resistance	Phenotypic multi-drug resistance	*MAR index
22	(55)	XDR	8 classes and 15 antimicrobials Penicillins: AX, AM Aminoglycosides: APRA, CN, N, S Cephalosporin: CTX, CE Phosphonic acid: FF Macrolides: E, SP Amphenicols: FFC Sulfonamides: STX Tetracyclines: TE, DO	0.83
8	(20)	XDR	8 classes and 14 antimicrobials Penicillins: AX, AM Aminoglycosides: CN, S Cephalosporin: CTX, CE Phosphonic acid: FF Fluroquinolones: NOR Macrolides: E, SP Amphenicols: FFC Sulfonamides: STX Tetracyclines: TE, DO	0.77
6	(15)	XDR	8 classes and 14 antimicrobials Penicillins: AX, AM Aminoglycosides: CN, N, S Cephalosporin: CTX, CE Phosphonic acid: FF Fluroquinolones: CIP Macrolides: E, SP Sulfonamides: STX Tetracyclines: TE, DO	0.77
2	(5)	XDR	8 classes and 12 antimicrobials Penicillins: AX, AM Aminoglycosides: CN, S Cephalosporin :CTX, CE Phosphonic acid: FF Polymixin: CT Macrolides: SP Sulfonamides: STX Tetracyclines: TE, DO	0.67
2	(5)	MDR	6 classes and 7 antimicrobials Penicillins: AX, AM Aminoglycosides: S Cephalosporin: CE Phosphonic acid: FF Macrolides: SP Sulfonamides: STX	0.39

* Multiple antibiotic resistance

Table 5: Distribution of virulence and biofilm genes in *P. aeruginosa* isolates

Genes		No. of *P. aeruginosa* isolates	%*	Chi-square P-value
Virulence genes	*opr*L	19	100	18.914
	*tox*A	15	78.9	0.01532
	*exo*U	15	78.9	
	*las*B	0	0	
Quorum sensory genes	*las*I	19	100	
	*las*R	17	89.5	
Toxin/antitoxin system	*hig*BA	19	100	
Two-component regulatory system	*ppr*A	17	89.5	
	*ppr*B	19	100	
Biofilm genes	*Psl*A	19	100	13.351
	*Fli*C	16	84.2	0.001261
	*Pel*A	2	10.5	

* Percentage according to the total number of confirmed PCR isolates (n=19)

