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TESIS

ANTIBIOFILM EFFECTS OF CORTICOSTEROID AND NON-STEROIDS ANTI-INFLAMMATORY DRUGS COMBINED WITH CURRENT ANTIBIOTICS AGAINST *Staphylococcus aureus* AND *Pseudomonas aeruginosa*

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Abstract

With the spread of drug-resistant bacteria and the lack of effective antibiotics to treat them, the development of new therapeutic methods and strategies is essential. In this study, we evaluated the antibacterial and anti-biofilm activity, as well as the transcription level of biofilm-associated genes, when clinical isolates of *S. aureus* and *P. aeruginosa* were treated with dexamethasone sodium phosphate (DXP), the NSAIDs ibuprofen (IBP), and acetylsalicylic acid (ASA) in combination with ciprofloxacin, gentamicin, cefepime, imipenem, and meropenem**.** Minimal inhibitory concentration (MIC), minimal biofilm inhibitory concentration (MBIC), and minimum biofilm eradication concentration (MBEC) of ciprofloxacin, gentamicin, cefepime, imipenem, and meropenem with/without sub-MIC of IBP, ASA, and DXP were determined by the microbroth dilution method. qPCR was used to determine expression levels of *icaA* and *algD* in *S. aureus* and *P. aeruginosa*, respectively, at sub-MIC of IBP, ASA, and DXP. Our results showed that the DXP did not have antibacterial or antibiofilm activity. IBP decreased the level of MIC, MBIC, and MBEC to antibiotic agents in both clinical isolates, except for cefepime in *P. aeruginosa*. Among MRSA isolates, ASA decreased MIC to gentamicin, cefepime, and imipenem, and it was able to reduce MBIC of imipenem and meropenem. Among carbapenem-resistant *P. aeruginosa*, ASA decreased MIC to cefepime, imipenem, and meropenem, MBIC to cefepime and meropenem, and MBEC to cefepime. DXP increased the level of MIC to ciprofloxacin, gentamicin, and cefepime in both clinical isolates. The MBIC to ciprofloxacin, gentamicin, and cefepime increased among both clinical isolates. MBEC of MRSA isolates to ciprofloxacin and cefepime increased, as did MBEC to ciprofloxacin, gentamicin, and meropenem among carbapenem-resistant *P. aeruginosa*. qPCR results showed that sub-MIC of IBP and ASA could significantly decrease the transcription level of *algD* in *P. aeruginosa*, and sub-MIC of IBP could significantly decrease

the transcription level of *icaA* in *S. aureus*. DXP significantly increased the expression level of *algD* and *icaA* genes in *S. aureus* and *P. aeruginosa*, respectively. In contrast to DXP, our results showed that ASA and IBP have significant effects on decreasing MIC, MBIC, and MBEC levels to some antibiotics and can down-regulate the expression of biofilm-related genes such as *icaA* and *algD*. Therefore, NSAIDs represent appropriate candidates to the design of new antibacterial and anti-biofilm therapeutics.

Keywords: Antibiotics, Biofilm, Anti-inflammatory drugs, *icaA*, *algD*

Chapter 1:

Introduction and Literature review

Staphylococcus aureus (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are opportunistic human pathogens capable of causing severe infections in hospital settings. These bacteria are usually multiple antibiotic-resistant, therefore the selection of antibiotics for treatment of infections by these bacteria is very hard. The ability of bacteria to produce an extracellular slime and constitutive biofilm enables these organisms to withstand the host immune response and to make clinical treatment extremely difficult because biofilm formation protects bacteria from antimicrobial agents and immune response. Biofilm formation has an important role in the pathogenicity of bacteria and biofilm has low permeability to antimicrobial agents.

