The effects of *Staphylococcus aureus* on the β-lactamase enzymes and virulence factors of *Pseudomonas aeruginosa* in lung disease

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Abstract

Background

Pseudomonas aeruginosa and Staphylococcus aureus often lead to serious lung infections. This study aimed to investigate the role of Staphylococcus aureus in the expression of the β-lactamase enzymes and virulence factors of P. aeruginosa in the polymicrobial infections of the respiratory tract.

Results

It was demonstrated that S. aureus reduced the viability of P. aeruginosa , particularly in the biofilm state. In addition, S. aureus decreased β-lactam resistance but increased resistance to tobramycin in the biofilm condition. Furthermore, S. aureus showed a positive effect on reducing resistance to meropenem, doripenem, ertapenem, and tobramycin (except PA-2) and on increasing pathogenicity in P. aeruginosa . Moreover, the gene expression levels for lasR/I and algD were increased in biofilm conditions. The levels of lasI were more prominent in the virulent strain than the β-lactamase producing strain. Furthermore, the expression of KPC was increased in all strains of P. aeruginosa.

Conclusion

It was concluded that S. aureus has an inhibitory effect in polymicrobial infections by suppressing the β-lactamase genes and viability of P. aeruginosa and also cooperates with the biofilm-producing P. aeruginosa strains to increase pathogenicity and resistance to tobramycin.

Background

Lung infections are usually colonized by several microorganisms that interact with each other constituting complex polymicrobial communities [1, 2]. Two of the organisms that are frequently co-isolated from this type of infection are *Pseudomonas aeruginosa* and *Staphylococcus aureus* [3]. In vitro studies suggest that *P. aeruginosa* prospers better than *S. aureus* by multiplying faster and acting as its antagonist [4]. This might be related to the fact that *P. aeruginosa* seems to induce the biofilm production of *S. aureus* [5, 6]. Biofilms are structured communities that secrete and encase themselves within a protective matrix [1]. In addition, the presence of both pathogens is a sign of poor prognosis for the patients. Evidence shows that their virulence increases and antibiotic treatments become less efficient during co-infections [6, 7].

As a matter of fact, *P. aeruginosa* inhibits the electron chain transport of respiration using 4-hydroxy-2-heptylquinoline-N-oxide, kills *S. aureus* to acquire iron using the LasA protease, and produces rhamnolipids to disperse the *S. aureus* biofilm [8, 9]. *S. aureus*, on the other hand, increases its resistance to the inhibition of respiration by forming small colony variants and ferments carbon sources to lactic acid as the end product [10]. *P. aeruginosa* establishes a biofilm within the lung of the susceptible patient coupled with the overproduction of the exopolysaccharide alginate [11, 12]. Moreover, the pathogenicity

and antibiotic resistance of *P. aeruginosa* increases in polymicrobial infections [13]. Two of the microorganisms that are frequently co-isolated from this type of lung infection are *Staphylococcus aureus* and *P. aeruginosa* although they have a competitive relationship [14]. Nevertheless, causative microbial agents vary in the different levels of co-existing with mild infections generally being considered as monomicrobial and moderate and severe infections being considered as polymicrobial. Various studies have demonstrated that the dual-microbial infection of *P. aeruginosa* and *S. aureus* is more virulent and resistant than those of single species.

Using cell culture as an in vivo condition is one of the best methods for assessing the performance of *P. aeruginosa* and *S. aureus* in co-culture [14]. Co-culture models are a step towards bridging the gap between in vitro models and in vivo systems [5, 8]. Furthermore, accessory cells or support cells may aid in target cell growth, differentiation, and resistance to challenge. The cell line utilized in the present study is the widely used A549 type II alveolar epithelial cell model [1, 15]. The A549 cell line is a transformed variant of type II alveolar epithelia commonly used as a model of toxicology and drug delivery in the pulmonary epithelium [15]. Moreover, resistance to beta-lactams increases in the co-infections of *S. aureus* and *P. aeruginosa*. Furthermore, some virulence factors of *P. aeruginosa* (such as biofilm) play a more beneficial role in enhancing beta-lactamase resistance[16].

In this study, the resistance to beta-lactam and the pathogenicity of *P. aeruginosa* were evaluated in an in vivo model using the A549 respiratory cell line in co-culture with *S. aureus*.

Results

Molecular confirmation of the standard strains

Sequencing was done for *S. aureus* ATCC25923 with the accession number CP009361.1 (SA-1), for *P. aeruginosa* PAO1 with the accession number AE004091.2 (PAO1), for *P. aeruginosa* NCTC13359 (as the biofilm-producing strain) with the accession number LR590473.1 (PA-2), for *P. aeruginosa* NCTC13618 (as the toxin producer) with the accession number LR590474.1 (PA-3), and for *P. aeruginosa* NCTC12903 (as the MDR strain) with the accession number LR590474.1 (PA-4).

Long-term competition on the A549 cell line

The viability of *P. aeruginosa* in biofilm and planktonic co-culture with *S. aureus* was monitored by plate counts. At the beginning of the experiment, the relative abundance of the *P. aeruginosa* strains was slightly in favor of PAO1. In the biofilm state of co-culture, the survival of PA-2 and PA-3 was less than that of PA-4 (Figure 1, A1 and A3). Moreover, in the planktonic form, the PA-4 strain survived longer (Figure 1, A2 and A4). In the biofilm state of co-culture, the PAO1 and PA-4 strains had a higher cell density than the PA-2 and PA-3 strains at 48 hours of growth (Figure 1).

Antibiotic susceptibility pattern of the recovered *P. aeruginosa* strains

Figure 2 illustrates the antibiotic patterns of the *P. aeruginosa* strains. Resistance to all antibiotics increased after co-culture in the biofilm and planktonic conditions. Antibiotic susceptibility decreased more efficiently in the planktonic form compared with the biofilm one. Antibiotic resistance to meropenem and imipenem was more effectively increased in the beta-lactamase-producing strain compared with the biofilm and toxin producers. After two days, resistance to imipenem increased in the toxin-producing strain (PA-3) so that the MIC of imipenem ranged from 8 µg/mL to 3 µg/mL. Besides, in the MDR strain, increased resistance to meropenem was more than the other antibiotics. In addition, a decrease of imipenem, meropenem, and doripenem zone inhibitions was more observed in the pathogenic strains compared with the MDR strain. (Figure 2).

