JOURNAL OF ADVANCED MEDICAL AND PHARMACEUTICAL RESEARCH

Research Article

Potential antivirulence activity of sub-MIC of aspirin either alone or combined with certain antibiotics against *Pseudomonas aeruginosa* isolates

Received 12th May 2024,
Accepted 27th May 2024
Published 6th June 2024

DOI: 10.21608/JAMPR.2024.288946.1071
jampr.journals.ekb.eg

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ABSTRACT

One of the main problems for public health is the emergence of multidrug resistant (MDR) *Pseudomonas aeruginosa.* This work aimed to evaluate the potential anti-virulence activities of aspirin, either alone or in combination with antibiotics, against MDR *P. aeruginosa* isolates. In the present work, antibiotic susceptibility testing revealed a high incidence of resistance among the tested *P. aeruginosa* isolates against aminoglycosides, antipseudomonal fluoroquinolones, and beta-lactam antibiotics. The MIC values of aspirin against tested isolates ranged from 1000 to ≥ 8000 µg/ml. The screening of our *P. aeruginosa* isolates for various virulence factors showed that 71% of the isolates produced biofilm. The percentage of isolates that exhibited hemolytic activity, pyocyanin production, and swarming motility was 93.5%, 71%, and 57.5%, respectively. The tested *P. aeruginosa* isolates produce different enzymes including protease (83%) and lipase (61%). Also, they harbored various toxin genes, such as *toxA* (93%), *exoT* (93%), *exoY* (87%), *exoS* (67%), and *exoU* (32%). Treatment with aspirin (1/4 MIC), either alone or in combination with various antibiotics, resulted in significant reductions (*P-value* ≤ 0.05) in pyocyanin production, biofilm production, hemolytic activity, and swarming motility among *P. aeruginosa* tested isolates. Also, the treated isolates with aspirin showed significant reductions in the expression of genes: *exoS* (51.6%), *exoY* (42.7%), and *exoT* (33.9%). In conclusion, aspirin could be repurposed as a potential anti-virulent that interferes with *P. aeruginosa* pathogenicity rather than inhibiting microbial growth.

Keywords: aspirin, biofilm, combinations, toxins, virulence.

1. INTRODUCTION

Pseudomonas aeruginosa is a common hospital-acquired pathogen. *P. aeruginosa* frequently affects hospitalized patients, especially those who are immunocompromised or have neutropenia. *P. aeruginosa* infections are frequent in intensive care units. HIV-infected individuals and patients with cystic fibrosis, especially those in advanced stages, are more vulnerable to community-acquired *P. aeruginosa* infections. Treatment of *Pseudomonas* infections becomes difficult due to the spread of multidrug resistance and the production of several virulence factors.[1] "Virulence" is a quantitative indicator of a microorganism's pathogenicity, which can be expressed as the ratio between the number of individuals exhibiting clinical

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illness and the total number of people exposed to the microorganism. These characteristics enable microorganisms to colonize a host and increase their ability to cause disease.[2]

Numerous cell-associated and secreted virulence traits mediate *P. aeruginosa* pathogenesis. The cell-associated virulence factors involve lipopolysaccharide, which suppresses the host immune response and facilitates the establishment of persistent infections;[3] secretion systems, which are implicated in the conveyance of effector macromolecules to the host cells, and flagella, which aid in motility.[4] Secreted factors like protease and elastase cause the hydrolysis of collagen and other host proteins, disrupting host tissue structure,[5] while low-molecular-weight toxins, including exotoxin A, target several sites of the cell machinery.[6] Quorum sensing is a communication mechanism between cells that involves the production of small chemicals, through which bacteria can detect their population density. *Three QS systems are present in P. aeruginosa: LasI-LasR* and *RhlI-RhlR*, two LuxI/LuxR-type QS circuits that regulate virulence factor expression, and the *Pseudomonas* quinolone signal (PQS) system, a third system that is non-LuxI/LuxR-type.[7]

An important strategy for combating disease caused by certain pathogens is to interfere with pathogenesis. The antivirulence strategy is expected to reduce the pathogen's capacity to cause disease instead of suppressing growth.[8] It has been reported that some natural and synthetic compounds have antivirulence properties. Aspirin is commonly prescribed as an analgesic and antipyretic agent. It was found that the treatment of endocarditis with intravenous aspirin significantly reduced bacterial densities in target tissues (kidneys and vegetation).[9] Our study explored the antivirulence activity of aspirin at 1/4 MIC, either alone or in combination with several antibiotics, against *P. aeruginosa*.

2. METHODS

2.1. Collection of tested bacterial isolates

One hundred *P. aeruginosa* isolates were collected from the culture collection of Microbiology and Immunology department, Faculty of Pharmacy, Tanta University. Various clinical samples were also collected from outpatients and inpatients in different departments of Tanta University Hospital. Each clinical specimen was inoculated on MacConkey agar as well as *Pseudomonas* cetrimide agar. The recovered colonies were further identified morphologically and biochemically. The reference strain employed was *P. aeruginosa* (ATCC 27829).

2.2. Susceptibility of *P. aeruginosa* isolates to aspirin and various antibiotics

Antimicrobial susceptibility testing of all *P. aeruginosa* isolates against aspirin and 14 antibiotics representing 8 different classes was performed using the following authentic powders from Oxoid, UK: aspirin (Asp), amikacin (AK), aztreonam (ATM), cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (CT), fosfomycin (FOS), gentamicin (CN), imipenem (IPM), levofloxacin (LEV), meropenem (MEM), piperacillin/tazobactam (TZP), polymyxin b (PB), and tobramycin (TOB). The minimum inhibitory concentration (MIC) determination of aspirin and antibiotics against *P. aeruginosa* isolates was performed by the agar dilution method, except for colistin and polymyxin B, where the broth microdilution method was used to determine their MIC values.[10] MDR **(**multidrug-resistant) isolates were identified as exhibiting resistance to one or more antibiotics in three or more antimicrobial classes.

2.3. Calculation of the Multiple antimicrobial resistance (MAR) indices

The MAR indices of bacterial isolates were calculated using the subsequent equation based on their resistance patterns: [12]

$$\text{MAR index} = \frac{\text{Number of antibiotics to which the isolate was resistant}}{\text{Total number of antibiotics to which the isolate was subjected}}$$

2.4. Screening of virulence factors among all tested isolates

Biofilm formation was investigated by inoculating the tested isolates onto congo red agar plates. Black colonies indicate biofilm formation.[13] The isolates were inoculated on *Pseudomonas* cetrimide agar for visual analysis of bluish-green pyocyanin production.[14] Hemolytic activity was investigated by inoculating isolates on 5% blood agar plates. A positive hemolytic reaction was indicated by lysis zones around the colonies.[15] The tested isolates were screened for protease enzymes by inoculating onto 10% skim milk agar plates. The protease activity was confirmed by halo zones around the bacterial colonies.[16] The tested isolates were screened for the lipase enzyme using tween 80 agar plates. Precipitation around the growth indicated lipase-producing isolates.[17] As previously mentioned,[18] the isolates were tested for the swarming migration distance assay. All plates were incubated at 37 °C for 24 and 48 hours before examination.

