Chapter

Evaluation of the Synergistic Effect of Amikacin with Cefotaxime against *Pseudomonas aeruginosa* and Its Biofilm Genes Expression

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Abstract

A total of 100 broiler chickens were examined for the presence of *Pseudomonas aeruginosa* by standard microbiological techniques. Susceptibility pattern for amikacin and cefotaxime was performed by Kirby-Bauer and microdilution assays. Then, checkerboard titration in trays was applied and FIC was measured to identify the type of interaction between the two antibiotics. The ability of isolates to form in vitro biofilm was detected by two methods, one qualitative (CRA) and the other quantitative (MTP), followed by investigating the effect of each antibiotic alone and in combination on the expression of biofilm genes. The overall isolation percentage of P. aeruginosa was 21%. Resistance to each antibiotic was more than 50%; the range of cefotaxime MIC was 8–512 μg/ml, while amikacin MIC range was 1–64 μg/ml. The FIC index established a synergistic association between tested two drugs in 17 (81%) of isolates and the remaining represent partially synergism. The qualitative technique showed that only 66.6% of the isolates were considered biofilm producers, while the quantitative technique showed that 90.4% of the isolates were biofilm producers. Further to RT-PCR investigation, significant repression against biofilm-forming genes (*filC*, *pelA*, and *pslA*) was observed for the combination of antibiotics when compared with monotherapy.

Keywords: *P. aeruginosa*, cefotaxime, amikacin, combination therapy, biofilm, gene expression

1. Introduction

The infection with *Pseudomonas aeruginosa* is responsible for humanity in poultry and clinical signs including respiratory signs and septicaemia. *P. aeruginosa* produces dyspnea and cheesy deposits on the serous surfaces lining the air sacs and peritoneal cavity and also congestion of the internal organs, perihepatitis, and pericarditis [1]. *Pseudomonas* species are not related to disease entity except *Pseudomonas aeruginosa* that has been associated with infection in both man and animals. The disease of pseudomonas induces a significant economic loss to the farm by causing high mortality of newly hatched chickens and death of embryo at

a later stage [2]. Furthermore, *Pseudomonas aeruginosa* shows innate resistance to almost antibiotics in recent years [3, 4].

Due to this intrinsic resistance to antibiotics, its ability to easily develop new resistance, its ability to create biofilms, and the recent decline in drug discovery programs, *P. aeruginosa* infections have become an urgent worldwide health concern [3, 5]. Recent efforts to focus on this rising challenge comprise repositioning screens to recognize commercially permitted drugs with novel antimicrobial activity [6–9] and combinatorial drug screens to categorize combinations of traditional antibiotics and novel repositionable modulators [10, 11].

Concomitant use of antibiotics (combination therapy) is recommended for severe infections when *P. aeruginosa* is the suspected pathogen, to prevent the development of resistance during treatment and to achieve a wide spectrum of activity. In addition to preventing the development of resistance, the combined use of antibiotics (as cephalosporins and aminoglycosides) may have synergistic effects and may reduce the occurrence of side effects, since each drug is used at a lower dose than would be used for monotherapy [12].

Concerning bacterial biofilms, Batoni et al. [13] and Grassi et al. [14] proved a strong interaction between the effectiveness of combination therapy and biofilms formed by *P. aeruginosa*. Therefore, the present study concerned the effect of cefotaxime, amikacin singly, and in combination besides validating the activity of them on biofilm expression of the obtained *P. aeruginosa* isolates.

2. Material and methods

2.1 Sampling and isolate characterization

A total of 500 samples of the liver, heart, kidney, spleen, and lung (100 each) was aseptically collected from 100 freshly dead and diseased with respiratory manifestations broiler chickens from different ages and localities in Sharkia province, Egypt, from November 2018 to February 2019. All samples were subjected to conventional methods for isolation and identification of pseudomonas recommended by the Health Protection Agency [15]. *Pseudomonas aeruginosa* isolates were further identified with API20E kits (BioMérieux, France).

2.2 Antibiotic susceptibility testing

2.2.1 Disk diffusion method

The antimicrobial susceptibility test of the isolates was performed by Kirby-Bauer disk diffusion test [16]. In brief, each test isolate was swabbed uniformly onto the surface of Mueller-Hinton agar plates. Antibiotic sterile disks including cefotaxime (CTX: 30 μg) and amikacin (AK: 30 μg) were then placed on to the agar surface of the plate. Following incubation, the inhibition zones, in millimeters, were measured in duplicate and scored as sensitive, intermediate, and resistant categories by the critical breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) [17].

2.2.2 Preparation of antibiotic stock solution

Standard powder forms of cefotaxime and amikacin were stored at 4°C till usage. The stock solution of each antibiotic was prepared by weighing and consequently

dissolving suitable amounts of the antibiotics reaching a concentration of 1000 μg/mL in Mueller-Hinton broth.

2.2.3 Determination of the minimum inhibitory concentration (MIC)

MIC values of antibiotics were determined by the microdilution method following the recommendations of Papich [18]. Stock solutions of antibiotics were prepared and added to the bottom of a 96-well microtiter plate (Nunc Inc., Roskilde, Denmark). 100 mL of this solution was added to the first well of the 96-well plate and serially diluted. 100 mL of an overnight culture of *P. aeruginosa* was added to each well at a final concentration of 5×10^5 CFU/mL (colony-forming units per milliliter). The microtiter plates were incubated at 35°C for 24 h and the MIC determined as the lowest concentration of antibiotics showing no visible bacterial growth.

2.2.4 Test for synergism

The synergistic effect of the antibiotic combinations was detected using a checkerboard dilution assay [19]. The initial concentration of each drug should be fourfold greater than the desired concentration (MIC concentration) and then diluted twofold. In a screw cap test tube, 0.25 mL of broth of each two drugs to be tested was added to 0.5 mL of broth containing a suspension of the organism to be tested to reach the final volume of 1 mL. The inoculum of the bacterial suspension (in 0.5 mL of broth) should be approximately 2×10^5 colony-forming unit (CFU) to produce a final inoculum of 1×10^5 CFU per mL after the addition of an equal volume of the antimicrobial solutions. Each test composed of 36 tubes set horizontally and vertically, 6 rows in one direction contained twofold serial dilutions of antibiotic 1, and 6 rows in the other direction contained twofold serial dilutions of antibiotic 2; two additional rows contained twofold serial dilution of antibiotic 1 or antibiotic 2 alone. The tubes were incubated at 37°C for 24 and 48 h, the tubes were read as those showing turbidity (+) and those showing no turbidity (−). A fractional inhibitory concentration index was used to interpret the results.