Virulence factors of the recovered *P. aeruginosa* strains

The co-culture of PAO1 and SA-1 showed no change in biofilm production after six days (Figure 3A). The LasA and LasB elastases in the PA-2 and PA-3 strains were enhanced after two days. In the PA-4 strain, the amounts of LasA and LasB elastases decreased after three days (Figure 3B). In the A549 cell line, pyocyanin production was increased in the co-cultures of PA-2 and SA-1 and PA-3 and SA-1 strains. Moreover, the highest amount of pyocyanin was produced on the fifth and sixth days. In the PAO1 strain, pyocyanin production decreased (Figure 3D). The production of pyoverdine showed a significant change on day six in the A549 cell co-culture conditions in all the *P. aeruginosa* strains (except for PA-4). Nevertheless, after six days, the PA-4 strain showed a little change in the production of pyoverdine (Figure 3E).

Gene expression of *P. aeruginosa* after co-culture in different states

After the biofilm co-culture of *P. aeruginosa and S. aureus*, the activities of *lasR* and *lasI* were up-regulated in *P. aeruginosa* in response to the presence of *S. aureus* (Figure 4Ac, Bc). The expression level of the *lasR* gene was higher in the co-culture of the PA-2 and SA-1 strains than that of the PA-4 and SA-1 strains (Figure 4Ab). However, the expression of the *lasR* gene in the planktonic state was lower than that of the biofilm form at different times (Figure 4Aa). The up-regulations of *lasI* and *algD* were observed in the biofilm co-cultures of PA-2 and SA-1 and PA-3 and SA-1 strains. Hence, the SA-1 strain had the greatest effect on the expressions of *lasI* gene (Figure 4Ba and 4Ca). However, the activity levels of *lasI* were more remarkable in the virulent strains than the KPC-producing strain (Figure 4Bb). The results of this experiment revealed that the SA-1 strain induced more virulence genes in the PA-2, PA-3, and PA-4 strains than the PAO1 strain (Figure 4Cc-Dc). The down-regulation of the *KPC* gene was observed in the biofilm and planktonic co-culture. Furthermore, the activity of the *mexR* gene did not change significantly in the different strains of *P. aeruginosa* (Figure 4Da).

Analysis of the data

The chi-square test showed a statistical significance between the numbers of mono-cultured and co-cultured bacteria ($p < 0.001$). According to the Student's t-test and $\chi 2$ test analysis (Figure 1), the biofilm co-culture containing SA-1+PAO1, SA-1+PA-2, and SA-1+PA-3 significantly decreased viability ((P:0.0004),

(P0.0006), and (P:0.0009), respectively). However, after analyzing the t-test results, there was no significant difference between the viability results of the PA-4 mono-culture and the SA-1+PA-4 co-culture (P:0.059), indicating that no synergism occurred. All the other co-culture combinations had a strong and impressive effect on viability.

According to the Student's t-test and χ2 test analysis (Figure 2), the production of carbapenemase in the combinations containing SA-1+PA-2 and SA-1+PA-3 declined considerably. However, after analyzing the t-test results, there was a notable difference between the observed and expected results, indicating that SA-1 had blocked the antibiotic resistance activity in all the *P. aeruginosa* strains. Furthermore, all the other co-culture combinations had a remarkable effect on tobramycin resistance. The Tukey analysis found the same results. Figure 2 also shows significant t-test results for co-cultures containing SA-1+PA-4. However, these results demonstrated a significant positive difference, indicating that antagonism occurred while SA-1 was in the co-culture. The Tukey analysis found the same results.

As shown in Figure 4, the co-culture containing *S. aureus*+*P. aeruginosa* produced significant t-test results so that *S. aureus* remarkably suppressed resistance activity in the biofilm model (P<Q0.0001). According to the Tukey results, the *S. aureus*+*P. aeruginosa* combination significantly increased biofilm production and virulence activity in the biofilm co-culture of *P. aeruginosa*. However, *S. aureus* in the planktonic co-culture had no significant effect on the virulence activity of *P. aeruginosa*. Figure 4 demonstrates that there was a significant positive difference between the observed and expected resistance to carbapenem antibiotics in the *S. aureus*+*P. aeruginosa* combination (P=0.0004). This indicates that the increase in the KPC expression was because of *S. aureus*. The one-away ANOVA analysis showed that each biofilm co-culture had a significant effect on the antibiotic resistance and production of the virulence factors of *P. aeruginosa*. The results suggested that there was a strongly significant difference between the virulence gene expressions of *S. aureus* and *P. aeruginosa* (P< 0.0006).

Discussion

The scientists who study the virulence of *P. aeruginosa* usually focus on its virulence factors to describe its pathogenic potential. However, the synthesis of these virulence factors requires a functional metabolism that furnishes the energy necessary for the lifestyle and virulence of this microorganism. This study showed that the virulent strains (PA-2 and PA-3) co-cultured with SA-1 in planktonic and biofilm conditions survived less than the PA-4+SA-1 strains (MDR). However, the PAO1 strain exhibited better survival than the PA-4 strain. Meanwhile, the *P. aeruginosa* populations in the biofilm co-culture were more than those of the planktonic form. Thus, SA-1 has a much more notable inhibitory effect on the PA-2 and PA-3 strains in the A549 cell culture. Our findings are in agreement with the studies of Alves *et al*. [16] and Hotterbeekx *et al*. [13] who suggested that *S. aureus* had a more inhibitory effect on the pathogenic strains of *P. aeruginosa*. Because of the depletion of oxygen and the suppression of growth in polymicrobial populations, *P. aeruginosa* changes its metabolic pathway for its survival and pathogenicity. Frapwell *et al*. [10] and Ali Mirani *et al*. [11] demonstrated that in the biofilm co-culture of *P. aeruginosa* with *S. aureus*, the viability of *P. aeruginosa* was reduced after three days which was the most

important reason for the type of metabolic pathway and the occurrence of genetic mutations in these bacteria. According to Orazi and O'Toole [17], the interaction between *P. aeruginosa* and *S. aureus* in co-culture conditions alters the metabolic pathway of *P. aeruginosa* and the bacterium shifts to fermentative growth and reduced antibiotic resistance. Although the biofilm-producing strain (PA-2) was more inhibited by *S. aureus* than most other *P. aeruginosa* strains, it remains to be seen how its pathogenicity and resistance changed.