2.5. Screening of toxin producers among tested isolates

The Multiplex Polymerase Chain Reaction (PCR) technique was employed to screen isolates for the exoenzyme genes (*exoS, exoU, exoT*, and *exoY*), while uniplex PCR was employed to screen for the *toxA* gene. PCRs were conducted using a Thermo Fisher Thermal Cycler (USA). To extract the total DNA of the selected isolates, a number of fresh bacterial colonies were suspended in sterile water and denaturated at 98°C for 15 min, followed by centrifugation for 30 seconds at

13,000 rounds per minute (rpm). The PCR conditions include an initialization step for 5 min at 94ºC and subsequent 36 cycles of denaturation at 94ºC for 40 sec, annealing for 40 sec at 58ºC (for *exoS*, *exoU*, *exoT*, and *exoY*) and at 55ºC (for *toxA*), extension for 60 sec at 72ºC, and a final extension stage at 72ºC for 420 sec, which can be stored at 4°C.[19,20] The sequences of used primers are demonstrated in **Table 1**. A horizontal gel electrophoresis device (Mupid CO., Japan) was utilized to run the PCR results on a 1.5% agarose gel. A 100-bp DNA ladder, ZELLX (Nippon Genetics Europe GmbH, Germany), was used to determine the size of the DNA fragments.

Table 1. Sequences of PCR primers and products for the detection of exoenzymes and exotoxin A

Gene	Nucleotide sequence	Amplicon size (bp)
exo S	F- GCG AGGTCAGCAGAGTATCG R- TTC GGCGTCACTGTG GATGC	118
exo T	F- AAT CGCCGTCCAACTGCA TGC G R- TGT TCGCCGAGGTACTGCTC	152
exo U	F- CCG TTG TGGTGCCGTTGAAG R- CCA GATGTTCACCGACTCGC	134
exo Y	F- CGG ATTCTATGG CAG GGA GG R- GCCCTTGATGCACTCGACCA	289
toxA	F- GGTAACCAGCTCAGCCACAT R- TGATGTCCAGGTCATGCTTC	352

2.6. Estimation of aspirin/antibiotic combinations

The MICs determination of aspirin/antibiotic combinations against *P. aeruginosa* isolates was performed by the agar dilution method, except for aspirin/colistin and aspirin/polymyxin B combinations, where the broth microdilution method was used to determine their MIC values.[10,21] The fractional inhibitory concentration index (FICI) for each aspirin/antibiotic combination against the selected bacterial isolates was computed using the following formulas:[22]

$$\text{FICI} = \frac{\text{MIC of drug A in combination with drug B}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination with drug A}}{\text{MIC of drug B alone}}$$

The interaction was considered as antagonism (**A**) when FIC > 4, synergism (**S**) when FIC ≤ 0.5, and indifference (**I**) when FIC > 0.5 to 4.[21]

2.7. Effect of aspirin/antibiotic combinations on various virulence factors

To investigate the effect of aspirin/antibiotic combinations on *P. aeruginosa* virulence, the treatment of bacterial isolates with 1/4 MIC of aspirin and antibiotic, either alone or in combination, was done as described by Roudashti *et al.*[23]

2.7.1. Biofilm assay

The crystal violet assay was used to investigate the effect of aspirin/antibiotic combinations on the biofilm production by selected isolates.[24] One hundred µl of bacterial culture in LB medium in the absence or presence of 1/4 MIC of tested aspirin/antibiotic combinations were introduced in wells of microtitration plates and incubated for 24 hours at 37 ºC. After emptying the microtitration plates, the wells were carefully washed twice. The wells were filled with about 125 µl of crystal violet solution (0.1%) for 15 min to stain the biofilm, followed by three washings. Then, the wells were filled with 125 µl of 30% glacial acetic acid. The absorbance (OD_{550}) was measured using acetic acid solution (30%) as a blank. The biofilm production by the tested isolates was categorized following the protocol outlined previously.[25]

2.7.2. Pyocyanin assay

The method was conducted in accordance with the Essar et al.[26] P. aeruginosa cultures in LB medium, either in the presence or absence of 1/4 MIC of aspirin/antibiotic combinations, were centrifuged at 4000 rpm for 10 min. Pyocyanin was extracted by mixing 10 ml of culture supernatant with 6 ml of chloroform. After transferring the chloroform layer into a sterile tube, this layer was mixed with 3.2 ml of 1N HCl. The OD_{520} of the HCL layer was measured. The pyocyanin concentration was calculated according to the following equation:

$$\text{Pyocyanin concentration } \mu g/ml = \text{Absorbane (at 520 nm)} \times 17.07$$

2.7.3. Hemolytic activity assay

The hemolytic capacity of bacterial isolates was quantitatively evaluated.[27] Briefly, 0.6 ml of the supernatant of the bacterial culture was mixed with 0.6 ml of RBCs suspension (2%) for 2 hours at 37°C. Then, these mixtures were centrifuged at 4 °C. The hemoglobin release was measured at OD_{540}. The previous procedures were also performed using 0.6 ml sterile LB instead of bacterial supernatant (negative control) or 0.6 ml LB with 0.1% SDS (positive control). The percent hemolysis was calculated according to the following formula:

$$\% = \frac{(X - B)}{(T - B)} * 100$$

where X represents the OD_{540} of the analyzed sample, T represents the OD_{540} of the positive control, and B represents the OD_{540} of the negative control.

2.7.4. Total proteases' assay

Total proteases activity was evaluated quantitatively.[28] One milliliter of 2% casein in a 0.05 M phosphate buffer-0.1 M NaOH solution was added to 1 ml of the culture supernatant. After incubation of the mixture for 10 minutes at 37°C, 2 ml of trichloroacetic acid (0.4 M) were introduced to the reaction mixture in order to halt the reaction. The mixture was then incubated at room temperature for 30 minutes, followed by centrifugation. Five ml of sodium carbonate solution (0.4 M) and 1 ml of Folin's reagent were combined with 1 ml of the supernatant. The absorbance (OD_{660}) of the resultant blue-colored chromophore was measured. Tyrosine solutions ranging from 0 to 60 µg/ml in 0.2 N HCL were used to establish a standard curve. The amount of enzyme sufficient to generate 0.5 µg/ml of tyrosine under the same testing conditions was interpreted as one unit of protease activity. A calibration curve for tyrosine served as the basis for the estimations.

2.7.5. Lipase enzyme assay

According to Molinari *et al*.,[29] 10 microliters from treated and untreated bacterial cultures were transferred to the surface of tween 80 agar, followed by incubation at 37°C for 24 h. A precipitation zone surrounding the inoculation site indicated lipase activity. The zone of precipitation diameter (mm) was measured in treated and untreated cultures and compared.

2.7.6. Swarming motility assay

According to Diggle *et al*.,[30] overnight untreated and treated cultures (2 µl) were inoculated onto the surface of dry swarming agar plates and then incubated in an upright position for 24 h at 37°C. The swarming zone diameters were measured in millimeters (mm).