las and *rhl* genes are the main genes in systems that control the expression of the *P. aeruginosa* genome. These genes control the expression of nearly 10% of the *P. aeruginosa* genome including virulence and biofilm genes. In *P. aeruginosa* the Las and Rhl systems have an important role in the production of elastase, protease, hemolysin, biofilm formation and motility. *P. aeruginosa* utilizes quorum-sensing to regulate expression of many virulence genes and biofilm formation. Quorum-sensing (QS) signal molecules termed autoinducers (AI) have an important role in the biofilm formation in *P. aeruginosa*. The las system is comprised of the transcriptional activator LasR and the AI synthase enzyme LasI, which directs the synthesis of the signal molecules in QS. In *P.*

aeruginosa alginate has an important role in biofilm production and is a polymer consisting of β-D-mannuronic acid and α-l-guluronic. *algACD* operon is responsible for the synthesis of alginate. AlgD is an enzyme (GDP-mannose dehydrogenase) encoded by *algD* that catalyzes the production of GDP-mannuronic acid from GDP-mannose that is important elements for biofilm formation.

In *S. aureu*s some operon and genes named *icaABCD*, *agr*, and *sigB* genes systems have an important role in biofilm formation. In *Staphylococcus spp*, biofilm formation has demonstrated that bacteria cell aggregation and biofilm accumulation are mediated by the products of the *ica operon* [*icaR*; regulatory and *icaADBC*; biosynthetic genes], which comprises four genes *icaA*, *icaB*, *icaC* and *icaD* and a regulator gene *icaR*, which seems to function as a repressor. The production of *ica* genes is polysaccharide intercellular adhesin (PIA). The PIA has different roles such as intercellular adhesin, resistance to antibiotic agents, and resistance to phagocytosis or immune response. PIA is composed of b-1,6-linked N-acetylglucosamine residues (80–85%) and an anionic fraction with a lower content of non-N-acetylated D-glucosaminyl residues that contain phosphate and ester-linked succinate (15–20%). Some studies have shown that *ica* operon are necessary for biofilm formation and virulence and response to anaerobic growth.

There are different types of anti-inflammatory drugs including non-steroidal (NSAIDs; aspirin, ibuprofen, diclofenac, naproxen, etc.) and corticosteroidal anti-inflammatory drugs (dexamethasone, betamethasone, and hydrocortisone) that are commonly used for ameliorate fever and other symptoms of acute and chronic infections. The use of a combination of anti-inflammatory drugs with antibiotics agents in the treatment of infections can have different effects on biofilms. It was demonstrated that anti-inflammatory drugs are effective on biofilm formation alone and with combination of some antibiotic agents in bacteria.

There are low data or reports about the mechanism effect of anti-inflammatory on transcriptional level of genes related to biofilm formation, therefore, we will determine the in vitro interactions between aspirin, dexamethasone, and Ibuprofen in combination with commonly used antibacterial agents, including gentamicin (GM), ciprofloxacin (CIP), cefepime (FEP), imipenem (IPM) and meropenem (MEM). The combinatorial treatments and interactions will be studied against planktonic and biofilm forms of *P. aeruginosa* and *S. aureus* including the expression level of related biofilm genes; *icaA* and *algD* to decipher antibiofilm mechanisms of action.

1-1. Mechanism of antibiotics (Antibacterial agents)

The mechanisms of action of antibacterial agents can be discussed under six headings:

- Cell wall synthesis inhibitors: β-lactams (penicillins, cephalosporins, carbapenems, and monobactams) and glycopeptides (vancomycin & teicoplanin).
- DNA and RNA synthesis inhibitors:
- DNA synthesis inhibitors: Earlier quinolones (nalidixic acid), fluoroquinolones (ciprofloxacin).
- RNA synthesis inhibitors: rifampin
- Protein synthesis inhibitors:
- Bind to 30S unit of bacterial ribosomes: tetracycline, tigecycline, aminoglycosides (Gentamicin & Amikacin).
- Bind to 50S unit of bacterial ribosomes: chloramphenicol, erythromycin.
- Inhibition of cell membrane function: polymixins (A & E).
- Inhibiting the synthesis of essential metabolites: sulfonamides.
- Other antibacterial agents with specialized uses: dapsone, metronidazole, and isoniazid (1).