Our observations indicated that the SA-1 strain had an inhibitory effect on the antibiotic resistance of PA-2 and PA-3 strains, while such effect was observed slightly less in the MDR strain of *P. aeruginosa* (PA-4). Increased resistance to tobramycin in the biofilm-producing strains of *P. aeruginosa* in co-culture with *S. aureus* was reported in the study of Beaudoin *et al*. [3]. Unlike the other strains, the PA-2 strain demonstrated an increase in tobramycin resistance. Furthermore, the antibiotic inhibition of *P. aeruginosa* strains in the planktonic state was more than that of the biofilm co-culture, although this sensitivity was lower in the MDR strains as expected based on the studies of Chan *et al*. [18] and DeLeon *et al*. [14] on the co-culture of *P. aeruginosa* with *S. aureus*. Yang *et al.* [5] also suggested that biofilm formation may be a beneficial survival characteristic in the co-culture. The biofilm can physically protect bacteria from antimicrobial agents particularly those with large polar molecules. This study demonstrated that, after six days, resistance to imipenem decreased in the PA-2 and PA-3 strains so that the MIC of meropenem ranged from 8 µg/mL to 4 µg/mL. Besides, increased resistance to meropenem in the MDR strain was more than those of the other antibiotics. Moody [19], Frapwell *et al*. [10], and Tognon *et al*. [9] showed that *S. aureus* reduced the antibiotic resistance of *P. aeruginosa* in co-culture conditions. Therefore, there was a significant correlation between SA-1 and the viability of *P. aeruginosa* strains (p:0.0001, p:0.0002, and p:0.0001 for PA-2, PA-3 and PA-4, respectively) so that the *S. aureus* co-culture had the lowest effect on the virulence factors and the highest impact on the antibiotic resistance of *P. aeruginosa*. Our findings are in agreement with those of Frapwell *et al*. [10].

P. aeruginosa is a denitrifying bacterium and thus possesses the ability to convert nitrate (NO3-) into nitrogen gas (N2). In response to *S. aureus*, *P. aeruginosa* differentially expresses nitrogen metabolism enzymes. Increasing nitrogen has an inducible effect on the pathogenesis of *P. aeruginosa* and augments the production of such factors as elastase, pyocyanin, biofilm, and toxins. Moreover, *P. aeruginosa* has many quorum-regulated virulence factors including elastase, pyocyanin, pyoverdine, and elastase. The studies by Kim *et al*. [20], Radlinski *et al*. [21], and Yang *et al*. [5] confirmed that the virulence of *P. aeruginosa* was altered by interaction with *S. aureus*. Based on the present study, the co-culture of PAO1+SA-1 showed no change in biofilm production after six days. However, SA-1 augmented LasA and LasB elastases in the PA-2 and PA-3 strains after two days. An enhanced production of pyocyanin in the PA-2+SA-1 and PA-3+SA-1 co-cultures was confirmed. Furthermore, *S. aureus* reduced the production of pyoverdine in all strains of *P. aeruginosa* after three days except for PA-4. Abisado *et al*. [22] and Yang *et al*. [5] demonstrated that *S. aureus* had a more significant inhibitory effect on the virulence and biofilm production of *P. aeruginosa* in an in vivo co-culture. Koley *et al*. [23] recently showed that pyocyanin created a redox potential gradient in the biofilm called 'electro line' which increased iron availability which is essential for the development of biofilm. In the interaction of bacteria, *S. aureus* acts as a potent iron

supplier for *P. aeruginosa*. Iron plays a crucial role in enhancing the Pseudomonas quinolone signal (PQS) activity of *P. aeruginosa*. Moreover, it induces biofilm production and quorum sensing (QS) [24]. This change provides the basis for the increased pathogenicity of *P. aeruginosa* in fermentative conditions. Another important issue addressed in the current study (also confirmed by the studies of Armbruster *et al*. [7], and Alves *et al*.[16] is that the production of pyoverdine and pyocyanin increased after the co-culture of the PA-3 and SA-1 strains. According to the study of Hotterbeekx *et al*. [13], there was a significant relationship between the increased pathogenicity of *P. aeruginosa* and the effects of *S. aureus*. They found that in polymicrobial infections, *S. aureus* increased the virulence factors of *P. aeruginosa*, which confirms our results.

In the current study, some differences were observed in the virulence factors and the KPC expression was significant among PAO1, PA-2, PA-3, and PA-4. In the biofilm state of co-culture, the expression of *lasR* was escalated in the PA-2+SA-1 strains. However, the expression of this gene decreased in PA-4+SA-1. The expression of the *lasR* gene in the planktonic state was lower than that of the biofilm form at different times. The increased expressions of the *lasI* gene in the co-cultures of PA-2+SA-1, PA-3+SA-1, and PA-4+SA-1 on the fourth and fifth days showed the most prominent effect on the ΔCT*lasI* gene. Woods *et al*. [8] observed that the expressions of the *lasR* and *lasI* genes of *P. aeruginosa* in the co-culture with *S. aureus* increased. These genes play a significant role in controlling QS in bacteria. With the increase of QS, the expression of antibiotic resistance genes decreases. In this study, it was demonstrated that the levels of *lasI* gene in biofilm- and toxin-producing strains were more pronounced than that of the carbapenem-resistant strain. The *algD* gene was highly expressed in the co-cultures of the PA-2+SA-1 and PA-3+SA-1 strains, however, no increase was observed in the PA-4+SA-1 co-culture. The PA-3+SA-1 strain was also not expressed on the sixth day. Similar results were reported by Limoli *et al*. 25] and Tognon *et al*. [9]. At first, the KPC gene expression in the biofilm and planktonic co-culture was reported. The fold change of KPC was increased in all the strains of *P. aeruginosa* and the highest activity of this gene was for the PA-4+SA-1 strain. In other words, all the strains showed the increased activity of carbapenemase from the third day onward. DeLeon *et al*. [14] used the term 'mutual benefit' for *P. aeruginosa* and *S. aureus* in cell culture conditions. Although these bacteria inhibit each other's viability, pathogenicity, and antibiotic resistance, with some genetic changes, the surviving population becomes more infectious and more resistant.