2.7.7. Expression of toxin genes

The extraction of total RNA from untreated and 1/4 MIC aspirin-treated *P. aeruginosa* isolates was performed using the Purelink® RNA Mini Kit (Thermo Scientific, USA). The Power cDNA Synthesis Kit (iNtRON Biotechnology, Korea) was used to synthesize complementary DNA. Power SYBR® Green Master Mix (Thermo Scientific, USA) was utilized, and the real-time quantitative polymerase chain reaction (RT-qPCR) was conducted in a thermocycler Rotor-Gene Q (Qiagen). Primers' sequences are described in **Table 2**. Compared with the expression of the *ropD* gene, the expression of virulence genes was comparatively normalized. Using the $2^{-\Delta\Delta CT}$ technique, the gene expression level was determined in both untreated and aspirin-treated isolates.

Table 2. RT-PCR primers and products for detection of toxin genes of the tested isolates

Gene type	Gene Symbol	Sequence (5` to 3`)	Amplicon size (bps)	Ref.
Reference gene	*ropD*	F- CGAACTGCTTGCCGACTT	131	31
		R- GCGAGAGCCTCAAGGATAC		
Virulence genes	*toxA*	F- GACAACGCCCTCAGCATCACCAG	150	32
		R- CGCTGGCCCATTCGCTCCAGCGCT		
	exoU	F- CCGTTGTGGTGCCGTTGAAG	134	32
		R- CCAGATGTTCACCGACTCGC		
	exoS	F- CCATCACTTCGGCGTCACT	129	31
		R- GAGAGCGAGGTCAGCAGAG		
	exoT	F- AATCGCCGTCCAACTGCATGCG	152	32
		R- TGTTCGCCGAGGTACTGCTC		
	exoY	F- TGCCATAGAATCCGTCCTC	145	31
		R- GATGACCGCCGATTATGAC		

2.8. Statistical analysis

Statistical Package for the Social Sciences (SPSS) software version 27.0 (IBM Corp., Armonk, NY, USA) was used to analyze the obtained results statistically. The information was displayed as mean ± standard deviation. The two groups were compared using the student's t-test. All *P*-values were two-tailed. The *P*-value was considered statistically significant and highly significant when it was less than 0.05 and 0.001, respectively.

3. RESULTS

3.1. Bacterial strains

Three hundred twelve *P. aeruginosa* isolates were recovered from the outpatient clinic and inpatients of different departments of Tanta University Hospital, as well as from the culture collection of the Microbiology and Immunology Department, Faculty of Pharmacy, Tanta University. Bacterial isolates grown on *Pseudomonas* cetrimide agar, that were gram-negative and oxidase-positive were identified as *P. aeruginosa.* The collected isolates were stored at -80°C in TSB containing 10% (v/v) glycerol for further studies.

3.2. Susceptibility of *P. aeruginosa* isolates to different antimicrobials

Analysis of the antimicrobial resistance of 312 recovered *P. aeruginosa* strains against the 14 tested antibiotics demonstrated that 247 were assigned as multi-drug resistant (MDR). From these MDR isolates, 200 isolates were selected in order to perform further investigation. Tobramycin

and levofloxacin showed the highest incidence of resistance (80%). Moderate incidences of resistance were reported against cefepime (59.5%), meropenem (55.5%), imipenem (52%), and aztreonam (42%). On the other hand, polymyxin B and colistin showed the highest activity against our bacterial isolates **(Table 3)**.

Table 3. Incidences of resistance of *P. aeruginosa* isolates to different antimicrobials

Antimicrobial category	Antimicrobial agent	No (%) of resistant isolates (n=200)
Antipseudomonal penicillins + β-lactamase inhibitors	Piperacillin/Tazobactam	154 (77)
Antipseudomonal cephalosporins	Ceftazidime	157 (78.5)
	Cefepime	119 (59.5)
Monobactams	Aztreonam	84 (42)
Antipseudomonal carbapenems	Imipenem	104 (52)
	Meropenem	111 (55.5)
Polymyxins	Colistin	4 (2)
	Polymyxin B	2 (1)
Aminoglycosides	Gentamicin	150 (75)
	Tobramycin	160 (80)
	Amikacin	156 (78)
Antipseudomonal Fluoroquinolones	Ciprofloxacin	148 (74)
	Levofloxacin	160 (80)
Phosphonic acids	Fosfomycin	48 (24)

3.3. Antimicrobial resistance patterns and MAR indices of *P. aeruginosa* isolates

P. aeruginosa isolates exhibited 23 different patterns that belonged to 9 major ones. All *P. aeruginosa* isolates were resistant to 4-12 out of 14 tested antimicrobial agents. P VIIIa was the most prevalent pattern, exhibited by 25 isolates. It was found that all isolates exhibited a MAR index value > 0.2. The antimicrobial resistance patterns and MAR indices of the tested *P. aeruginosa* isolates are shown in **Supplementary**

Table 1.3.4. MICs of aspirin against tested isolates:

The MICs of aspirin were determined against MDR *P. aeruginosa* isolates (n = 200). Aspirin showed slight antimicrobial activity. The MICs of aspirin were 1000 µg/ml, 2000 µg/ml, 4000 µg/ml, and 8000 µg/ml for 2, 22, 78, and 91 tested isolates, respectively. Seven isolates showed MIC values >8000 µg/ml for aspirin.

3.4. Screening for virulence factors among tested MDR isolates

The tested MDR isolates were screened for different virulence traits, namely biofilm formation, pyocyanin production, hemolysis, protease, lipase production, and swarming motility. The incidences of these different virulence factors among all selected isolates are demonstrated in **Table 4**. The number of antibiotic resistance markers was found to be in a positive relationship with the biofilm-forming capacity and in a negative relationship with pyocyanin production by the tested isolates **(Figures 1 and 2)**. No association was detected between the number of antibiotic resistance markers and either hemolytic activity, protease, lipase production, or swarming motility of the tested isolates.

Table 4. Prevalence of virulence factors of tested isolates (n = 200)

Virulence factors	Incidence n (%)
Biofilm	142 (71)
Pyocyanin	142 (71)
Hemolysis	187 (93.5)
Protease	166 (83)
Lipase	122 (61)
Swarming	115 (57.5)

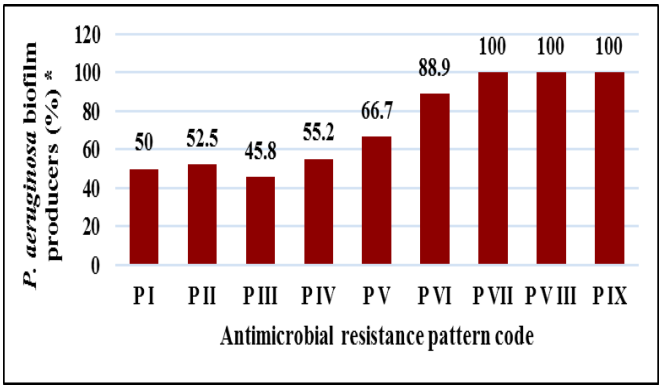

Figure 1. Biofilm-producing isolates among different antimicrobial resistance patterns. * The percentage was computed relative to the number of *P. aeruginosa* isolates exhibiting the corresponding major resistance pattern.