2.2.5 Estimation of FIC index

FIC of each agent was calculated by dividing the MIC of the drug in combination by the MIC of the drug alone. The sum of both FICs (ΣFIC = FIC of antibiotic A + FIC of antibiotic B) in each well was used to categorize the combined activity of antimicrobial agents at the given concentrations as synergistic (ΣFIC <= 0.5), partially synergistic (ΣFIC >0.5 and < 1), additive (ΣFIC = 1), indifferent (ΣFIC >1 and < 4), and antagonistic (ΣFIC > = 4) [20].

2.3 Phenotypic characterization of biofilm production

2.3.1 Congo red agar test

Freeman et al. [21] have described a simple qualitative method to detect biofilm production by using a Congo red agar (CRA) medium. CRA medium was prepared with brain heart infusion agar (Oxoid, UK) 37 g/L, sucrose 50g/L, and Congo red indicator (Oxoid, UK) 8 g/L. The first Congo red dye was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 min) separately from the other medium constituents. Then, it was added to the autoclaved brain heart

infusion agar with sucrose at 55°C. In this test, the Congo red dye was used as a pH indicator, showing black coloration at pH ranges between 3.0 and 5.2. Plates with the Congo red agar medium were seeded and incubated in an aerobic environment for 24–48 h at 37°C. Isolates were interpreted according to their colony phenotypes. Black colonies with dry constancy and rough surface and edges were suspected as a positive sign of slime formation, while both black colonies with a smooth, round, and shiny surface and red colonies of dry texture and rough edges and surface were suspected as intermediate slime producers. Red colonies with smooth, round, and shiny surfaces were indicators for negative slime formation.

2.3.2 Quantitative detection of biofilm by microtiter plate method

The biofilm assay is performed by using flat-bottom microtiter plates (Techno Plastic Products, Switzerland) as described by O'Toole [22]. *P. aeruginosa* isolates were grown at 37°C in tryptic soy broth (TSB; Oxoid, UK). The bacterial cells were then pelleted at 6000 g for 10 min, and the cell pellets were in 5 mL of fresh medium. The optical densities (ODs) of the bacterial suspensions were measured using a spectrophotometer (Model 6305, Jenway Ltd., Essex, UK) and normalized to an absorbance of 1:00 at 600 nm. The cultures were diluted 1:40 in fresh TSB, and 200 μL of cells were aliquoted into a 96-well polystyrene microtiter plate and inoculated for 24 h at 37°C without agitation. After incubation at 37°C for 24 h, the planktonic cells were aspirated, and the wells were washed three times with sterile phosphate-buffered saline (PBS). The plates were inverted and allowed to dry for an hour at room temperature.

For biofilm quantification, 200 μL of 0.1% aqueous crystal violet solution was added to each well, and the plates were allowed to stand for 15 min. The wells were subsequently washed three times with sterile PBS to wash off the excess crystal violet. Crystal violet bound to the biofilm was extracted with 200 μL of an 80:20 (v/v) mixture of ethyl alcohol and acetone, and the absorbance of the extracted crystal violet was measured at 545 nm on ELISA reader (stat fax 2100, USA). A negative control, crystal violet binding to wells was measured for wells exposed only to the medium with no bacteria. All biofilm assays were performed in triplicate. The interpretation of biofilm production was according to the criteria described by Stepanović et al. [23]. Based on these criteria, optical density cutoff value (ODc) is defined as an average OD of negative control +3 × SD (standard deviation) of the negative control. The ability to produce biofilm of each *P. aeruginosa* isolate was classified according to the following criteria: $OD \leq ODc$ = not a biofilm producer, $ODc < OD \leq 2x\ ODc$ = weak biofilm producer, $2x\ ODc < OD \leq 4x\ ODc$ = moderate biofilm producer, and $4x\ ODc < OD$ = strong biofilm producer.

2.4 Molecular evaluation

2.4.1 DNA extraction

DNA extraction from isolates was performed using the QIAamp DNA Mini Kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Concisely, 10 μL of proteinase K and 200 μL of lysis buffer were added to 200 μL of the sample suspension and incubated at 56°C for 10 min. Then, 200 μL of 100% ethanol was added to the lysate followed by washing and centrifugation according to the manufacturer's recommendations. Nucleic acid was eluted with 100 μL of elution buffer.

2.4.2 PCR amplification of biofilm virulence genes

The obtained DNA was examined for the presence of biofilm in a 25 μL reaction comprising 12.5 μL of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 μL of each primer of 20 pmol concentration, 4.5 μL of water, and 6 μL of DNA template. The reaction was implemented in an Applied Biosystems 2720 Thermal Cycler for the investigation of the presence of biofilm genes. The properties of all used primers, as well as amplicon length and cycling conditions, were synopsized by Ghadaksaz et al. [24] and listed in **Table 1**.

2.4.3 Analysis of the PCR products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (AppliChem, Germany, GmbH) in 1× TBE buffer at room temperature using gradients of 5 V/cm. For gel analysis, 20 μL of the products were loaded in each gel slot. A GelPilot 100 bp DNA ladder (Qiagen, Germany, GmbH) and GeneRuler 100 bp ladder (Fermentas, Germany) were used to verify the size of fragments. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data were assessed through computer software.

2.4.4 Quantitative analysis of biofilm gene expression

Biofilm gene expression was analyzed by quantitative real-time PCR (qRT-PCR), and the 16S rRNA housekeeping gene of *Pseudomonas aeruginosa* served as internal control with primer sequence F: GGGGGATCTTCGGACCTCA, R: TCCTTAGAGTGCCCACCCG to normalize the expressional levels between samples. Primers were utilized in a 25 μL reaction containing 12.5 μL of the 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 μL of RevertAid Reverse Transcriptase (200 U/μL) (Thermo Fisher), 0.5 μL of each primer of 20 pmol concentration, 8.25 μL of water, and 3 μL of RNA template. The reaction was performed in a Stratagene MX3005P real-time PCR machine with specific conditions mentioned in **Table 2**. To estimate the variation of gene expression on the RNA of the different samples, the Ct of each sample was compared with that of the positive control group according to the "ΔΔCt" method stated by Yuan et al. [25].

2.5 Statistical analysis

Data analysis was performed by SPSS version 22 for windows. A t-test was used to detect statistical differences of the experiments including antibiotic combination treatment versus single antibiotic therapy. Moreover, one-way ANOVA was used for contrasting the influence of these remedies on the fold change of biofilm gene expression. A $P \leq 0.05$ value was suspected as statistically significant.