Limitation

A limitation of the current study is that many variables can affect the growth of the bacterium in the A549 cell line. Therefore, it is suggested that in future studies, the nutritional status of the cell be taken into account when evaluating QS-based gene expression. QS-based gene regulation models based on the studies of planktonic cells must be modified to explain the behavior of biofilm gene expression since gene expression in biofilms is dynamic. In addition, determining the physiological differences between biofilm and planktonic cultures is critical for understanding *P. aeruginosa* infections (such as those found in the cystic fibrosis lung) or for removing problematic biofilms from tissue infections.

Conclusion

Our findings demonstrated that an initial foundation is needed to explain how factors other than cell density can control the expressions of quorum sensing-regulated genes and carbapenemase genes. Since biofilm formation, toxicity, and carbapenem resistance cause the up- or down-regulation of quorum sensing regulated genes (*lasR/lasI*), it is not inconceivable that globally regulated genes can be controlled by more than one factor. Even though this conclusion is novel, it is not surprising. Furthermore, in the co-culture in the A549 cell line, a significant relationship was observed among the viability of *P. aeruginosa*, the activity of pathogenic enzymes, incubation time, resistance to carbapenem, and the expression of virulence genes. Carbapenemase enzymes played a more critical role than pathogenic enzymes in maintaining bacterial growth. Hence, in respiratory infections, resistance to carbapenem antibiotics in *P. aeruginosa* can provide a basis for the development and spread of co-infection with *S. aureus*. Besides, the production of pathogenic enzymes and biofilms by *P. aeruginosa* changes the metabolic pathways of the bacteria and causes the emergence of pathogenic strains.

Materials And Methods

Preparation of the standard strains

Some standard strains including *P. aeruginosa* PAO1, *S. aureus* ATCC25923, *P. aeruginosa* NCTC13359 (a strong biofilm-former and KPC-producing strain), *P. aeruginosa* NCTC13618 (a toxin and KPC-producing strain), and *P. aeruginosa* NCTC 13620 (a KPC-producing strain) were used in this study. All the strains were derived from clinical isolates and incubated at 37 °C unless described otherwise (200 rpm). Trypticase Soy Broth (supplemented by 1% agar when needed) was applied to culture the bacterial strains. Mannitol salt agar and cetrimide agar were used to recover *S. aureus* and *P. aeruginosa*, respectively.

DNA extraction and Sanger sequencing

The bacterial DNA was extracted using the QIAamp® DNA Mini Kit (QiaGene Inc., Chatsworth, Calif., USA), following the manufacturer's instructions. To determine the sensitivity of the reaction, 100 µL of the serially diluted *S. aureus* reference strain was used during DNA extraction. The amplification of 16S rDNA was performed as follows. The bacterial DNA was amplified using three sets of primers including *mutL*, *aroC*, and *rpoD*, [26, 27]synthesized by Pishgam Research, Pte. Ltd., Iran (Table 1).

All the reactions were performed in a 25 µl volume containing 1 µl of 10 pmol of each primer, 12.5 µl 2X master mix (Ampliqon, Denmark), 2 µl DNA template, and 8.5 µl of deionized water (Sigma-Aldrich, USA). The amplification of the genes was performed using a C1001 Touch Thermal Cycler (BioRad, USA) with the following thermocycling program: initial denaturation was done at 95 °C for 5 minutes followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at 57 °C for 1 minute, extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes [10]. This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (No: IRUMSHA. REC. 1396.694). The PCR

products were sequenced using the service of Sequiserve company (Pishgam Co., Tehran, Iran). Using the Chromas sequence analysis software (version 2.6.5, Technelysium Pty Ltd., South Brisbane, AU), the sequencing results were analyzed by employing the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database (BLAST).

Co-culture of *P. aeruginosa* and *S. aureus* on the A549 cell line

The co-culture assays were done as described by the study of Anderson *et al*. [28]. Briefly, the bacterial strains were cultured on a TSB medium at 37 °C overnight and then centrifuged at 10000g for five minutes. The pellets were suspended in a minimal essential medium (MEM) supplemented by L-glutamine and the OD_{600} was adjusted to 0.1. 250 µl of each bacterial suspension was added to the A549 monolayer and incubated at 37 °C and 5% CO_2. In one- and five-hour time intervals after incubation, the supernatants were removed and replaced by fresh MEM+L-Glu. The plates were incubated for an additional six days and then the supernatants were collected, serially diluted in PBS, and plated on MSA and CA for eighteen hours to count CFU/mL. The established biofilms were treated with 0.1% Triton X-100 in PBS and shaken vigorously for thirty minutes. All the tests were done in triplicate.