2-1. Mechanism action of cefepime, imipenem, and meropenem

Cefepime (FEP), imipenem (IPM), meropenem (MEM) are beta-lactam antibiotics that kill bacteria by inhibiting cell wall synthesis. These antibiotics inhibit the synthesis of peptidoglycans or cell walls in bacteria by binding to penicillin-binding proteins (PBPs) (Figures 1-1 and 2-1). Cefepime is one of the fourth generation cephalosporins that has a very good effect against gram-positive and gram-negative bacteria. Meropenem is one of the broad-spectrum beta-lactams of the carbapenem family. This group of antibiotics plays an important role in the treatment of infections by multi-drug resistance bacteria (1, 2).

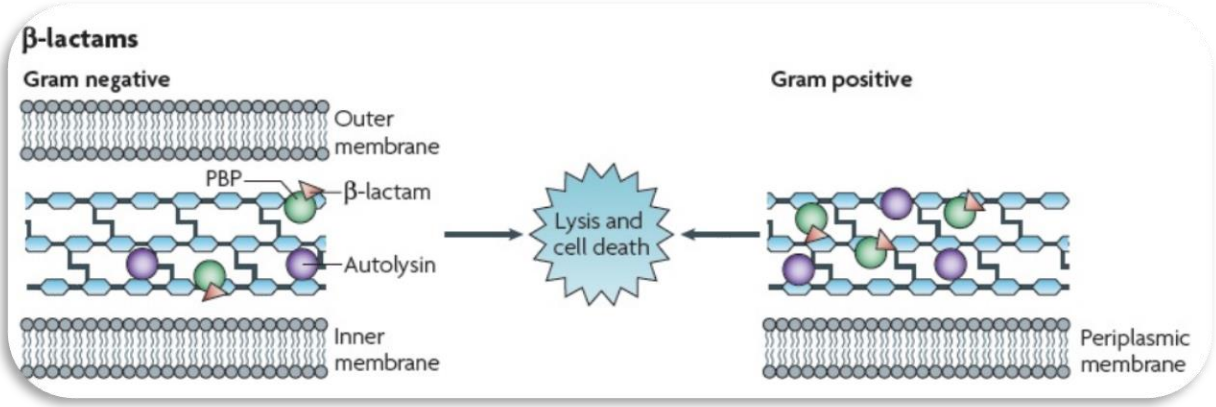

Figure 1-1. Interaction between beta-lactam antibiotic and target (PBPs). Beta-lactams antibiotics such as meropenem and cefepime inhibit transpeptidation by binding to PBPs on maturing peptidoglycan strands. The decrease in peptidoglycan synthesis and increase in autolysins leads to lysis and cell death (1).