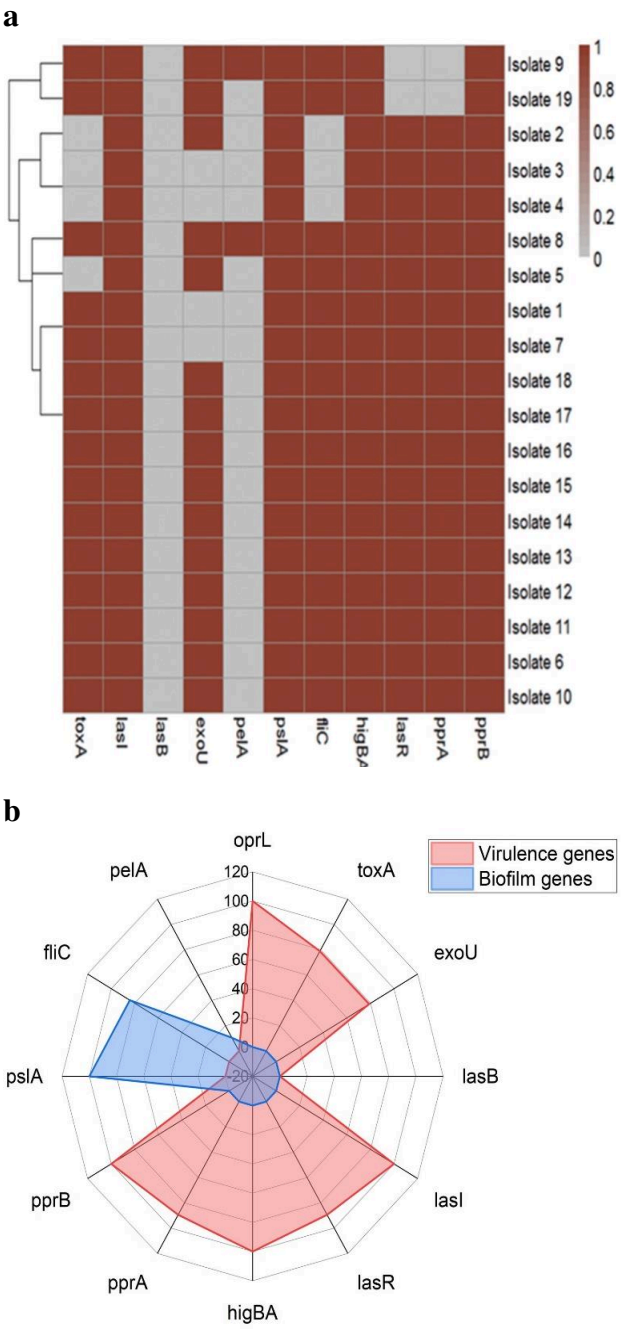

Fig. 1: The prevalence and distribution of virulence and biofilm genes between isolates. **a**) Heat-map showing the distribution of the virulence, and biofilm genes among the examined isolates. Dark red squares indicate the presence of genes; gray squares indicate an absence of genes, and (**b**) The radar chart illustrates the prevalence of various genes

Table 6: Association between biofilm formation and phenotypic antibiotic-resistant

Biofilm formation	XDR	MDR	Total
Strong biofilm producers	18	0	18
Moderate biofilm producers	14	0	14
Weak biofilm producers	6	0	6
Non-biofilm producers	0	2	2
Total	38	2	40

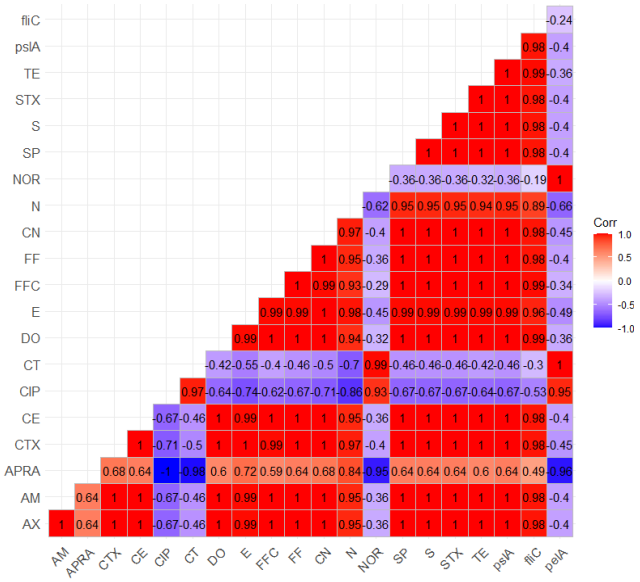

Fig. 2: Association between the phenotypic antimicrobial resistance, virulence, and biofilm genes

Discussion

Pseudomonas is an infection often found in the environment and can be an important concern in chicken farms (Badr *et al*., 2020). *P. aeruginosa* is associated with infections in both humans and animals (El-Demerdash and Bakry, 2020). In this study, *P. aeruginosa* was recovered from 25% of the samples collected from layer chickens. This prevalence is relatively higher than that obtained by Donnik *et al*. (2020), who isolated *P. aeruginosa* from oral and cloaca swabs of broiler chickens and laying hens. Approximately, 18.6% of all opportunistic pathogens found in chicken farms were *P. aeruginosa*. Settings, immunity, contamination degrees, strain type, and virulence play important roles in *P. aeruginosa* prevalence (Bakheet and Torra, 2020). In the present study, the clinical manifestations were observed in the affected birds and considered secondary to other viral or bacterial respiratory infections, similar to Badr *et al*. (2020), who recorded that severely stressed or immunodeficient birds and concurrent infections with viruses and other bacteria enhance susceptibility to *Pseudomonas* infection. In addition, inflammatory changes such as pericarditis, perihepatitis, and deposits of cheesy materials on air sacs and peritoneal cavities were seen in the autopsied birds; this is in accordance with Shukla and Mishra (2015). *P. aeruginosa* can inhibit cytokine production, reducing the host's ability to clear infection, which enhances the induction of variable clinical lesions in different organs (Hassan *et al*., 2020). This study showed the percentage of *P. aeruginosa* recovered from different organs as illustrated in Table 2 is substantially identical to that of Shukla and Mishra (2015), who observed that the highest rate of *P. aeruginosa* isolation was from the liver, lung, air sacs, nasal swab, and heart, except for the liver. On the contrary, Abd El-Tawab *et al*. (2014) reported a high

prevalence of *P. aeruginosa* from the liver, while heart samples were negative, and El-Demerdash *et al*. (2020) recorded the highest rate of *P. aeruginosa* from the liver and lowest rate from the heart. All recovered isolates showed the typical colonial characteristics of *pseudomonas* species and were observed as non-lactose fermenters on MacConkey's agar, with blue-green colonies on *Pseudomonas* agar. Biochemical reactions and beta hemolysis on sheep blood agar were consistent with those of pseudomonads (Quinn *et al*., 2002; Farghaly *et al*., 2017).