Biofilm assay of the recovered *P. aeruginosa* strains

A 500-µl sample of an overnight stationary phase broth culture was diluted 1:100 in a fresh and sterile broth media which was then grown to mid-exponential phase (00_{600} 0.7-1.0) at 37 ºC. 200 µl of this culture was pipetted into each well of a 96-well microtiter plate which was then incubated for four hours at 37 ºC. After incubation, the contents of the wells were gently aspirated. Each well was washed three times with 200 µl of sterile phosphate-buffered saline. 200 µl of safranin-O dye was then pipetted into each well to stain any resultant biofilm and the wells were then rinsed out with tap water. The plate was then dried in an incubator. Next, 200 µl of 70% ethanol was pipetted into each well and the plate was placed on a shaker at 100 revolutions per minute for fifteen minutes. The resultant solution in the microtiter plate wells was then read using a plate reader and the results were recorded [5]. All tests were done in triplicate.

Virulence factor production assay of the recovered *P. aeruginosa* strains

The productions of pyocyanin, pyoverdine, biofilm, LasA, LasB, and QS molecule were examined for the recovered strains as described in the studies of Oldak, El-Fouly, Limoli, and O'Toole [29-32]. All tests were done in triplicate.

Determination of the minimal inhibitory concentration of the recovered *P. aeruginosa* strains

Antibiotic disks (MAST, UK) and E-tests (Liofilchem, Italy) were used to examine the susceptibility of the bacteria to several antibiotics before and after their co-culture growth. The recovered bacteria from planktonic and biofilm conditions of co-culture were employed for antibiotic susceptibility testing using disk diffusion and MIC based on CLSI 2018. Antibiotic susceptibility was performed for imipenem,

meropenem, doripenem, and tobramycin. *P. aeruginosa* ATCC 27853 was used as the reference strain. All tests were done in triplicate.

RNA extraction and gene expression of the recovered *P. aeruginosa* strains

The total RNA was isolated during the log phase of the mono-cultures and over six periods during the log phase of the co-cultures. The strains were inoculated into LB broth (Merck, Germany) and then incubated at 37 °C. The RNA was extracted and cDNA synthesis was performed using the GeneAll RNA extraction kit and the GeneAll cDNA synthesis kit (GeneAll, Korea) according to the manufacturer's instructions. Quantitative real-time PCR was used to determine the expressions of *lasR*, *lasI, algD*, *mexR, and KPC* genes using the SYBR Green method and *rpoD* was employed as the reference gene. The primers used from different studies [2, 6, 26, 27, 33, 34] and are listed in Table 1. Each reaction contained 3 µL molecular grade water, 2 µL primers with a final concentration of 0.5 µM, and 10 µL SYBR Green master mix (Takara Bio, Inc., Otsu, Japan). The ABI StepOne-Plus LightCycler 96 (Applied Biosystems, Foster City, USA) was used. The cycling parameters included one denaturing cycle at 94 ^{o}C/15 minutes, followed by 40 three-step cycles of amplification (95 ^{o}C/30 seconds, 59 ^{o}C/30 seconds, and 72 ^{o}C/30 seconds). A melting curve was also drawn on the first run for each sample. The melting curve analysis was performed using a temperature range of 65 ^{o}C to 90 ^{o}C with a three-second interval. *P. aeruginosa* ATCC 27853 was used as the negative control. All tests were done in triplicate.

Statistical analysis

All the data were presented as mean ± SEM. For all the data collected, a two-way analysis of variance (ANOVA) was performed using GraphPad Prism 6.0 (Graph Pad Software, USA). When necessary, Tukey's test, the chi-square test, and the Student's t-test were applied to the data to determine the statistically significant changes by providing the adjusted p-values. All the presented p-values were adjusted for multiple comparisons. Gene expression analysis was performed using the REST® software (version 2009, Qiagen, Germany). The ΔΔCt method was used to determine the expression levels.

List Of Abbreviations

Minimal essential medium (MEM), Multidrug Resistant (MDR), Nnitrate (NO3-), Nitrogen gas (N2), Pseudomonas quinolone signal (PQS), Quorum Sensing (QS).

Declarations

Ethics approval and consent to participate: This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (Code No: IRUMSHA. REC. 1396.694) about the consent to participate is not applicable.

Consent for publication: Not Applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: No competing interests

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Author's contribution: Sanaz Dehbashi and Hamed Tahmasebi performed microbiological and molecular tests and write the manuscript. Mohammad Reza Arabestani supervised all of the stages of designing the study, conducting the research and writing the manuscript. Mohammad Yousef Alikhani plays a role in Project Administration.

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Tables

Table 1: Oligonucleotide sequences used in this study

Gene	Sequence	Reference
lasI	F: GAAATCGATGGTTATGACGC R: CGGCACGGATCATCATCTTC	[2]
lasR	F: AAGTGGAAAATTGGAGTGGAG R: GTAGTTGCCGACGACGATGAAG	[6]
mexR	F: TCAGAACCTGAAACAAGGTTG R: ATCGCCGGCGTTTTTCATTGTG	[33]
KPC	F: CGTCTAGTTCTGCTGTCTTG R: TTGTCATCCTTGTTAGGCG	[34]
algD	F: T GTCGCGCTACTACATGCGTC R: GTGTCGTGGCTGGTGATGAGA	[27]
rpoD	F: GGGCGAAGAAGGAAATGGTC R: CAGGTGGCGTAGGTGGAGAA	[26]