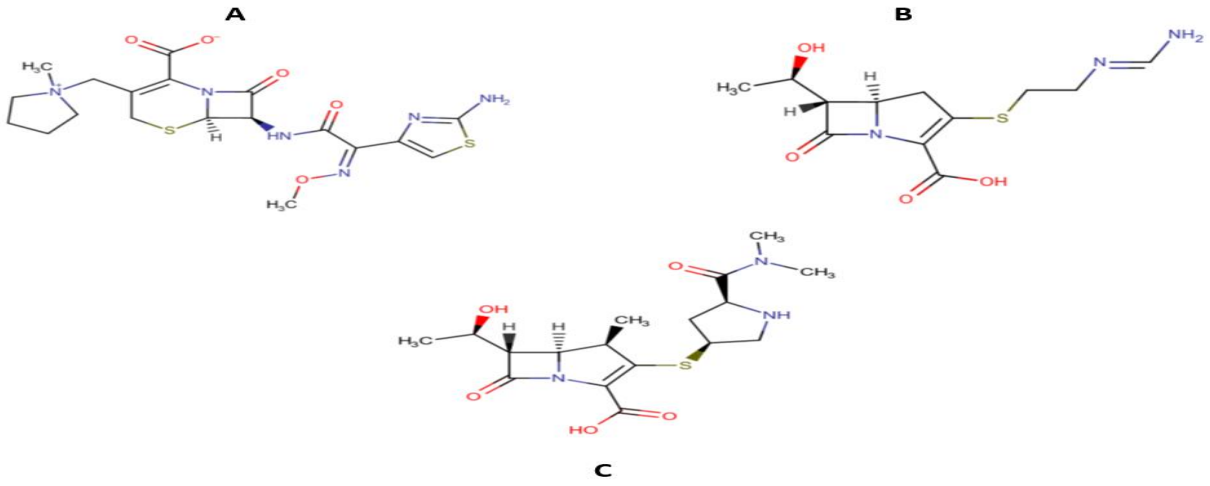

Figure 2-1. Molecular structure of cefepime (A), imipenem (B) and meropenem (C) as beta-lactam antibiotics (3).

3-1. Mechanism action of gentamicin

Gentamicin is one of the aminoglycoside antibiotics. This group of antibiotics binds to a small subunit of the ribosome (30S) to inhibit protein synthesis or mRNA mistranslation (Figures 3-1 and 4-1). Other members of this family include amikacin, streptomycin, and kanamycin (1, 2).

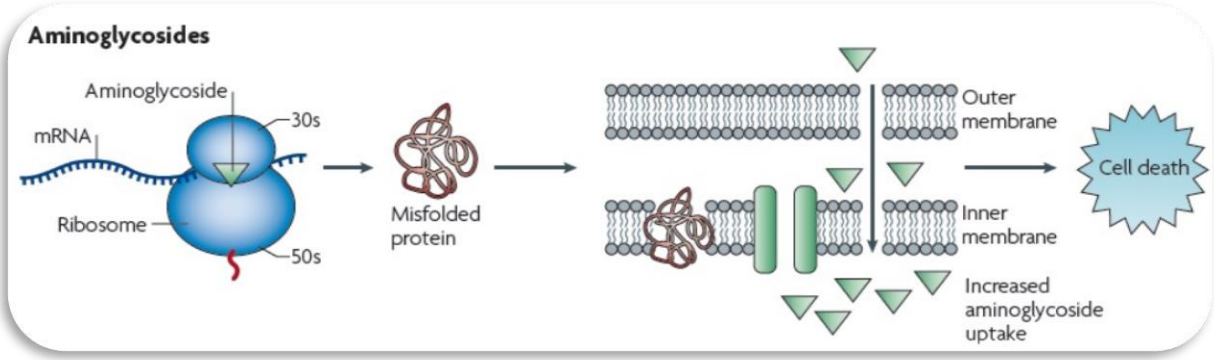

Figure 3-1. Aminoglycosides such as amikacin and gentamicin bind to the 30S subunit (small unit) of the ribosome and inhibit protein synthesis. Also, aminoglycosides antibiotics can cause mistranslated proteins. misincorporation of amino acids into elongating peptides. These inhibit protein synthesis and mistranslated proteins with misfolded of them were related to cell death (1).

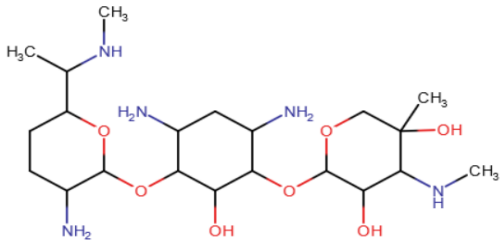

Figure 4-1. Molecular structure of gentamicin (3).

4-1. Mechanism action of ciprofloxacin

Ciprofloxacin is one of the antibiotics in the fluoroquinolone family. This group of antibiotics prevents DNA replication by binding to enzymes called topoisomerase (Figures 5-1 and 6-1) (1, 2). Table 1-1 shows the mechanism of action and metabolic pathways that are affected by antibiotics.