3. Results

3.1 The recovery rate of isolation and identification

Pseudomonas spp. were isolated from 34 of 100 examined broiler chickens (34%) as shown in **Table 3**. They were further identified by standard microbiological techniques, and an API giving an overall prevalence of 21% was identified as *Pseudomonas aeruginosa.*

Target gene	Primer sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension
				Secondary denaturation	Annealing	Extension	
PslA	TCCCTACCTCAGCAGCAAGC	656	94°C, 5 min	94°C, 30 s	60°C, 40 s	72°C, 45 s	72°C, 10 min
	TGTTGTAGCCGTAGCGTTTCTG						
PelA	CATACCTTCAGCCATCCGTTCTTC	786	94°C, 5 min	94°C, 30 s	60°C, 40 s	72°C, 45 s	72°C, 10 min
	CGCATTCGCCGCACTCAG						
FliC	TGAACGTGGCTACCAAGAACG	180	94°C, 5 min	94°C, 30 s	56.2 °C, 30 s	72°C, 30 s	72°C, 7 min

Table 1.
Primer sequences, target genes, amplicon sizes, and cycling conditions.

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Target gene	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)			Reference
			Secondary denaturation	Annealing	Extension	Secondary denaturation	Annealing	Final denaturation	
16S rRNA	50°C, 30 min	94°C, 15 min	94°C, 15 s	52°C, 30 s	72°C, 30 s	94°C, 1 min	52°C, 1 min	94°C, 1 min	Spilker et al. [26]
pslA				60°C, 30 s			60°C, 1 min		
pelA				60°C, 30 s			60°C, 1 min		Ghadaksaz et al. [24]
fliC				56.2 °C, 30 s			56.2 °C, 1 min		

Table 2.
Target genes and cycling conditions for SYBR green rt-PCR.

Sample	No. of examined samples	*Pseudomonas* spp. isolates		*P. aeruginosa* isolates	
		No.	Frequency	No.	Frequency
Freshly dead	28	11	39%	11	39%
Diseased chicks Young (1–10 days)	33	20	60%	9	27%
Old broilers (11–35 days)	39	3	7.6%	1	2.5%
Total	100	34	34%	21	21%

Table 3.
The incidence of Pseudomonas aeruginosa *isolated from examined samples.*

3.2 Antimicrobial activity

According to the disk diffusion method, 76.2% of isolates were resistant to cefotaxime, 14.3% were intermediate, and 9.5% were sensitive. Regarding amikacin, 57.2% of isolates were resistant, 9.5% were intermediate, and 33.3% were sensitive. Of interest, 57.2% of isolates were resistant to both tested antibiotics.

According to the microdilution assay, the range of cefotaxime MIC was 8–512 μg/mL, while the amikacin MIC range was 1–64 μg/mL as depicted in **Table 4**.

In the checkerboard technique, the interaction between the combination of cefotaxime and amikacin against *Pseudomonas aeruginosa* was predominantly synergistic, although there were few partially synergistic. Thus no growth or turbidity clearly illustrated the extensive activity of aminoglycoside which was enforced by the second drug: cefotaxime resulting in an antibacterial effect. The synergistic activities of the antimicrobial combinations are detailed in **Table 4**. The combination of amikacin and cefotaxime exerted synergetic effect against 17 isolates, and 4 isolates were partially synergistic. FIC index values ranged from 0.18 to 0.75. Statistical analysis of one sample test revealed no significant difference between synergism effects among all isolates indicating strong synergy between both antibiotics where P-value = 0.088. Antagonism was not detected against any isolate in our study.

3.3 Congo red test

About 66.6% of the isolates were positive for biofilm production with varying degrees. Out of 21 *P. aeruginosa* isolates, 19%, 28.6%, and 19% were strong, intermediate, and negative biofilm producers, respectively. The morphology of all types of colonies is illustrated in **Figure 1**.

3.4 Microtiter plate test (MTP)

Biofilm quantification analyses showed that 90.4% of the isolates were biofilm producers, indicating that this technique was more efficient than Congo red agar for the detection of biofilm production. The obtained isolates of this study had the following results for the categories of biofilm production: 9.6% were non-adherent, 33.4% weakly adherent, 42.8% moderately adherent, and 14.2% strongly adherent as shown in **Figure 2**.

A comparison of results obtained by the CRA method versus that of MTP assay is declared in **Table 5**. Out of 21 biofilm *P. aeruginosa* isolates by the CRA method, 19 isolates were positive by the MTP approach but with various levels of

Isolates no.	MIC of CTX	MIC of AK	MIC of CTX in combination	MIC of AK in combination	FIC of CTX	FIC of AK	Σ FIC	Interpretation
1	256	32	32	2	0.125	0.06	0.18	Synergistic
2	8	1	2	0.25	0.25	0.25	0.5	Synergistic
3	32	2	2	1	0.06	0.5	0.56	Partially synergistic
4	128	64	32	16	0.25	0.25	0.5	Synergistic
5	32	64	8	16	0.25	0.25	0.5	Synergistic
6	32	64	8	8	0.25	0.125	0.375	Synergistic
7	64	64	16	4	0.25	0.06	0.31	Synergistic
8	8	4	2	1	0.25	0.25	0.5	Synergistic
9	64	64	16	16	0.25	0.25	0.5	Synergistic
10	128	64	16	16	0.125	0.25	0.375	Synergistic
11	32	64	2	32	0.06	0.5	0.56	Partially synergistic
12	32	64	8	16	0.25	0.25	0.5	Synergistic
13	16	4	2	1	0.125	0.25	0.375	Synergistic
14	128	64	8	8	0.06	0.125	0.18	Synergistic
15	256	64	16	8	0.06	0.125	0.18	Synergistic
16	256	32	64	2	0.25	0.06	0.31	Synergistic
17	32	8	8	4	0.25	0. 5	0.75	Partially synergistic
18	16	4	4	2	0.25	0.5	0.75	Partially synergistic
19	16	4	2	1	0.25	0.25	0.5	Synergistic
20	256	64	64	16	0.25	0.25	0.5	Synergistic
21	512	64	64	8	0.125	0.125	0.25	Synergistic

Table 4.
MIC of CTX and AK alone and in combination and FIC index against P. aeruginosa *by the checkerboard method.*

production (3 strong, 7 moderate, 9 weak), and only 2 isolates were factual negative by both assays.

3.5 Detection of biofilm genes in strong biofilm *P. aeruginosa* isolates by conventional multiplex PCR

All strong biofilm producers *P. aeruginosa* isolates of code numbers (1, 4, 21) were harbored all examined biofilm genes and gave their characteristic bands as shown in **Figure 3**.