Figures

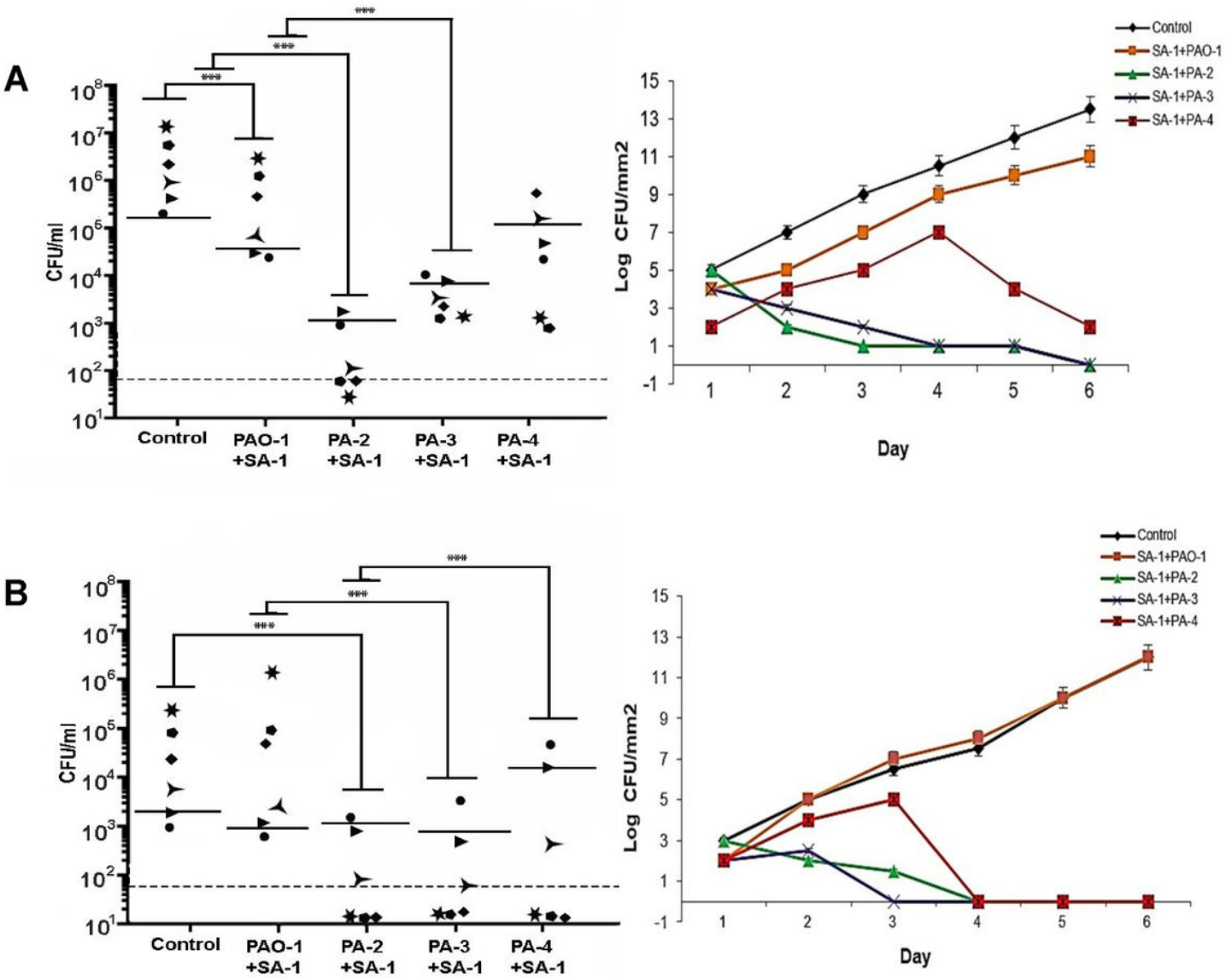

Figure 1

The biofilm (A) and planktonic (B) states of the co-culture of P. aeruginosa - S. aureus on the A549 cell line. 108 CFU/mL of PAO1 was obtained from corneas with and without the S. aureus co-culture. The viability of P. aeruginosa was measured as log10 (CFU/well) in the co-culture with S. aureus in a six-day period. PAO1: P. aeruginosa PAO1; SA-1: S. aureus ATCC 25923; PA-2: P. aeruginosa NCTC13359; PA-3: P. aeruginosa NCTC13618; PA-4: P. aeruginosa NCTC12903. The means of the colony counts in six different times (day 1 to day 6) are shown in the A549 cell line; star: sixth day; polygone: fifth day; diamond: fourth day; three-pointed star: third day; right arrowhead: second day; circle: first day. The error bars indicate the standard errors of the means in a representative triplicate time. The Student's t-test and $\chi 2$ test were used to test the differences between the groups. *: $p < 0.05$, **: $p < 0.001$, ***: $p < 0.0001$.