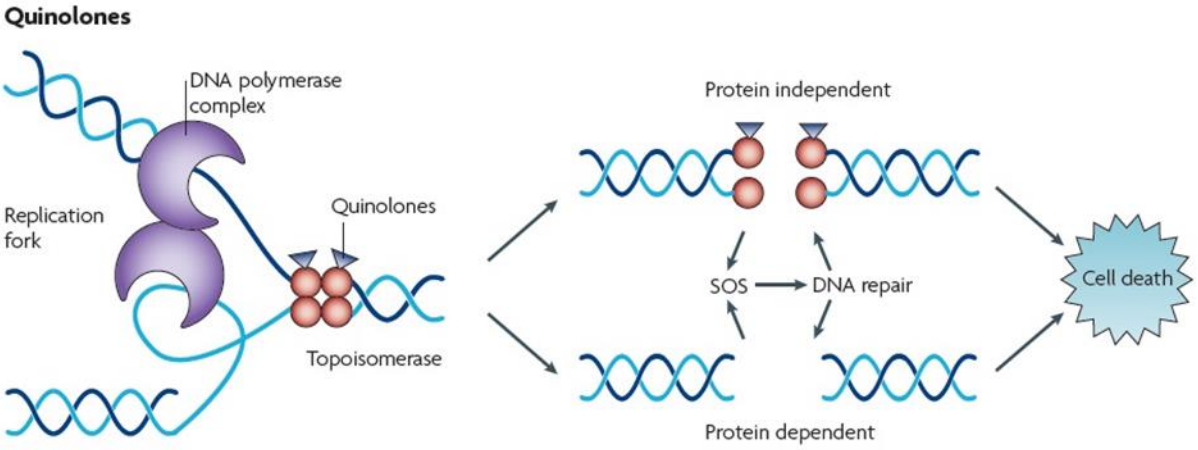

Figure 5-1. Fluoroquinolones antibiotics such as ciprofloxacin kill bacterial cells through inhabit DNA replication by interaction with topoisomerase enzyme and supercoil changing (1).

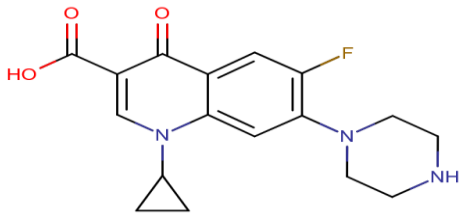

Figure 6-1. Molecular structure of ciprofloxacin (3).

Table 1-1. Antibiotic targets and pathways (1)(2)**.**

Antibiotic agents	Family or Type	Mechanism	Derivation	Species range	Primary target	Pathways affected
Ciprofloxacin	Fluoroquinolones	DNA synthesis inhibitor	Synthetic	Gram-positive, Gram-negative bacteria, anaerobic bacteria	Topoisomerase	DNA replication, SOS response, cell division, and ATP generation
Cefepime, imipenem & meropenem	β-lactams	Cell wall synthesis inhibitors	Natural and semi-synthetic	Aerobic and anaerobic bacteria (Gram-positive & Gram-negative)	PBPs	Cell wall synthesis, cell division, autolysin activity, SOS response, and TCA cycle
Gentamicin	Aminoglycosides	Protein synthesis inhibitors	Natural and semi-synthetic	Aerobic bacteria (Gram-positive & Gram-negative bacteria)	30S ribosome	Protein translation (mistranslation by tRNA mismatching), SOS response, and TCA cycle

PBPs: Penicillin-binding proteins, **SOS:** Global response to DNA damage in bacteria, **TCA:** Tricarboxylic acid.