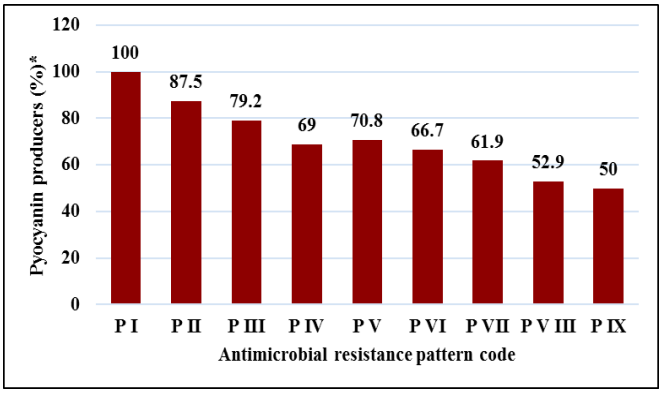

Figure 2: Pyocyanin-producing isolates among different antimicrobial resistance patterns.* The percentage was computed relative to the number of *P. aeruginosa* isolates exhibiting the corresponding major resistance pattern.

3.5. Screening for toxin production among tested isolates

Multiplex PCR was performed on the DNA extracted from 200 *P. aeruginosa* in order to detect the four secretion toxin genes; *exoS, exoU, exoT,* and *exoY*. Also, the exotoxin A gene (*toxA*) was detected using uniplex PCR. It was observed that 64 out of 200 isolates contained *exoU* but not *exoS,* while 134 isolates contained *exoS* but not the *exoU* gene. No isolate harbored both genes. On the other hand, two isolates (P43 and P163) contained neither of these genes. The prevalence of these toxin genes is demonstrated in **Table 5**. Representative electrophotographs are demonstrated in **Figures 3** and **4**.

Table 5. Prevalence of toxin genes among *P. aeruginosa* isolates (Total number = 200 isolates)

Toxin genes	Incidence n (%)
exoU	64 (32)
exoS	134 (67)
exoT	186 (93)
exoY	174 (87)
toxA	186 (93)

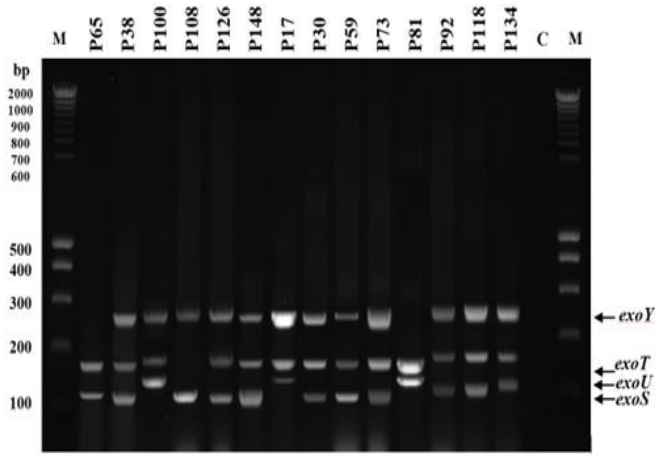

Figure 3. A representative electrophotograph showing the detection of exoenzyme toxin genes of tested isolates using multiplex PCR technique. The genes were *exoS* (118 bp), *exoU* (134 bp), *exoT* (152 bp), and *exoY* (289 bp). Lane M; 100 bp DNA ladder, lane C; negative control, the remaining lanes showed the amplified DNA products of tested isolates.

3.6. Effect of aspirin on the susceptibility of tested isolates to various antimicrobials

Based on the fractional inhibitory concentration indices (FICI) of 42 tested *P. aeruginosa* isolates, it was found that aspirin antagonized the action of imipenem and meropenem against 78.6% and 73.8%, respectively, of the tested isolates **(Table 6)**. It is to be noted that the MIC of aspirin alone was 2000–8000 µg/ml against the tested *P. aeruginosa* isolates.

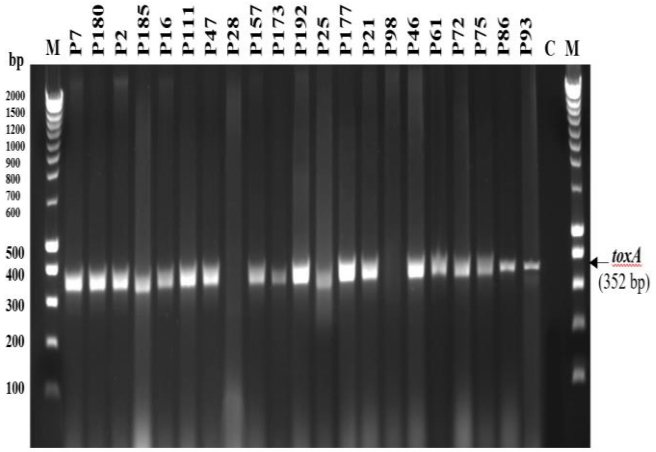

Figure 4: A representative electrophotograph showing the detection of *toxA* gene of tested isolates using uniplex PCR technique. Lane M; 100 bp DNA ladder, lane C; negative control, the remaining lanes showed the amplified DNA products of tested isolates.

Table 6. MICs and FICIs of aspirin and tested antibiotics, either alone or in combinations, against *P. aeruginosa* isolates

Antibiotic	MIC (µg/ml) data*				FICI data		Effect** (%)
	MIC_A range	MIC_{bp}	MIC_{A+B} range	MIC_{B+A} range	FICI range	$FICI_{50}$	
Piperacillin/ Tazobactam	16/4-512/4	≤ 16/4	4/4-1024/4	1000->8000	1-4	3	I (100%)
Ceftazidime	2-512	≤ 8	2-512	2000->8000	1.5-4	3	I (100%)
Cefepime	2-256	≤ 8	2-256	2000->8000	1-4	2.5	I (100%)
Aztreonam	2-256	≤ 8	2-512	1000->8000	1.5-4	3	I (100%)
Imipenem	0.5-128	≤ 2	1-512	2000->8000	4.5-10	6	A (78.6%) I (21.4%)
Meropenem	0.5-128	≤ 2	1-256	1000->8000	3-8.5	5	A (73.8%) I (26.2%)
Colistin	0.5-8	≤ 2	0.5-16	1000->8000	0.75-4	2	I (100%)
Polymyxin B	0.5-8	≤ 2	0.25-16	2000->8000	0.75-4	2	I (100%)
Gentamicin	2-256	≤ 4	2-256	2000->8000	1-4	2	I (100%)
Tobramycin	1-512	≤ 4	1-512	2000->8000	1.5-4	2.5	I (100%)
Amikacin	8-1024	≤ 16	4-1024	2000->8000	0.75-4	2.5	I (100%)
Ciprofloxacin	0.5-256	≤ 1	0.25-256	1000->8000	0.75-3	2	I (100%)
Levofloxacin	1-128	≤ 2	0.5-128	1000->8000	0.75-3	2	I (100%)
Fosfomycin	16-1024	≤ 64	16-1024	2000->8000	1.5-4	2	I (100%)

***MIC_A**: The MIC of antibiotic alone, MIC_{bp}: MIC breakpoints for susceptible of the antibiotics to *P. aeruginosa* (**CLSI, 2017**), **MIC_{A+B}**: The MIC of antibiotics in the presence of aspirin, **MIC_{B+A}**: The MIC of aspirin in the presence of antibiotic. ** **A**: antagonism, **I**: indifference.