3.6 Quantitative assessment effect of each antibiotic alone and in combination on biofilm gene expression

By RT-PCR, comparing the amount of examining biofilm gene products before and after each treatment with a sub-inhibitory concentration (SIC) of each antibiotic alone and combination, results revealed that the amount of examining

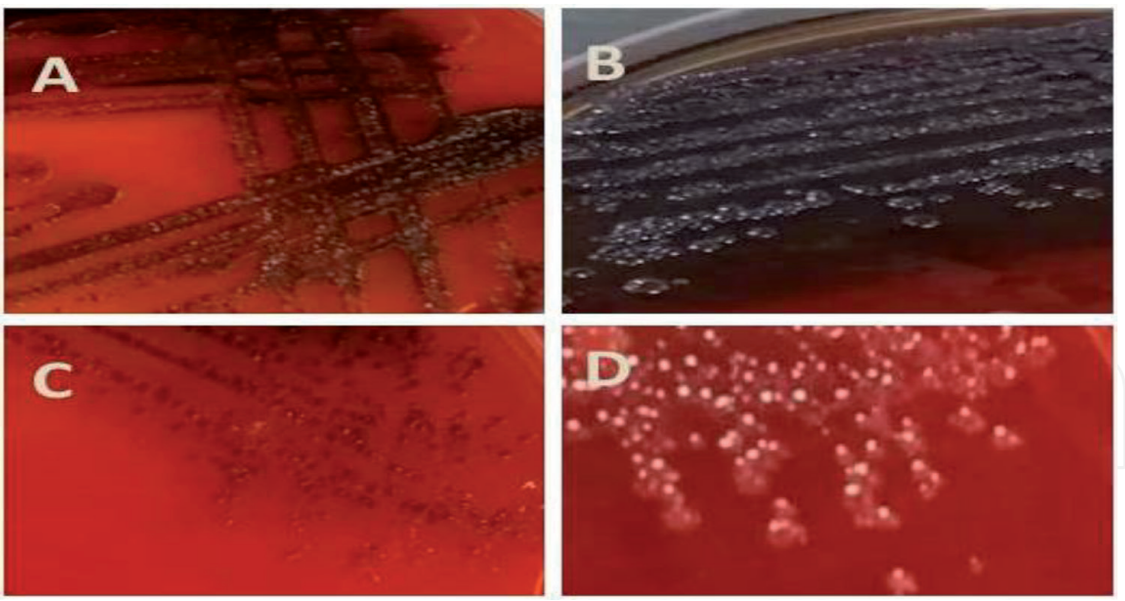

Figure 1.
Investigation of biofilm producer P. aeruginosa *using CRA method: (A) dry black colonies, (B) smooth black colonies, (C) dry red colonies, and (D) smooth red colonies.*

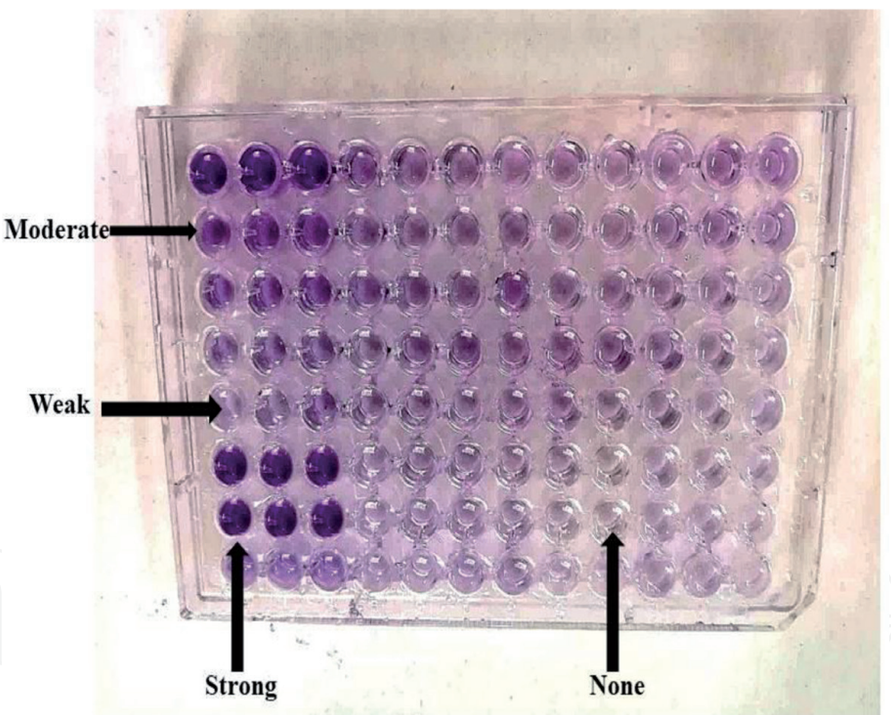

Figure 2.
Microtiter plate method showing none, strong, moderate, and weak biofilm producers differentiated by crystal violet stain in 96-well tissue culture plate.

gene products was relatively increased in untreated samples with drugs than those treated, which leads to high threshold cycle (Ct) value in treated than untreated. Interestingly, we found that drug combination was more effective in significantly reducing the expression of biofilm genes than each antibiotic alone.

Statistical data assessed that fold changes in *pslA*, *pelA*, and *filC* gene expression after treatment with SIC of cefotaxime and amikacin alone were (0.599:0.752:0.597

Sample code no.	CRA	No.	MTP			
			Strong	Moderate	Weak	None
1, 16, 20, 21	Dry black	4	2	2	0	0
4, 7, 9, 10, 14, 15	Smooth black	6	1	3	2	0
3, 5, 6, 17	Dry red	4	0	2	2	0
2, 8, 11, 12, 13, 18, 19	Smooth red	7	0	0	5	2

Table 5.
CRA versus MTP methods for detection of biofilm formation by P. aeruginosa.

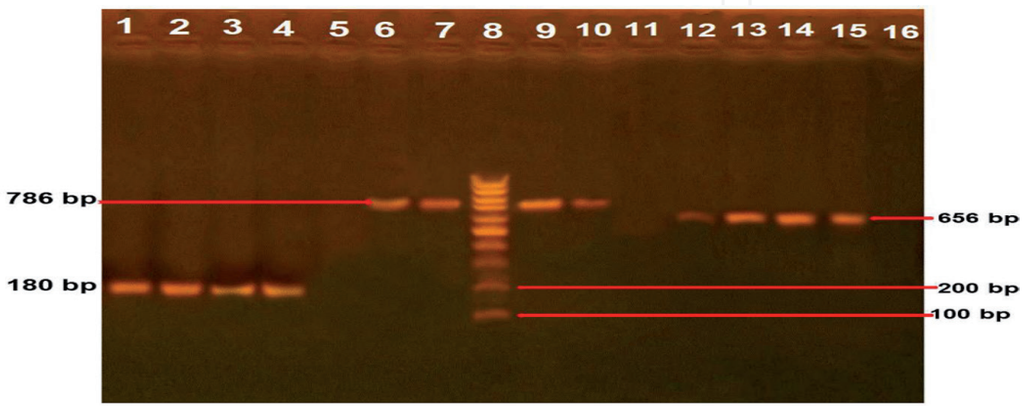

Figure 3.
Agarose gel electrophoresis of biofilm genes: Lanes 1, 6, and 12, positive controls; lanes 5, 11, and 16, negative controls; lane 8, DNA ladder (100 bp); lanes 2–4, positive isolates for filC *gene; lanes 7, 9, and 10, positive isolates for* pelA *gene; and lanes 13–15, positive isolates for* pslA *gene.*