5-1. Mechanism of resistance to antibiotics

Bacteria could resist antibiotics through various mechanisms. The mechanisms of resistance to antibacterial agents can be discussed under headings (1, 2):

1. Enzymatic destruction or inactivation of the antibiotic

2. Prevention of penetration to the target site

3. Alteration of the antibiotic's target site

4. Efflux pumps systems

5. Biofilm formation

1-5-1. Resistance to beta-lactam antibiotics (cefepime, imipenem, and meropenem)

So far, four main mechanisms of resistance to beta-lactam antibiotics have been identified. These mechanisms include the following (1, 2):

1-1-5-1. Beta-lactam ring hydrolysis by β-lactamases enzymes production: Many bacteria by production of extended-spectrum beta-lactamases (ESBL), metallo-β-lactamase (MBL), and AmpC β-lactamases can be resistant to beta-lactam antibiotics. ESBLs, MBLs, and AmpC β-lactamases can hydrolyze different beta-lactam antibiotics such as penicillins, cephalosporins, carbapenems, and monobactams (Figure 7-1).

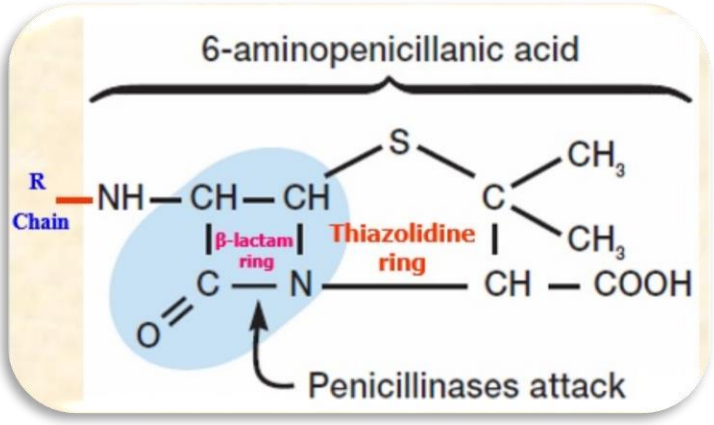

Figure 7-1. Hydrolyzing of beta-lactam ring by the penicillinase enzyme (as beta-lactamase). Beta-lactam antibiotics are inactivated by hydrolysis of the beta-lactam ring (3).

2-1-5-1. Prevention of penetration to the cell

Preventing antibiotics from entering the bacterial cell is another mechanism of resistance to beta-lactam antibiotics (Figure 8-1). In this type of resistance mechanism, the drug entry channel is reduced or lost. For example, OprD is a porin channel in the outer membrane of the *P. aeruginosa*, which is a channel for entry of carbapenem antibiotics. Factors such as inactivation of *oprD* with insertion elements (ISs), point mutations, and decreased expression of the *oprD* increase carbapenem resistance in *P. aeruginosa*.