3.7. Effect of aspirin on biofilm production by tested isolates

Twenty-three biofilm-producing isolates representing different antimicrobial resistance patterns were selected to determine the potential anti-biofilm activity of aspirin and/or antibiotics. The untreated 23 *P. aeruginosa* isolates were categorized as moderate producers (14 isolates, 60.9%) and strong producers (9 isolates, 39.1%). Aspirin at 1/4 MIC caused significant reductions in biofilm production by *P. aeruginosa* isolates, where the mean absorbance (OD_{550}) decreased from 0.71 to 0.39 after treatment with 1/4 MIC of aspirin. It inhibited biofilm production in 7 (30.4%) *P. aeruginosa* isolates (6 moderate producers and one strong producer). Also, aspirin reduced the degree of biofilm produced by other isolates, where 7 strong biofilm-producing (30.4%) and 8 moderate biofilm-producing isolates (34.8%) were changed to moderate biofilm-producing and weak biofilm-producing isolates, respectively. Moreover, one strong biofilm producer (3%) was changed to a weak biofilm producer after treatment with 1/4 MIC of aspirin, as shown in **Figure 5.**

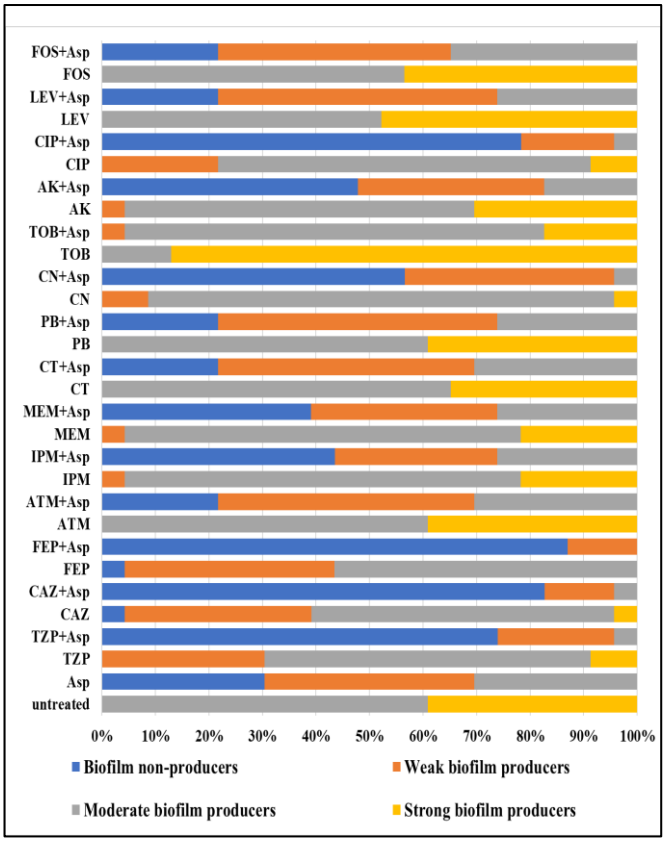

Figure 5. Effect of 1/4 MICs of aspirin and/or antibiotics on the degree of biofilm production (non/weak/moderate/strong producer) by tested *P. aeruginosa* isolates.

Concerning antibiotics, 1/4 MIC of amikacin, gentamicin, piperacillin/tazobactam, ceftazidime, cefepime, and ciprofloxacin showed significant inhibition in biofilm production by tested *P. aeruginosa* isolates. However, tobramycin significantly induced biofilm production by tested isolates. All tested aspirin/antibiotic combinations significantly reduced biofilm production by tested isolates, and the maximal reduction (highest t-value) was exerted by 1/4 MIC aspirin/amikacin **(Table 7).**

Table 7. Effect of tested antibiotics, either alone or combined with aspirin on biofilm production by tested isolates.

Antibiotic	Effect on biofilm production					
	Antibiotic alone			In combination with aspirin		
	Absorbance (OD_{550})	Paired t-test		Absorbance (OD550)	Paired t-test	
	Mean ± SD	t	*P*-value*	Mean ± SD	t	*P*-value*
Untreated	0.71 ± 0.15			0.39 ± 0.09	12.119	<0.001
Piperacillin/ Tazobactam	0.49 ± 0.17	7.898	<0.001	0.21 ± 0.05	16.320	<0.001
Ceftazidime	0.45 ± 0.17	8.339	<0.001	0.21 ± 0.06	15.572	<0.001
Cefepime	0.43 ± 0.16	10.562	<0.001	0.20 ± 0.03	16.289	<0.001
Aztreonam	0.71 ± 0.16	0.555	0.585	0.33 ± 0.13	13.058	<0.001
Imipenem	0.64 ± 0.19	1.848	0.078	0.29 ± 0.13	11.924	<0.001
Meropenem	0.64 ± 0.21	1.964	0.062	0.29 ± 0.14	11.363	<0.001
Colistin	0.71 ± 0.17	0.498	0.623	0.33 ± 0.14	13.452	<0.001
Polymyxin B	0.71 ± 0.15	0.033	0.974	0.32 ± 0.14	11.887	<0.001
Gentamicin	0.59 ± 0.14	8.964	<0.001	0.23 ± 0.07	16.177	<0.001
Tobramycin	1.03 ± 0.18	15.057	<0.001	0.62 ± 0.16	2.515	0.020
Amikacin	0.63 ± 0.15	6.626	<0.001	0.26 ± 0.10	17.173	<0.001
Ciprofloxacin	0.48 ± 0.17	7.933	<0.001	0.22 ± 0.07	14.855	<0.001
Levofloxacin	0.70 ± 0.16	0.707	0.487	0.31 ± 0.13	13.731	<0.001
Fosfomycin	0.70 ± 0.16	0.449	0.657	0.32 ± 0.13	13.722	<0.001

* *P*-value <0.05 refers to statistically significant, *P*-value <0.001 refers to statistically highly significant; green color; significant decrease in biofilm formation, white color; indifference, red color; significant increase in biofilm formation.

3.8. Effect of aspirin on pyocyanin production by tested isolates

Twenty-two pyocyanin-producing isolates representing different antimicrobial resistance patterns were selected to test the effect of 1/4 MIC of aspirin or antibiotic, either alone or in combination, on pyocyanin production. Pyocyanin production by tested isolates was highly significantly reduced upon treatment with 1/4 MIC of aspirin. Statistical analysis of the effect of tested antibiotics at 1/4 MIC on pyocyanin production showed that 7 out of 14 tested

antibiotics exerted highly significant reductions in pyocyanin production **(Table 8)**. Interestingly, all tested aspirin/antibiotic combinations exerted significant reductions in pyocyanin production by tested isolates. The maximum reduction (highest t-value) was reported in the case of aspirin/cefepime combination **(Table 8)**.

Table 8. Effect of tested antibiotics, either alone or combined with aspirin on pyocyanin production by selected isolates.