Genes	Isolate no.	Fold change		
		Cefotaxime	Amikacin	Cefotaxime-amikacin combination
PslA	1	0.5212	0.3209	0.0890
	2	0.6830	0.3121	0.1869
	3	0.5946	0.4118	0.1216
PelA	1	0.7371	0.4506	0.2535
	2	0.8526	0.3276	0.2253
	3	0.6690	0.2852	0.1550
FliC	1	0.6071	0.3322	0.2176
	2	0.5471	0.2643	0.1708
	3	0.6373	0.2932	0.0884

Table 6.
Results of RT-PCR showing expression of biofilm genes in P. aeruginosa *isolates before and after treatment with SIC of each antibiotic alone and in combination.*

fold) and (0.348:0.354:0.296 fold), respectively, which were significantly higher ($P \leq 0.05$) than a fold change in same gene expression after combination treatment (0.132:0.211:0.158 fold) as shown in **Table 6** and **Figure 4.**

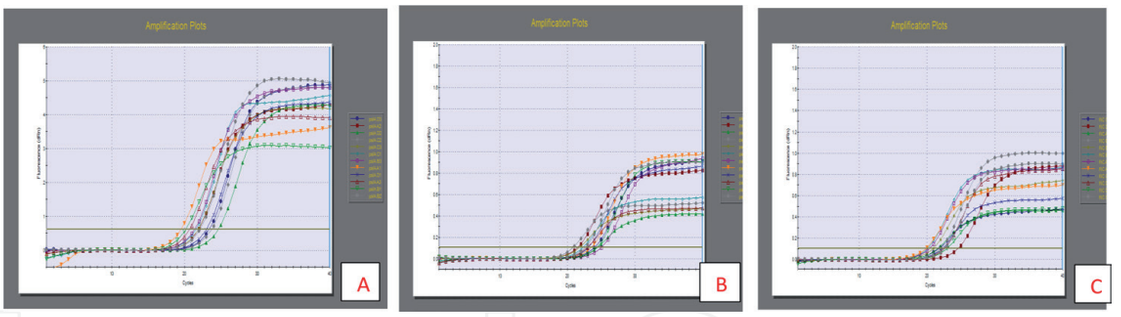

Figure 4.
Expression curves of each biofilm gene after different treatments by SYBR green RT-PCR, (A) PslA gene, (B) PelA gene and (C) FilC gene.

4. Discussion

Pseudomonas aeruginosa is considered to be an opportunistic organism that produces respiratory infection, sinusitis, keratitis/keratoconjunctivitis, and septicemia, and it becomes an infection when it is introduced into tissues of susceptible hosts [27]. The bacterium is widely distributed in the environment, as it can utilize a wide range of materials for its nutrients while only requiring a limited amount of nutrients to survive [28]. Moreover, biofilm production has been considered to be an important determinant of pathogenicity in *P. aeruginosa* infections [29]. The formation of biofilms facilitates chronic bacterial infections and reduces the efficacy of antimicrobial therapy [29–31]. The situation is getting very concerning, the World Health Organization has declared it to be a "critical priority pathogen," on which research and development of novel antibiotics should be focused [32]. For this reason, this work designed to find repositionable candidate's antibiotics against *P. aeruginosa* biofilms, which are disreputable for their intensified drug resistance.

Here we isolated 21 *P. aeruginosa* from 100 broiler chickens suffering from respiratory manifestations (21%). These findings were close to that (20%) reported earlier in India [1]. Many studies showed different prevalence rates of *P. aeruginosa* isolates in broilers worldwide: in Iraq, a low rate of 6% was reported [33], while in Nigeria, a high rate of 75% was reported [34]. These differences in prevalence rates may reflect the considerable disparity in the sampling scheme, sample types, pseudomonas detection protocol, and geographic location.

In the current investigation, all the isolates were tested against cefotaxime and amikacin to determine the antibiotic susceptibility patterns. A high-resistance rate was detected for both antibiotics at which 76.2% were resistant to cefotaxime and 57.2% to amikacin. This might be due to the indiscriminate use of antibiotics in the feed of broiler breeders or other environmental possibilities [35].

The increased observance of multiple resistances (mainly to beta-lactam antibiotics) in pseudomonas isolates is making it increasingly difficult to treat infections caused by this pathogen. Resistance to antimicrobials in pseudomonas strains develops via several mechanisms, including the production of specific enzymes (b-lactamases, enzymes that modify aminoglycosides, for example), changes in cell-membrane permeability, and active efflux systems [36].

Interpretative reading was used to detect the bactericidal activity of each antibiotic against isolates with cefotaxime MICs of 8–512 and amikacin MICs of 1–64. These data are reinforced by findings from other countries, including Kuwait [37], Canada [38], China [39], and the USA [40].

Synergy testing has shown evidence of an interaction of two antibiotics in combination against pseudomonas bacterial isolates where statistical analysis provides important insights into drug synergism where the FIC index calculations

exemplified a significant synergism of both drugs achieving an enhanced overall effect which is substantially greater than the sum of their ones. These results were consistent with the previous studies of Saiman [41], Dundar and Otkun [42], and Hawkey et al. [43]. The possible explanation for this synergism is the ability of beta-lactam cefotaxime to penetrate the outer membrane of pseudomonas bacteria which thereby increases the permeability of the bacterium to the aminoglycoside amikacin binding to 30S ribosome inhibiting the protein synthesis, thus leading to a synergistic effect in the in vitro studies [44].

To investigate the effect of a synergistic combination of the repositionable drugs against *P. aeruginosa* biofilms, we detected their effect on the expression of screened biofilm genes.

In this study, biofilm production was examined qualitatively, depending on colony morphology of 21 *P. aeruginosa* isolates inoculated on Congo red agar. Some differences between researches were apparent concerning the interpretation of CRA test results. In that respect, both bright black colonies [45] and black colonies [46] were considered as a positive result. However, Cucarella et al. [47] described the dry crystalline surface (rough colony morphology) as a positive result, disregarding the color (black or pink). Such discrepancy when interpreting the results may be possible since the test itself was not originally designed for investigating *P. aeruginosa* isolates as reported by Freeman et al. [21]. In this investigation, according to Osman et al. [48], isolates that produced black/rough colonies were verified as strong biofilm-forming, while isolates producing red/smooth colonies were described as non-biofilm formers. The smooth black and dry red colonies were respected as indefinite findings.