For antimicrobial sensitivity, the isolates showed complete resistance (100%) to ampicillin, amoxicillin, streptomycin, cephradine, spiramycin, fosfomycin, and trimethoprim-sulfamethoxazole and strong resistance (90-95%) against gentamycin, ceftriaxone, erythromycin, tetracyclin, and doxycycline, where 95% of the isolates were XDR. The isolates showed sensitivity to colistin sulfate. These results are in agreement with Shahat *et al*. (2019) who reported complete resistance of *P. aeruginosa* isolates from broiler chickens to penicillin, ampicillin, tetracycline, sulphamethazone, and erythromycin and sensitivity to ciprofloxacin and norfloxacin. Eraky *et al*. (2020) reported resistance of *P. aeruginosa* (100%) to erythromycin, doxycycline, and amoxicillin/clavulanic acid. In the same line, Tawakol *et al*. (2018), and Hassan *et al*. (2020) isolated *P. aeruginosa* from broiler chickens that were 100% sensitive to colistin sulfate and 100% resistant to doxycycline, ampicillin, cefoxitin, and sulfamethoxazole-trimethoprim. MDR and XDR *P. aeruginosa* strains were reported in many studies (Chakraborty *et al*., 2020; Dong *et al*., 2022; Algammal *et al*., 2023). The high resistance of *P. aeruginosa* to most antibiotics may be attributed to the low permeability of the bacterial outer membrane lipoprotein (oprL) and the ability of some bacteria to produce antibiotic-inactivating enzymes like β-lactamases (Abd El-Ghany, 2021). A few antibiotics can cross the outer membrane via a porin channel, which explains why certain *Pseudomonas* spp. are sensitive to them. Most human *Pseudomonas* infection is related to occupational contact with poultry and its products and the emergence of multidrug-resistant bacteria is considered a serious public health threat that leads to high morbidity and mortality mainly in patients admitted to intensive care units (Morales *et al*., 2016).

Biofilm microorganisms are resistant to disinfectants, antibiotics, and the host immune system, thereby, as a challenge to eradicate. They disrupt medicine, agriculture, the food industry, and the household environment (Meliani and Bensoltane, 2015). In the current study, 95% of isolates were biofilm producers with different degrees (strong, moderate, and weak) by Tube method. This result is consistence with Tawakol *et al*. (2018) who recorded that all isolates of *P. aeruginosa* recovered from broilers were biofilm producers by the TM technique. Mohamed *et al*. (2022) recorded that 83% of *P. aeroginosa* isolated from broilers chickens were able to produce biofilm. The capacity of bacteria to produce biofilm is due to the primary production of exopolysaccharide (EPS) that promotes preserving micro-colonies and building up resistance to various environmental stresses and disinfectants. *fil*C and *psl*A genes correlate positively in a strong way with phenotypic antimicrobial resistance, as shown in Fig. 2, attributed to bacteria grown in a biofilm being much more resistant to antibiotics and other chemotherapeutics than bacteria grown in planktonic form (Khan *et al*., 2014).

Conventional *P. aeruginosa* identification requires time and technician labor (Qin *et al*., 2003). *P. aeruginosa* is also misidentified with other Gram-negative bacilli (Al-Ahmadi and Roodsari, 2016). Thus, polymerase chain reaction assists in recognizing *P. aeruginosa* quickly and accurately (Anuj *et al*., 2009). *opr*L gene encodes a protein in the inner and outer membranes that are necessary for epithelial cell invasion and can be used in identifying *P. aeruginosa* in clinical specimens due to their limited abundance (Abd EL-Dayem *et al*., 2021). As mentioned in Table 5, all recovered isolates were positive for *opr*L gene. For QS genes, *las*I was successfully amplified in all isolates (19/100%), while *las*R was in 17/89.5%. These results are in agreement with Bakheet and Torra (2020), who recorded *las*I and *las*R genes with the percentage of 100% and 75%, respectively. Quorum sensing systems aid *P. aeruginosa* spread and result in septicemia, acute and chronic lung infections, and deaths by connecting to eukaryotic cells and encouraging virulence factors production (Smith and Iglewski, 2003). There is a significant strong effect of QS gene (*las*R) on *psl*A (biofilm gene), *las*B, *hig*BA, *ppr*A, and *ppr*B by simple regression (rs=1) as *P. aeruginosa*'s QS system regulates virulence factor synthesis and pathogenic characteristics including tissue attachment and biofilm formation. Deletions of one or more QS genes result in a reduction in the virulence of *P. aeruginosa* (Sonbol *et al*., 2022).