Antibiotic	Effect on pyocyanin production					
	Antibiotic alone			In combination with aspirin		
	Concentration (µg)	Paired t-test		Concentration (µg)	Paired t-test	
	Mean ± SD	t	*P*-value*	Mean ± SD	t	*P*-value*
Untreated	10.14 ± 3.24			2.79 ± 0.75	13.492	<0.001
Piperacillin/ Tazobactam	5.29 ± 2.16	12.769	<0.001	2.29 ± 0.55	13.327	<0.001
Ceftazidime	4.66 ± 2.64	15.043	<0.001	2.02 ± 1.07	16.048	<0.001
Cefepime	3.50 ± 1.04	13.779	<0.001	1.56 ± 1.02	16.508	<0.001
Aztreonam	10.12 ± 3.30	0.319	0.753	2.69 ± 0.75	13.752	<0.001
Imipenem	5.18 ± 1.67	14.026	<0.001	2.01 ± 0.76	14.186	<0.001
Meropenem	5.07 ± 1.60	13.767	<0.001	2.07 ± 0.62	13.501	<0.001
Colistin	9.74 ± 3.80	1.343	0.194	2.66 ± 0.73	13.598	<0.001
Polymyxin B	9.62 ± 3.91	1.230	0.232	2.63 ± 0.73	13.724	<0.001
Gentamicin	5.56 ± 1.71	13.235	<0.001	2.04 ± 1.10	16.418	<0.001
Tobramycin	10.48 ± 2.92	1.804	0.086	2.72 ± 0.65	12.877	<0.001
Amikacin	10.20 ± 3.08	0.573	0.573	2.50 ± 0.70	13.963	<0.001
Ciprofloxacin	6.02 ± 2.90	10.918	<0.001	1.94 ± 1.03	16.021	<0.001
Levofloxacin	10.48 ± 3.32	1.933	0.067	2.43 ± 0.63	13.739	<0.001
Fosfomycin	10.38 ± 3.21	1.352	0.191	2.35 ± 0.55	13.497	<0.001

* *P*-value <0.05 refers to statistically significant, *P*-value <0.001 refers to statistically highly significant; green color: a significant decrease in pyocyanin production, white color: indifference

3.9. Effect on RBCs hemolysis by tested bacterial isolates

The effect of aspirin and/or antibiotics on the percentage of RBCs hemolysis was evaluated using 23 β-hemolytic *P. aeruginosa* isolates, representing different antimicrobial resistance patterns. Aspirin, ceftazidime, cefepime, imipenem, or ciprofloxacin at 1/4 MIC exhibited significant reductions in hemolytic activity **(Table 9)**. All tested aspirin/antibiotic combinations exerted significant reductions in the hemolytic activity of tested *P. aeruginosa* isolates. The maximum reduction (highest t-value) was observed in aspirin/ciprofloxacin, where the mean % hemolysis decreased from 58.69% to 9.55% (**Table 9).**

Table 9. Effect of tested antibiotics, either alone or combined with aspirin on hemolytic activity exerted by selected *P. aeruginosa* isolates.

Antibiotic	Effect on hemolytic activity					
	Antibiotic alone			In combination with aspirin		
	% Hemolysis	Paired t-test		% Hemolysis	Paired t-test	
	Mean ± SD	t	*P*-value*	Mean ± SD	t	*P*-value*
Untreated	58.69 ± 13.87			15.47 ± 12.87	30.080	<0.001
Piperacillin/ Tazobactam	57.75 ± 14.37	0.967	0.344	14.39 ± 13.20	23.033	<0.001
Ceftazidime	43.47 ± 13.63	25.779	<0.001	4.95 ± 7.66	24.953	<0.001
Cefepime	37.96 ± 13.79	34.506	<0.001	2.64 ± 5.75	22.787	<0.001
Aztreonam	57.56 ± 15.04	1.206	0.241	14.40 ± 13.35	25.283	<0.001
Imipenem	43.23 ± 14.70	27.025	<0.001	5.00 ± 8.43	24.448	<0.001
Meropenem	57.84 ± 12.92	0.731	0.473	13.96 ± 11.87	23.403	<0.001
Colistin	58.24 ± 11.44	0.457	0.652	14.59 ± 10.54	24.994	<0.001
Polymyxin B	58.37 ± 13.30	0.347	0.732	15.19 ± 11.57	27.747	<0.001
Gentamicin	58.50 ± 12.82	0.173	0.864	15.20 ± 12.49	21.869	<0.001
Tobramycin	57.87 ± 14.57	0.688	0.499	15.07 ± 13.30	22.081	<0.001
Amikacin	58.25 ± 13.09	0.414	0.683	14.94 ± 12.36	22.613	<0.001
Ciprofloxacin	51.22 ± 13.81	22.296	<0.001	9.155 ± 10.36	30.446	<0.001
Levofloxacin	58.44 ± 15.51	0.222	0.826	16.33 ± 14.00	24.271	<0.001
Fosfomycin	58.86 ± 13.29	0.141	0.889	16.54 ± 11.68	25.987	<0.001

* *P*-value <0.05 refers to statistically significant, *P*-value <0.001 refers to statistically highly significant; green color: a significant decrease in hemolytic activity, white color: indifference

3.10. Effect on the activity of total proteases

The total protease activity of the selected 23 isolates was evaluated in the presence of 1/4 MIC of aspirin or antibiotics, either alone or in combination. The statistical analysis of the result showed that 1/4 MIC of aspirin as well as seven antibiotics caused significant reductions in the activity of total proteases of the tested isolates. The effects of tested aspirin/antibiotic combinations on the total protease activity of tested *P. aeruginosa* isolates are presented in **Table 10**. The maximal reductions (highest t-value) were observed in the aspirin/imipenem, combination.

3.11. Effect on the activity of lipase

The lipolytic activity of selected twenty lipase-producing *P. aeruginosa* isolates representing different antimicrobial resistance patterns was determined in the absence and presence of 1/4 MIC of aspirin or antibiotics, either alone or in combination. Aspirin didn't cause any significant change in the lipase activity of the tested isolates. On the other hand, gentamicin and ciprofloxacin at 1/4 MIC reduced the lipolytic activity **(Table 11)**.

Table 10. Effect of tested antibiotics, either alone or combined with aspirin on protease activity exerted by selected *P. aeruginosa* isolates.