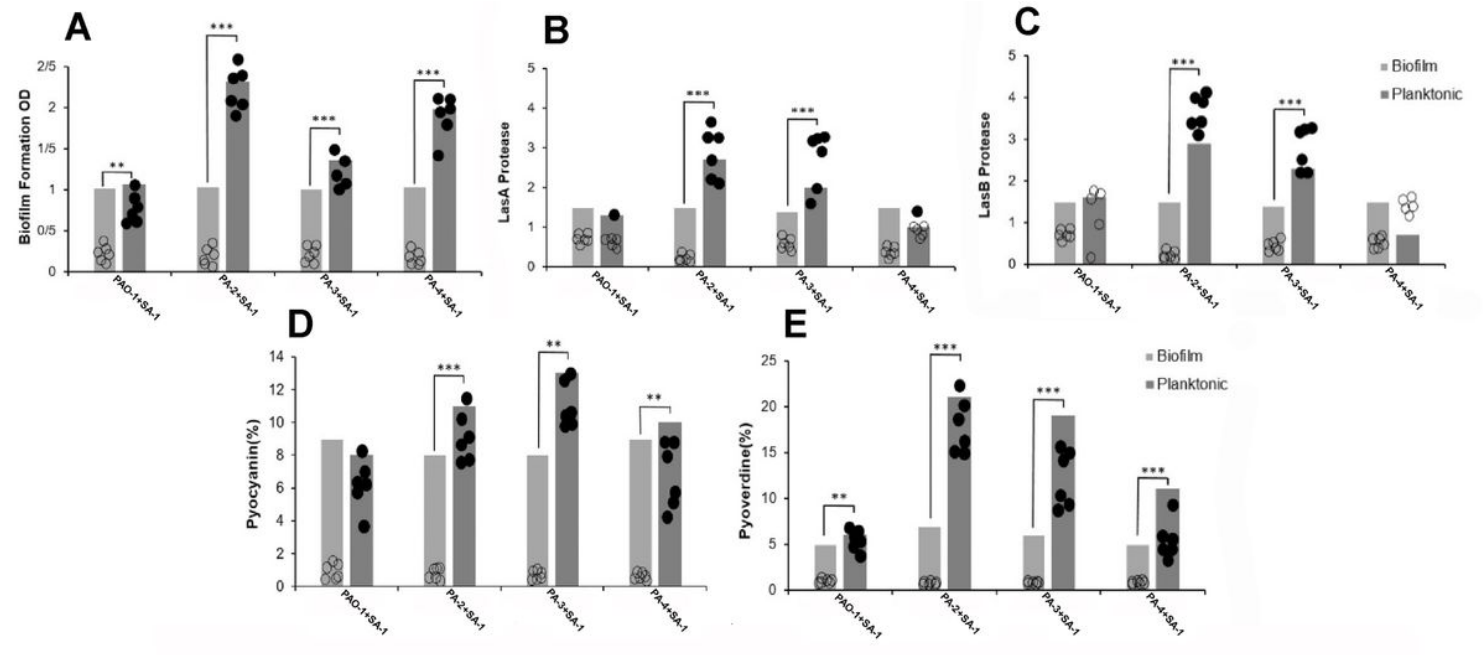

Figure 2

The role of the co-culture of P. aeruginosa - S. aureus on the A549 cell line in biofilm and planktonic formations, LasA protease, LasB protease, pyocyanin, and pyoverdine. The biofilm formation of P. aeruginosa (A), the amounts of the total LasA (B) and LasB proteases (C), the percentages of pyoverdine (D) and pyocyanin (E) in the P. aeruginosa - S. aureus co-culture on the A549 cell line on day 6. PAO1: P. aeruginosa PAO1; SA-1: S. aureus ATCC 25923; PA-2: P. aeruginosa NCTC13359; PA-3: P. aeruginosa NCTC13618; PA-4: P. aeruginosa NCTC12903. The filled circles represent detectable +/+ results, while the clear circles represent undetectable -/- results. Each circle represents a time and the numbers are indicated under each bar. The means and SDs from the triplicate experiments using the Student's t-test and $\chi 2$ test showed the differences between the groups. *: $p < 0.05$, **: $p < 0.001$, ***: $p < 0.0001$. No stars: the non-statistical correlations.

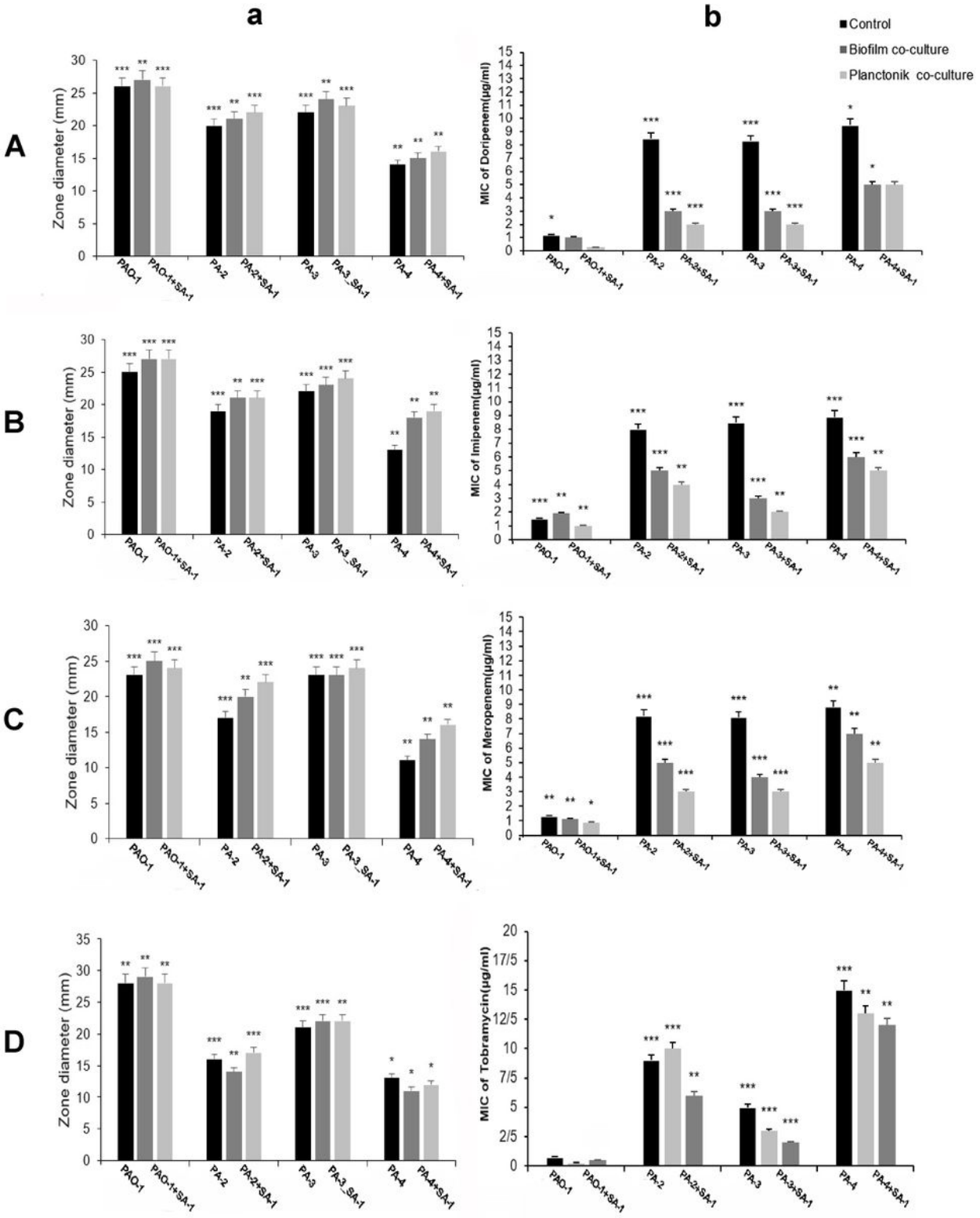

Figure 3

The effect of the P. aeruginosa + S. aureus co-culture on the A549 cell line on the inhibition zone (a) and the MIC (b) of antibiotics. The MICs of doripenem (A), imipenem (B), meropenem (C), and tobramycin (D) on the P. aeruginosa strains. a1: control; a2: PAO1+SA-1; b1: control; b2: PA-2 + SA-1; c1: control; c2: PA-3 + SA-1; d1: control; d2: PA-4 + SA-1. Each circle represents a time and the numbers are indicated under each bar. The means and SDs from the triplicate experiments using the Student's t-test and χ2 test

showed the differences between the groups. *: p < 0.05, **: p < 0.001, ***: p < 0.0001. No stars: the non-statistical correlations.