The qualitative technique revealed that only 66.6% of the isolates were considered biofilm producers, while the biofilm quantitative technique (MTP method) revealed that 90.4% of the isolates were biofilm producers, indicating that the quantitative technique was more efficient than the qualitative technique for the detection of biofilm production. There was also high biofilm production by the evaluated tested isolates of *P. aeruginosa*.

Biofilms are surface-associated communities embedded within an extracellular matrix [49]. The extracellular matrix consists of polysaccharides, proteins, nucleic acids, and lipids and is a distinguishing feature of biofilms, capable of functioning as both a structural scaffold and a protective barrier [45]. Extracellular polysaccharides are a crucial component of the matrix and carry out a range of functions including promoting attachment to surfaces and other cells, building and maintaining biofilm structure, as well as protecting the cells from antimicrobials and host defenses [50, 51].

P. aeruginosa produces at least two extracellular polysaccharides that can be important in biofilm development and is accompanied by gene regulation [52–54].

Conventional PCR was carried out for detection of *pelA* and *pslA* genes which were involved in the formation of polysaccharide components of biofilm among tested isolates and were expressed heavily in all of them (100%). These data matched with previous studies of Wei and Ma [55], Vasiljević et al. [56], and Emami et al. [57].

Moreover, Suriyanarayanan et al. [58] mentioned that the effects of *fliC* phosphorylation on biofilm attachment and dispersal led to two conclusions. Both initial attachment and detachment during the dispersal stage were delayed by the loss of *fliC* phosphorylation in static and dynamic flow biofilms. As each of these processes still proceeded in the lack of phosphorylation, it suggested that *fliC* phosphorylation regulates the timing and rate of these processes without affecting biofilm architecture. These investigations were parallel with our results where *fliC* detected in all tested isolates.

Regarding the qRT-PCR results, the suppressing effects in fold change of previously mentioned biofilm gene expression were detected for drug combination in comparison with each antibiotic alone. Exposure to each antibiotic caused a decreased level of biofilm expression ranging between 0.1- and 0.7-fold changes,

while the repression was strong and most significant with amikacin-cefotaxime combination treatment with fold change reaching 0.08, i.e., the consequence of treatment on the average expression profile among all biofilm involving genes constituting the bacterial communities studied. As described in this paper and by others [59–61], sub-MICs of combinations have potent effects on attenuating biofilm formation which are totally different from each antibiotic alone.

5. Conclusion

The treatment of biofilm-related *P. aeruginosa* infections in the poultry industry has become an important part of antimicrobial chemotherapy because biofilms are not affected by therapeutic concentrations of antibiotics permitting attachment of other pathogens. Our study proved that using a combination of antimicrobial agents including cefotaxime and amikacin represents a profound synergism, significant antibiofilm, and a suitable candidate in combatting this fierce infection.

Conflict of interest

The authors manifested that they have no conflicts of interest.

Abbreviations

FIC	fractional inhibitory concentration
MIC	minimum inhibitory concentration
CRA	Congo red agar
MTP	microtiter plate
RT-PCR	reverse transcriptase-polymerase chain reaction

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References

[1] Shukla S, Mishra P. *Pseudomonas aeruginosa* infections in broiler chicks in Jabalpur. International Journal of Environmental Research. 2015;**6**:37-39. Available from: https://www.researchgate.net/publication/304704794_Pseudomonas_aeruginosa_Infection_in_Broiler_Chicks_in_Jabalpur

[2] Kebede F. Pseudomonas infection in chickens. Journal of Veterinary Medicine and Animal Health. 2010;**2**:55-58. Available from: https://academicjournals.org/journal/JVMAH/article-full-text-pdf/663013539683

[3] Wilkinson GF, Pritchard K. In vitro screening for drug repositioning. Journal of Biomolecular Screening. 2015;**20**:167-179. DOI: 10.1177/1087057114563024

[4] Dandachi I, Sokhn ES, Dahdouh EA, Azar E, El-Bazzal B, Rolain JM, et al. Prevalence and characterization of multi-drug-resistant gram-negative bacilli isolated from lebanese poultry: A nationwide study. Frontiers in Microbiology. 2018;**9**:1-11. DOI: 10.3389/fmicb.2018.00550

[5] Shrivastava SR, Shrivastava PS, Ramasamy J, et al. World health organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Journal of Medical Society. 2018;**32**:76. Available from: http://www.jmedsoc.org/temp/JMedSoc32176-3543534_095035.pdf

[6] Siles SA, Srinivasan A, Pierce CG, Lopez-Ribot JL, Ramasubramanian AK. High-throughput screening of a collection of known pharmacologically active small compounds for identification of *Candida albicans* biofilm inhibitors. Antimicrobial Agents and Chemotherapy. 2013;**57**:3681-3687. DOI: 10.1128/AAC.00680-13

[7] Rangel-Vega A, Bernstein LR, Mandujano Tinoco E-A, Garcia-Contreras S-J, Garcia-Contreras R. Drug repurposing as an alternative for the treatment of recalcitrant bacterial infections. Frontiers in Microbiology. 2015;**6**:282. DOI: 10.3389/fmicb.2015.00282

[8] Torres NS, Abercrombie JJ, Srinivasan A, Lopez-Ribot JL, Ramasubramanian AK, Leung KP. Screening a commercial library of pharmacologically active small molecules against *Staphylococcus aureus* biofilms. Antimicrobial Agents and Chemotherapy. 2016;**60**:5663-5672. DOI: 10.1128/AAC.00377-16

[9] Yssel AEJ, Vanderleyden J, Steenackers HP. Repurposing of nucleoside-and nucleobase-derivative drugs as antibiotics and biofilm inhibitors. The Journal of Antimicrobial Chemotherapy. 2017;**72**:2156-2170. DOI: 10.1093/jac/dkx151

[10] Delattin N, De Brucker K, Vandamme K, Meert E, Marchand A, Chaltin P, et al. Repurposing as a means to increase the activity of amphotericin B and caspofungin against *Candida albicans* biofilms. The Journal of Antimicrobial Chemotherapy. 2013;**69**:1035-1044. DOI: 10.1093/jac/dkt449

[11] den Driessche F, Brackman G, Swimberghe R, Rigole P, Coenye T. Screening a repurposing library for potentiators of antibiotics against Staphylococcus aureus biofilms. International Journal of Antimicrobial Agents. 2017;**49**:315-320. DOI: 10.1016/j.ijantimicag.2016.11.023

[12] Nazli E, Zer Y, Eksi F. In vitro efficacy of various antibiotic combinations against *Pseudomonas aeruginosa* isolates. The Journal of International Medical Research. 2015;**43**:217-225. DOI: 10.1177/0300060514553490