Antibiotic	Effect on protease activity					
	Antibiotic alone			In combination with aspirin		
	Unit activity	Paired t-test		Unit activity	Paired t-test	
	Mean ± SD	t	P-value*	Mean ± SD	t	P-value*
Untreated	43.52 ± 10.06			27.37 ± 3.66	9.92	<0.001
Piperacillin / Tazobactam	43.02 ± 8.65	0.508	0.616	30.34 ± 3.8	7.837	<0.001
Ceftazidime	29.57 ± 6.87	7.68	<0.001	18.72 ± 4.52	9.804	<0.001
Cefepime	44.35 ± 14.42	0.344	0.734	36.66 ± 14.8	2.205	0.038
Aztreonam	43.98 ± 12.16	0.447	0.66	31.08 ± 6.85	8.708	<0.001
Imipenem	32.86 ± 11.14	7.044	<0.001	24.82 ± 5.13	11.313	<0.001
Meropenem	29.02 ± 10.18	7.263	<0.001	21.83 ± 4.55	10.51	<0.001
Colistin	44.03 ± 13.42	0.352	0.728	34.14 ± 7.58	8.414	<0.001
Polymyxin B	43.07 ± 10.53	0.446	0.66	26.61 ± 8.88	4.482	<0.001
Gentamicin	23.94 ± 5.11	8.737	<0.001	22.99 ± 4.61	9.382	<0.001
Tobramycin	35.29 ± 5.02	6.084	<0.001	26.54 ± 2.56	8.38	<0.001
Amikacin	35.74 ± 5.57	3.864	<0.001	26.96 ± 4.66	6.734	<0.001
Ciprofloxacin	33.82 ± 5.58	4.871	<0.001	24.81 ± 4.29	7.771	<0.001
Levofloxacin	44.85 ± 14.56	0.723	0.478	31.72 ± 8.31	7.154	<0.001
Fosfomycin	43.34 ± 10.79	0.183	0.857	32.08 ± 6.1	8.296	<0.001

* *P*-value <0.05 refers to statistically significant, *P*-value <0.001 refers to statistically highly significant; green color: a significant decrease in protease activity, white color: indifference.

3.12. Effect on swarming motility

The swarming motility of the tested 20 isolates, representing different antimicrobial resistance patterns, significantly decreased after treatment with 1/4 MICs of aspirin or ten antibiotics **(Table 12)**. A subinhibitory concentration (1/4 MIC) of the aspirin/gentamicin combination exerted a maximal reduction (highest t-value) in swarming zone diameter.

3.13. Effect on toxin genes expression

The expression of four virulence genes (*toxA, exoU, exoT,* and *exoY*) in one selected *P. aeruginosa* isolate (P7) and the *exoS* gene in the P185 isolate was evaluated in the presence and absence of 1/4 MIC of aspirin. The housekeeping primer *rpoD* was used as a normalizer. Melting curves were constructed to evaluate the specificity of the primers used. As shown in Figure 6, aspirin at 1/4 MIC significantly reduced the expression of the *exoS, exoT,* and *exoY* genes by 51.6%, 33.9%, and 42.7%, respectively.

Table 11. Effect of tested antibiotics, either alone or combined with aspirin on lipase activity of selected *P. aeruginosa* isolates.

Antibiotic	Effect on lipase activity					
	Antibiotic alone			In combination with aspirin		
	Zone diameter (mm)	Paired t-test		Zone diameter (mm)	Paired t-test	
	Mean ± SD	t	*P*-value*	Mean ± SD	t	*P*-value*
Untreated	24.90 ± 3.70			24.35 ± 5.19	0.398	0.695
Piperacillin / Tazobactam	24.35 ± 5.69	0.487	0.632	24.00 ± 6.00	0.573	0.573
Ceftazidime	24.85 ± 5.97	0.052	0.959	24.65 ± 6.76	0.168	0.868
Cefepime	24.80 ± 6.61	0.069	0.946	24.60 ± 6.44	0.185	0.855
Aztreonam	25.15 ± 5.37	0.383	0.706	24.80 ± 4.73	0.102	0.919
Imipenem	25.10 ± 5.75	0.242	0.811	24.45 ± 4.96	0.337	0.740
Meropenem	25.20 ± 7.83	0.185	0.855	24.80 ± 5.28	0.069	0.945
Colistin	25.10 ± 4.02	0.324	0.750	24.85 ± 3.92	0.050	0.961
Polymyxin B	24.75 ± 3.89	0.256	0.801	24.50 ± 4.03	0.413	0.684
Gentamicin	19.30 ± 1.53	7.201	<0.001	18.60 ± 3.66	8.963	<0.001
Tobramycin	25.20 ± 7.19	0.255	0.801	24.80 ± 5.15	0.091	0.928
Amikacin	24.20 ± 4.46	0.807	0.430	24.05 ± 3.82	0.750	0.463
Ciprofloxacin	22.00 ± 4.87	2.948	0.008	22.55 ± 6.16	1.395	0.179
Levofloxacin	25.10 ± 5.05	0.377	0.711	25.05 ± 4.20	0.273	0.788
Fosfomycin	25.00 ± 6.12	0.085	0.933	24.70 ± 4.99	0.165	0.870

P-value <0.05 refers to statistically significant, *P*-value <0.001 refers to statistically highly significant; green color: a significant decrease in lipase activity, white color: indifference.

Table 12. Effect of tested antibiotics, either alone or combined with either aspirin or eugenol, on swarming motility of selected *P. aeruginosa* isolates.

Antibiotic	Effect on swarming motility					
	Antibiotic alone			In combination with aspirin		
	Zone diameter (mm)	Paired t-test		Zone diameter (mm)	Paired t-test	
	Mean ± SD	t	P-value*	Mean ± SD	t	P-value*
Untreated	29.1 ± 8.1			15.03 ± 0.98	9.402	<0.001
Piperacillin / Tazobactam	18.1 ± 5.07	10.508	<0.001	9.35 ± 1.18	11.078	<0.001
Ceftazidime	20.8 ± 5.17	10.148	<0.001	10.9 ± 1.94	9.935	<0.001
Cefepime	27.4 ± 7.82	9.488	<0.001	18.65 ± 3.73	10.303	<0.001
Aztreonam	28.4 ± 6.44	1.022	0.32	14.35 ± 1.6	7.867	<0.001
Imipenem	24.3 ± 6.28	7.931	<0.001	10.7 ± 1.38	9.476	<0.001
Meropenem	16.95 ± 2.86	9.462	<0.001	9.85 ± 1.04	10.825	<0.001
Colistin	24.6 ± 6.43	8.252	<0.001	14.05 ± 1.82	9.514	<0.001
Polymyxin B	27.65 ± 4.67	1.346	0.194	13.65 ± 2.85	6.75	<0.001
Gentamicin	14.65 ± 1.39	9.115	<0.001	10.31 ± 1.56	11.156	<0.001
Tobramycin	19.85 ± 3.94	7.502	<0.001	10.55 ± 1.96	8.938	<0.001
Amikacin	22.6 ± 5.53	9.266	<0.001	12.7 ± 1.81	9.532	<0.001
Ciprofloxacin	19.4 ± 5.18	10.076	<0.001	10.65 ± 1.27	10.97	<0.001
Levofloxacin	29 ± 7.91	0.158	0.876	14.9 ± 2.43	8.465	<0.001
Fosfomycin	28.4 ± 8.75	0.67	0.511	14.8 ± 4.05	8.008	<0.001

* *P*-value <0.05 refers to statistically significant, *P*-value <0.001 refers to statistically highly significant; green color: a significant decrease in swarming motility, white color: indifference.