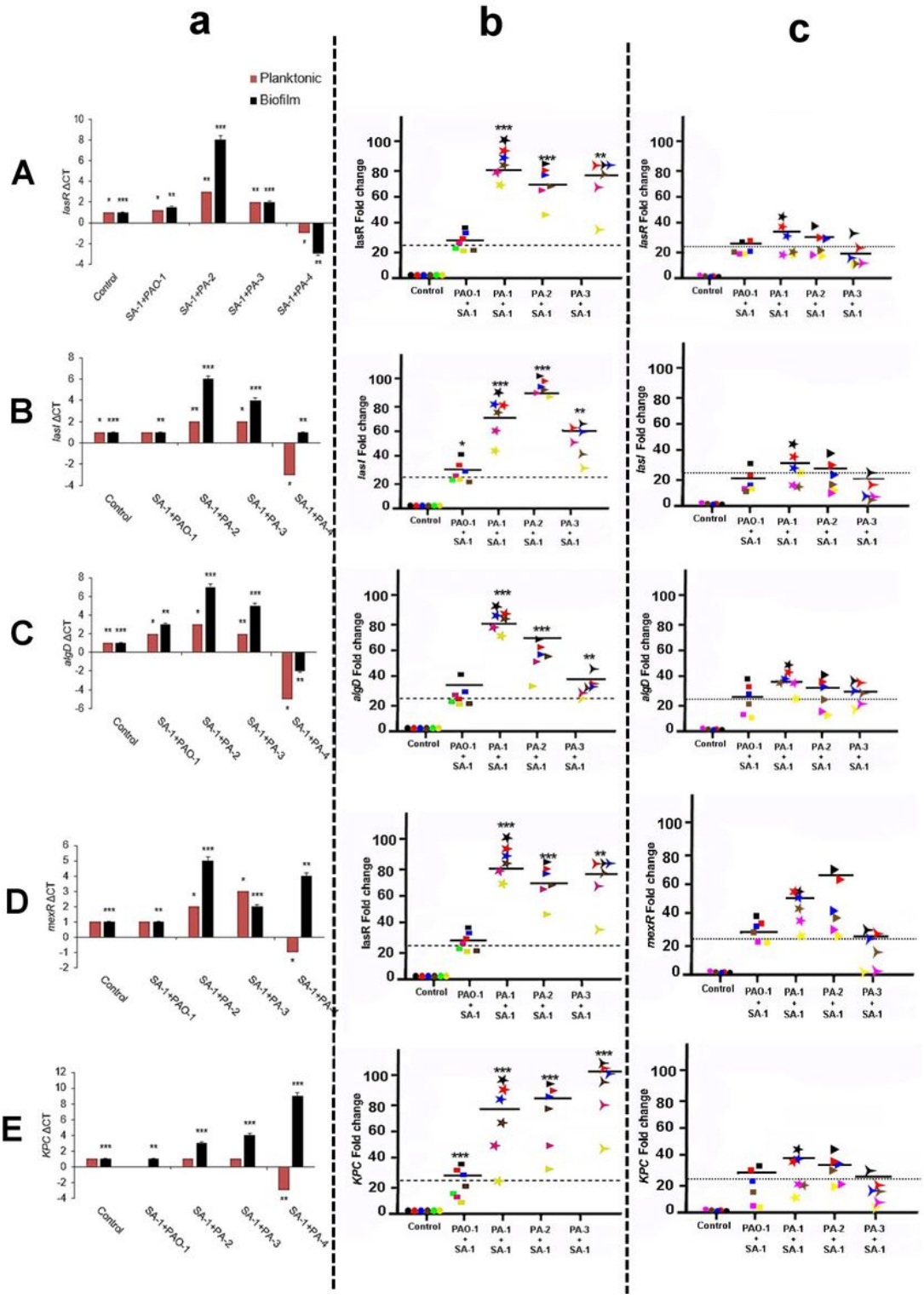

Figure 4

The expressions of lasR (A), lasI (B), algD (C), mexR (D), and KPC (E) genes in P. aeruginosa. a) The ΔCT values of the infected A549 cells with the P. aeruginosa - S. aureus co-culture normalized to aroC. The dotted horizontal lines represent the limit of detection. The means and SDs of the six experiments. b) The

fold changes of the biofilm form in the expressions of lasR, lasI, algD, mexR, and KPC for the infected A549 cells as determined by RT-qPCR. c) The fold changes of the planktonic form in the expressions of lasR, lasI, algD, mexR, and KPC for the infected A549 cells as determined by RT-qPCR normalized to aroC and compared to the uninfected control cells. The ΔRn and CT values of the P. aeruginosa error bars indicate the standard errors of the means from a representative triplicate time. The means of the colony counts in six different times (day 1 to day 6) are shown in the A549 cell line; star: sixth day; polygone: fifth day; diamond: fourth day; three-pointed star: third day; right arrowhead: second day; circle: first day. Black color: sixth day; red color: fifth day; blue color: fourth day; brown color: third day; purple color: second day; yellow color: first day. The Student's t-test and the ANOVA test were performed to test the differences between the groups. *: $p < 0.05$, **: $p < 0.001$, ***: $p < 0.0001$. No stars: the non-statistical correlations.