[13] Batoni G, Maisetta G, Esin S. Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria. Biochimica et Biophysica Acta - Biomembranes. 2016;**1858**:1044-1060. DOI: 10.1016/j.bbamem.2015.10.013

[14] Grassi L, Maisetta G, Esin S, Batoni G. Combination strategies to enhance the efficacy of antimicrobial peptides against bacterial biofilms. Frontiers in Microbiology. 2017;**8**:2409. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5770624/

[15] Health protection agency. Identification of pseudomonas species and other non-glucose fermenters. Standards for microbiology investigations (UK SMI). 2015;**3**:3-41. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/422699/ID_17i3.pdf

[16] Jorgensen JH, Turnidge JD. Susceptibility test methods: Dilution and disk diffusion methods. In: Manual of Clinical Microbiology, 11th ed. American Society of Microbiology; 2015. pp. 1253-1273. Available from: https://www.asmscience.org/content/book/10.1128/9781555817381.mcm11.ch71?crawler=true

[17] CLSI (Clinical and Laboratory Standards Institute). M100-S25 Performance Standards for Antimicrobial. 2015;**35**(3):1-240. Available from: http://file.qums.ac.ir/repository/mmrc/CLSI2015.pdf

[18] Papich MG. Antimicrobials, susceptibility testing, and minimum inhibitory concentrations (MIC) in veterinary infection treatment. The Veterinary Clinics of North America. Small Animal Practice. 2013;**43**:1079-1089. DOI: 10.1016/j.cvsm.2013.04.005

[19] Kim S-H, Park C, Chun H-S, Lee D-G, Choi J-K, Lee H-J, et al. Pilot screening to determine antimicrobial synergies in a multidrug-resistant bacterial strain library. Microbial Drug Resistance. 2016;**22**:372-378. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4939386/

[20] Orhan G, Bayram A, Zer Y, Balci I. Synergy tests by E test and checkerboard methods of antimicrobial combinations against Brucella melitensis. Journal of Clinical Microbiology. 2005;**43**:140-143. DOI: 10.1128/JCM.43.1.140-143.2005

[21] Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. Journal of Clinical Pathology. 1989;**42**:872-874. DOI: 10.1136/jcp.42.8.872

[22] O'Toole GA. Microtiter dish biofilm formation assay. Journal of Visualized Experiments. 2011;**47**:1-2. DOI: 10.3791/2437

[23] Stepanović S, Vuković D, Hola V, Bonaventura GDI, Djukić S, Ćirković I, et al. Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 2007;**115**:891-899. DOI: 10.1111/j.1600-0463.2007.apm_630.x

[24] Ghadaksaz A, Fooladi AAI, Hosseini HM, Amin M. The prevalence of some pseudomonas virulence genes related to biofilm formation and alginate production among clinical isolates. Journal of Applied Biomedicine. 2015;**13**:61-68. DOI: 10.1016/j.jab.2014.05.002

[25] Yuan JS, Reed A, Chen F, Stewart CN. Statistical analysis of real-time PCR data. BMC Bioinformatics. 2006;**7**:85. DOI: 10.1186/1471-2105-7-85

[26] Spilker T, Coenye T, Vandamme P, LiPuma JJ. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas*

species recovered from cystic fibrosis patients. Journal of Clinical Microbiology. 2004;**42**:2074-2079. DOI: 10.1128/jcm.42.5.2074-2079.2004

[27] Goldberg JB. Why is *Pseudomonas aeruginosa* a pathogen? F1000 Biology Reports. 2010;**2**:29. DOI: 10.3410/B2-29

[28] Abdelraouf K, Kabbara S, Ledesma KR, Poole K, Tam VH. Effect of multidrug resistance-conferring mutations on the fitness and virulence of Pseudomonas aeruginosa. The Journal of Antimicrobial Chemotherapy. 2011;**66**:1311-1317. DOI: 10.1093/jac/dkr105

[29] Wareham DW, Curtis MA. A genotypic and phenotypic comparison of type III secretion profiles of *Pseudomonas aeruginosa* cystic fibrosis and bacteremia isolates. International Journal of Medical Microbiology. 2007;**297**:227-234. DOI: 10.1016/j.ijmm.2007.02.004

[30] Parsek MR, Singh PK. Bacterial biofilms: An emerging link to disease pathogenesis. Annual Review of Microbiology. 2003;**57**:677-701. DOI: 10.1146/annurev.micro.57.030502.090720

[31] Finnan S, Morrissey JP, O'gara F, Boyd EF. Genome diversity of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients and the hospital environment. Journal of Clinical Microbiology. 2004;**42**:5783-5792. DOI: 10.1128/JCM.42.12

[32] Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis. The Lancet Infectious Diseases. 2018;**18**:318-327. DOI: 10.1016/S1473-3099(17)30753-3

[33] Noori TE, Alwan MJ. Isolation and identification of zoonotic bacteria from poultry meat. International Journal of Advanced Research in Biological Sciencesi. 2016;**3**:57-66. Available from: https://pdfs.semanticscholar.org/488e/4253f660528b486642056c4d74aa3b6cc742.pdf

[34] Abiala M, Olayiwola J, Babatunde O, Aiyelaagbe O, Akinyemi S. Evaluation of therapeutic potentials of plant extracts against poultry bacteria threatening public health. BMC Complementary and Alternative Medicine. 2016;**16**:417. DOI: 10.1186/s12906-016-1399-z

[35] Nazer AHK, Dadras H, Eskandari S. Aerobic bacteria isolated from eggs and day-old chicks and their antibacterial resistance in shiraz, Iran. Iranian Journal of Veterinary Research. 2006;**7**:20-30. Available from: https://www.sid.ir/en/Journal/ViewPaper.aspx?ID=43085

[36] Nathwani D, Raman G, Sulham K, Gavaghan M, Menon V. Clinical and economic consequences of hospital-acquired resistant and multidrug-resistant *Pseudomonas aeruginosa* infections: A systematic review and meta-analysis. Antimicrobial Resistance and Infection Control. 2014;**3**:32. DOI: 10.1186/2047-2994

[37] Alfouzan W, Dhar R, Nicolau D. In vitro activity of newer and conventional antimicrobial agents, including Fosfomycin and Colistin, against selected gram-negative bacilli in Kuwait. Pathogens. 2018;**7**:75. DOI: 10.3390/pathogens7030075

[38] Domalaon R, Sanchak Y, Koskei LC, Lyu Y, Zhanel GG, Arthur G, et al. Short proline-rich lipopeptide potentiates minocycline and rifampin against multidrug-and extensively drug-resistant *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy. 2018;**62**:e02374-e02317

[39] Kuti JL, Wang Q, Chen H, Li H, Wang H, Nicolau DP. Defining the potency of amikacin against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas*

aeruginosa, and *Acinetobacter baumannii* derived from Chinese hospitals using CLSI and inhalation-based breakpoints. Infection and Drug Resistance. 2018;**11**:783. DOI: 10.2147/IDR.S161636