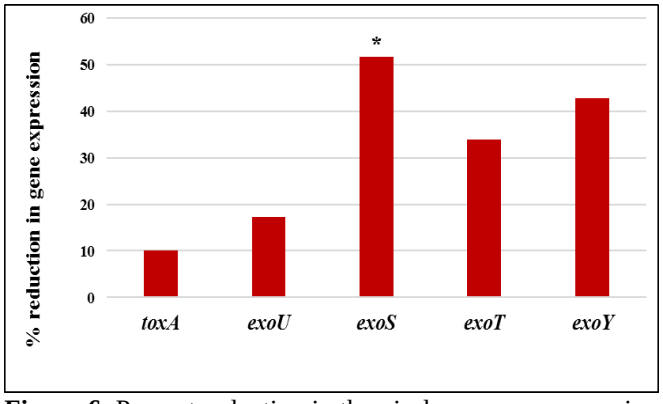

Figure 6. Percent reduction in the virulence gene expression of the tested *P. aeruginosa* isolates after treatment with aspirin. * *P*-value <0.05 refers to a statistically significant reduction

4. DISCUSSION

Antimicrobial resistance is anticipated to become the main cause of death in the coming decades.[33] This study was performed on 200 MDR *P. aeruginosa* isolates. High incidences of resistance were reported against aminoglycosides (75%–80%) and antipseudomonal fluoroquinolones (74%–80%). Also, El-Far *et al*.[34] reported high incidences of resistance of their MDR *P. aeruginosa* isolates against aminoglycosides in Cairo. Another study[35] in Ismailia reported moderate incidences of resistance of MDR *P. aeruginosa* isolates to fluoroquinolones (35.7%–46.4%), and this was explained by various patterns of antibiotic use among different governorates.

In the present study, resistance to beta-lactam antibiotics ranged between 42% and 77% of our tested isolates. Comparable results were recorded in Giza.[36] Colistin and polymyxin B exhibited the highest activity against our tested isolates, where only 2% and 1% of our isolates were colistin and polymyxin B resistant, respectively. Other studies performed in Giza[36] and Mansoura[37] recorded that all their *P. aeruginosa* strains were susceptible to polymyxin B and colistin. All our isolates exhibited a MAR index > 0.2, which means that all strains came from environments with excessive antibiotic usage.[38]

The spread of MDR pathogens indicates the necessity of the re-evaluation of antibiotic recommendation programs, the identification of new antibacterial strategies, and the reevaluation of old drugs as anti-virulence agents.[39] In this study, aspirin showed slight antibacterial activity, whose MICs ranged from 1000 to 8000 µg/ml. Also, Tabatabaeifar *et al*.[40] recorded nearly similar MIC values for aspirin.

The production of various virulence traits by our MDR isolates was determined. Biofilm production was recorded in 71% of our tested isolates. Comparable results were recorded in other studies in Mansoura,[37] Tanta,[41] and Alexandria,[42] Egypt. Interestingly, biofilm-forming capacity was directly proportional to the number of antibiotic resistance markers among our tested isolates. Also, Lin *et al.*[43] found higher levels of MDR *S. aureus* isolates among biofilm-producing isolates.

The current study detected pyocyanin production in 71% of our tested isolates. Comparable results were reported in other governorates in Egypt.[37,44] Fuse *et al.*[45] found that pyocyanin production was reduced in MDR isolates, a finding that was in accordance with our results, reporting an indirect relationship between the number of antibiotic resistance markers and pyocyanin production. The prevalence of other virulence factors exhibited by our tested isolates was 83% for protease, 61% for lipase, 57.5% for swarming, and 93.5% for hemolysis. The same findings were formerly reported.[37,46,47,48] The incidences of toxin genes in our *P. aeruginosa* isolates were also recorded (*toxA*, 93%; *exoU*, 32%; *exoS*, 67%; *exoT*, 93%; and *exoY*, 87%). These results were very close to those reported in other studies.[35,49]

In the current study, the FICI determination indicated that aspirin antagonized the action of imipenem and meropenem against 78.6% and 73.8% of the tested isolates, respectively. This finding was explained by the fact that salicylate suppressed the outer membrane protein D2 'OprD' synthesis (a unique porin channel for carbapenems).[50,51]

Aspirin completely inhibited biofilm production in 30.4% of our tested *P. aeruginosa* isolates and reduced the degree of biofilm produced by all other isolates (69.6%). In this respect, other investigators[31,52] found that aspirin and salicylic acid significantly reduced biofilm production in *P. aeruginosa.* It was explained by the fact that aspirin and salicylic acid inhibited quorum sensing at the transcriptional level as well as bacterial motility, which facilitates initial attachment and hence biofilm formation.[31] Also, salicylate inhibited the synthesis of extracellular polysaccharides necessary for the formation of biofilms.[53] Several tested antibiotics showed significant inhibition of biofilm formation in our tested isolates. This finding was explained by the inhibition of the quorum sensing-mediated system.[54] Also, these antibiotics might lower adhesion, motility, cell surface hydrophobicity (CSH), and consequently the formation of biofilms.[55] In the current study, all tested aspirin/antibiotic combinations caused significant reductions in biofilms produced by tested isolates. Consistent with our results, Belfield *et al.*[56] reported a synergistic inhibitory effect on biofilm production by *P. aeruginosa* when aspirin was combined with either gentamicin or ciprofloxacin.

Aspirin at 1/4 MIC, either alone or in combination with antibiotics, caused significant reductions in hemolytic activity, pyocyanin production, swarming motility, and protease enzyme activity in our tested isolates. Aspirin downregulated the *pqsA* gene, which could explain its quorum-sensing inhibition effect.[31,52,57,58] Because of being QS inhibitors, subinhibitory concentrations of several antibiotics significantly reduced hemolytic activity, pyocyanin production, swarming motility, and protease enzyme activity.[54,59- 63]

No significant effect of aspirin on lipase activity was observed in our study. However, 1/4 MIC of gentamicin or ciprofloxacin reduced the lipolytic activity in our *P. aeruginosa* isolates. Similar data was reported previously,[64] where lipase production in *P. aeruginosa* was significantly reduced by a sub-MIC of gentamicin. It might be explained by the interference with detection (signal/receptor interaction), as gentamicin can interact with the quorum sensing receptor (LasR) in *P. aeruginosa*.[63]

Concerning *P. aeruginosa* toxins, aspirin reduced the expression of the *exoT*, *exoY*, and *exoS* genes by 33.9%, 42.7%, and 51.6%, respectively. Comparable results were recorded previously. [31] It was explained by the QS inhibitory effect of aspirin, as the aryl group of aspirin might bind to the tyrosine moiety of the LasR receptor via strong π-π bonds, changing the LasR receptor's conformation. Another study[65] reported that salicylic acid caused a reduction in ExoS and ExoT toxin levels.

5. CONCLUSION

Aspirin significantly inhibited at least six *P. aeruginosa* functions, such as pyocyanin production, biofilm formation, hemolytic activity, proteolytic activity, swarming motility, and toxin production. Because aspirin interferes with microbial activity rather than inhibiting growth. Accordingly, this drug may not develop a selective pressure for resistance development. Aspirin reduces the effective doses of the available antibiotics, making it a useful antimicrobial adjuvant in *P. aeruginosa* infection treatment protocols. Aspirin could be a potential source of alternative antimicrobials or anti-virulence compounds. It is urgent to investigate the interaction between aspirin and virulence on molecular aspects as well as *in vivo* testing of aspirin and its combinations to combat MDR *P. aeruginosa* infections.

Conflict of Interest

The authors declare no conflict of interest

FUNDING

No funding was received for conducting this study.

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