Regarding the Biofilm genes, all recovered strains were positive for the *psl*A gene, 16 (84.2%) strains were positive for *fli*C gene and only 2 strains were positive for *pel*A gene by a percentage of 10.5%; this agrees with Eraky *et al*. (2020), who detected *P. aeruginosa psl*A gene in all isolated strains and *fli*C in 84.2% of isolates. These results varied greatly from that recorded by Tawakol *et al*. (2018), who detected *psl*A, *pel*A, and *fli*C genes in all of the examined samples, and Ghadaksaz *et al*. (2015), who detected *psl*A, *pelA*, and *fli*C genes with percentages of 45.2%, 83.7%, and 70.2%, respectively in *P. aeruginosa* isolated from clinical samples. The difference in results between different studies may be attributed to many factors such as the difference in type and number of samples collected in each study. Also, it may be due to differences in isolate's ability to form a biofilm (Abdelraheem *et al*., 2020). A total of 15 isolates were positive for *tox*A gene and *exo*U gene by a percentage of 78.9%, this is in accordance with Shahat *et al*. (2019), who recovered seven *P. aeruginosa* isolates that have different virulence genes such as *tox*A, *exo*U genes with an incidence rate of 71.42% for each of them.

Bakheet and Torra (2020) results that PCR-confirmed virulence genes in all *P. aeruginosa* isolates are in line with our findings. Exotoxin A (*tox*A), is considered the most toxic virulence factor contributing to *P. aeruginosa* pathogenicity (Dong *et al*., 2015), inhibits protein biosynthesis; it has a necrotizing activity on tissues, causing cell death and contributing to the colonization process (Michalska and Wolf, 2015). The abundance of *tox*A gene among *P. aeruginosa* clinical isolates demonstrates the crucial involvement of this virulence factor in chicken respiratory diseases (Tartor and El-Naenaeey, 2016). After entering the cytoplasm, *Exo*U disrupts eukaryotic membranes via phospholipase activity (Bahador *et al*., 2019). *Hig*BA was amplified successfully in all tested isolates (19/100%). *Hig*BA was the first TA system identified in *P. aeruginosa* clinical isolates. Pyocyanin, pyochelin, swarming motility, and biofilm formation are regulated by *Hig*B (Wood *et al*., 2016). TCSs genes (*ppr*A and *ppr*B) were amplified with percentages of 100% and 89.5%, respectively. TCSs use signal-response coupling to assist bacteria detect and respond to signals in varied environments. A previous investigation suggested that TCSs regulate *P. aeruginosa* virulence factors (Francis *et al*., 2017). Our results showed that there was a significant relationship between biofilm formation and antibiotic resistance, in line with (Yekani *et al*., 2017; Karami *et al*., 2020) and contrary to (Gajdács *et al*., 2021) who detected no correlation between biofilm formation, and antibiotic resistance. There was a statistically significant association identified in the current study between the antibiotic resistance pattern and the QS. Similar findings have been reported by (Haque *et al*., 2018). This could be explained by the QS system-regulated drug efflux pump and biofilm formation, which are thought to be significant mechanisms of antibiotic resistance in *P. aeruginosa* (Bäuerle *et al*., 2018; Zhao *et al*., 2020).

Multidrug-resistant *P. aeruginosa* infection is an essential concern in layers. The virulence and resistance to antimicrobial drugs and detergents that *P. aeruginosa* develops as an outcome of its QS system and biofilm formation are of increased concern. Eliminating the disease from chicken farms requires a reduction in virulence and drug resistance, both of which can be achieved by disrupting the QS system and biofilm multilayers.

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Conflict of interest

The authors declare that they have no conflict of interest.

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