[40] Sutherland CA, Verastegui JE, Nicolau DP. In vitro potency of amikacin and comparators against *E. coli, K. pneumoniae* and *P. aeruginosa* respiratory and blood isolates. Annals of Clinical Microbiology and Antimicrobials. 2016;**15**:39. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4912699/

[41] Saiman L. Clinical utility of synergy testing for multidrug-resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis. Paediatric Respiratory Reviews. 2007;**8**:249-255. Available from: https://www.ncbi.nlm.nih.gov/pubmed/17868923

[42] Dundar D, Otkun M. In-vitro efficacy of synergistic antibiotic combinations in multidrug resistant *Pseudomonas aeruginosa* strains. Yonsei Medical Journal. 2010;**51**:111-116. DOI: 10.3349/ymj.2010.51.1.111

[43] Hawkey PM, Warren RE, Livermore DM, McNulty CAM, Enoch DA, Otter JA, et al. Treatment of infections caused by multidrug-resistant gram-negative bacteria: Report of the British Society for Antimicrobial Chemotherapy/healthcare infection society/british infection association joint working party. The Journal of Antimicrobial Chemotherapy. 2018;**73**:iii2-iii78

[44] Spoorthi NJ, Vishwanatha T, Reena V, Divyashree BC, Aishwarya S, Siddhalingeshwara KG, et al. Antibiotic synergy test: Checkerboard method on multidrug resistant *Pseudomonas aeruginosa*. International Research Journal of Pharmacy. 2011;**2**:196-198. Available from: https://irjponline.com/admin/php/uploads/754_pdf.pdf

[45] Häussler S, Parsek MR. Biofilms 2009: new perspectives at the heart of surface-associated microbial communities 2010. DOI: 10.1128/JB.00332-10

[46] Jain A, Agarwal A. Biofilm production, a marker of pathogenic potential of colonizing and commensal staphylococci. Journal of Microbiological Methods. 2009;**76**:88-92. Available from: https://www.ncbi.nlm.nih.gov/pubmed/18851996

[47] Cucarella C, Tormo MA, Ubeda C, Trotonda MP, Monzón M, Peris C, et al. Role of biofilm-associated protein bap in the pathogenesis of bovine *Staphylococcus aureus*. Infection and Immunity. 2004;**72**:2177-2185. Available from: https://www.ncbi.nlm.nih.gov/pubmed/15039341

[48] Osman K, Alvarez-Ordóñez A, Ruiz L, Badr J, ElHofy F, Al-Maary KS, et al. Antimicrobial resistance and virulence characterization of *Staphylococcus aureus* and coagulase-negative staphylococci from imported beef meat. Annals of Clinical Microbiology and Antimicrobials. 2017;**16**:1-10. DOI: 10.1186/s12941-017-0210-4

[49] Rasamiravaka T, Vandeputte OM, Pottier L, Huet J, Rabemanantsoa C, Kiendrebeogo M, et al. *Pseudomonas aeruginosa* biofilm formation and persistence, along with the production of quorum sensing-dependent virulence factors, are disrupted by a triterpenoid coumarate ester isolated from Dalbergia trichocarpa, a tropical legume. PLoS One. 2015;**10**:e0132791

[50] Smith-Palmer A, Stewart J, Fyfe L. The potential application of plant essential oils as natural food preservatives in soft cheese. Food Microbiology. 2001;**18**:463-470. Available from: https://www.sciencedirect.com/science/article/pii/S0740002001904150

[51] Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. Annual Review of Microbiology. 2002;**56**: 187-209. Available from: https://www.ncbi.nlm.nih.gov/pubmed/12142477

[52] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: A common cause of persistent infections. Science. 1999;**284**:1318-1322. Available from: https://www.ncbi.nlm.nih.gov/pubmed/10334980

[53] Drenkard E. Antimicrobial resistance of Pseudomonas aeruginosa biofilms. Microbes and Infection. 2003;**5**:1213-1219. Available from: https://www.ncbi.nlm.nih.gov/pubmed/14623017

[54] Wei Q, Ma LZ. Biofilm matrix and its regulation in *Pseudomonas aeruginosa*. International Journal of Molecular Sciences. 2013;**14**:20983-21005. Available from: https://www.ncbi.nlm.nih.gov/pubmed/24145749

[55] Wei B, Cha S-Y, Kang M, Park I-J, Moon O-K, Park C-K, et al. Development and application of a multiplex PCR assay for rapid detection of 4 major bacterial pathogens in ducks. Poultry Science. 2013;**92**:1164-1170. Available from: https://academic.oup.com/ps/article/92/5/1164/1573914

[56] Vasiljević Z, Jovčić B, Ćirković I, DJukić S. An examination of potential differences in biofilm production among different genotypes of *Pseudomonas aeruginosa*. Archives of Biological Sciences. 2014;**66**:117-121. Available from: http://www.doiserbia.nb.rs/Article.aspx?ID=0354-46641401117V#.XhmKw9QzbIU

[57] Emami S, Nikokar I, Ghasemi Y, Ebrahimpour M, Ebrahim-Saraie HS, Araghian A, et al. Antibiotic resistance pattern and distribution of pslA gene among biofilm producing *Pseudomonas aeruginosa* isolated from waste water of a burn center. Jundishapur Journal of Microbiology. 2015;**8**(11):e23669. DOI: 10.5812/jjm.23669

[58] Suriyanarayanan T, Periasamy S, Lin MH, Ishihama Y, Swarup S. Flagellin FliC phosphorylation affects type 2 protease secretion and biofilm dispersal in *Pseudomonas aeruginosa* PAO1. PLoS One. 2016;**11**(10):1-19. DOI: 10.1371/journal.pone.0164155

[59] Gümücs D, Kalayci-Yüksek F, Yörük E, Uz G, Çelik E, Arslan C, et al. Alterations of growth rate and gene expression levels of UPEC by antibiotics at sub-MIC. Folia Microbiologia (Praha). 2018;**63**:451-457. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29327292

[60] Raymond B. Five rules for resistance management in the antibiotic apocalypse, a road map for integrated microbial management. Evolutionary Applications. 2019;**12**(6):1079-1091. DOI: 10.1111/eva.12808

[61] Shahat HS, Mohamed H, Al-Azeem A, Mohammed W, Nasef SA. Molecular detection of some virulence genes in *Pseudomonas aeruginosa* isolated from chicken embryos and broilers with regard to disinfectant resistance. SVU-International Journal of Veterinary Sciences. 2019;**2**:52-70. Available from: https://svu.journals.ekb.eg/article_40727_bc49d8a75991c214123ab7c8573d73a4.pdf