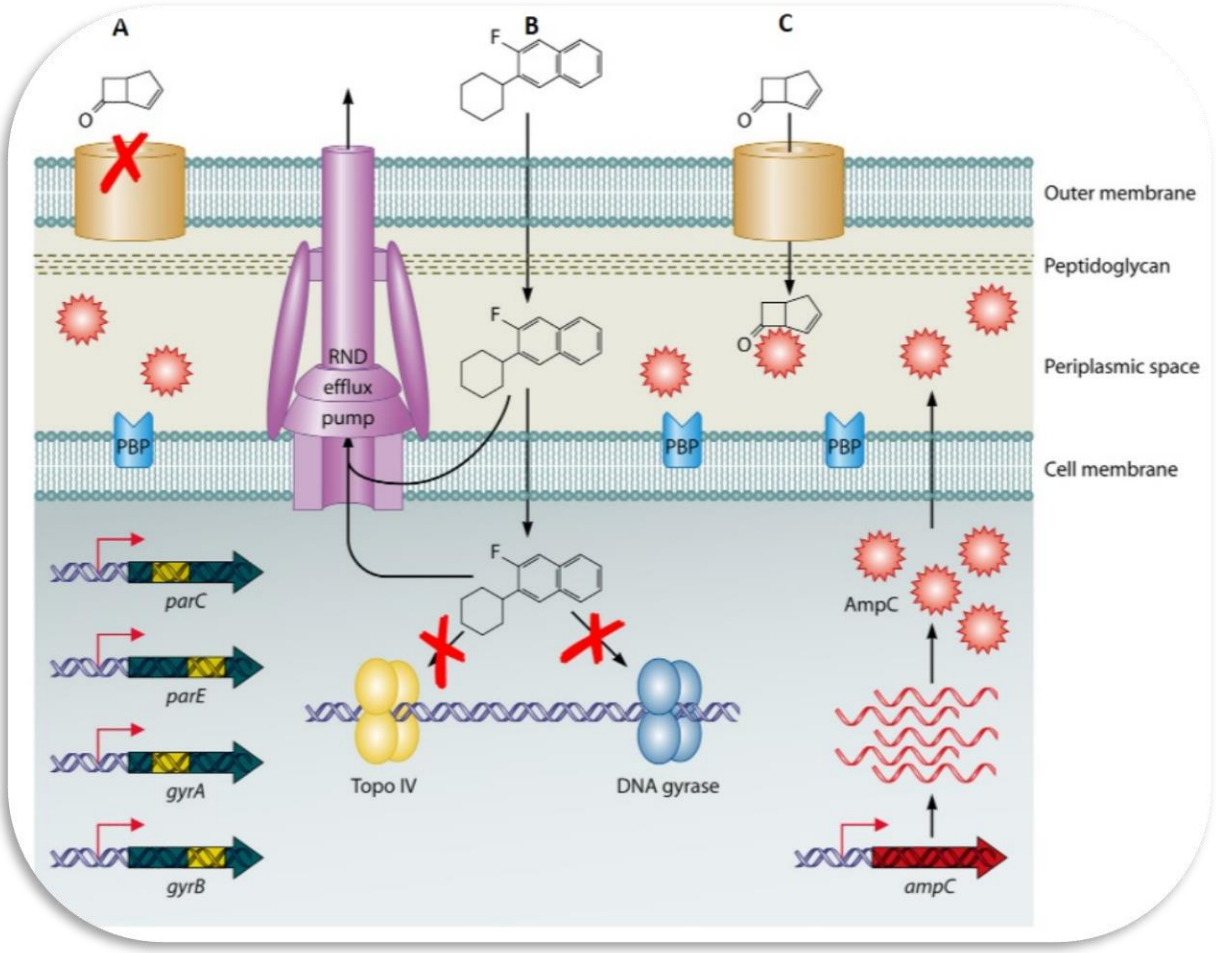

Figure 8-1. Resistance mechanisms to beta-lactams and fluoroquinolone antibiotics in a gram-negative bacterium. A: Resistance to beta-lactam antibiotics by blocking the entry of antibiotics (Prevention of penetration to the target site); B: Resistance to fluoroquinolone antibiotics such as ciprofloxacin by the change of target sites (Topo IV and DNA gyrase as topoisomerase enzymes) and efflux pumps activation; C: Hydrolyzing of beta-lactam ring by the AmpC β-lactamases (as beta-lactamase) (2).

3-1-5-1. Alteration of the antibiotic's target site

Changes in antibiotic targets are one of the most important mechanisms of resistance to antibiotics, including beta-lactam (Figure 9-1). Bacteria, especially gram-positive bacteria, become resistant to beta-lactam antibiotics by altering

penicillin-binding proteins (PBPs). One of the most well-known mechanisms of resistance to beta-lactam antibiotics by altering the PBPs is found in *Staphylococcus aureus*. A gene called *mecA* in *S. aureus* is responsible for producing a type of PBPs called PBP2a. This type of PBP is reluctant to bind to beta-lactam antibiotics, and as a result, bacteria containing PBP2a become resistant to antibiotics such as methicillin, penicillin, and other penicillin-like antibiotics. In *S. aureus* and other species of staphylococci, the *mecA* gene is carried on a transmissible genetic cassette called SCC*mec* (4, 5).

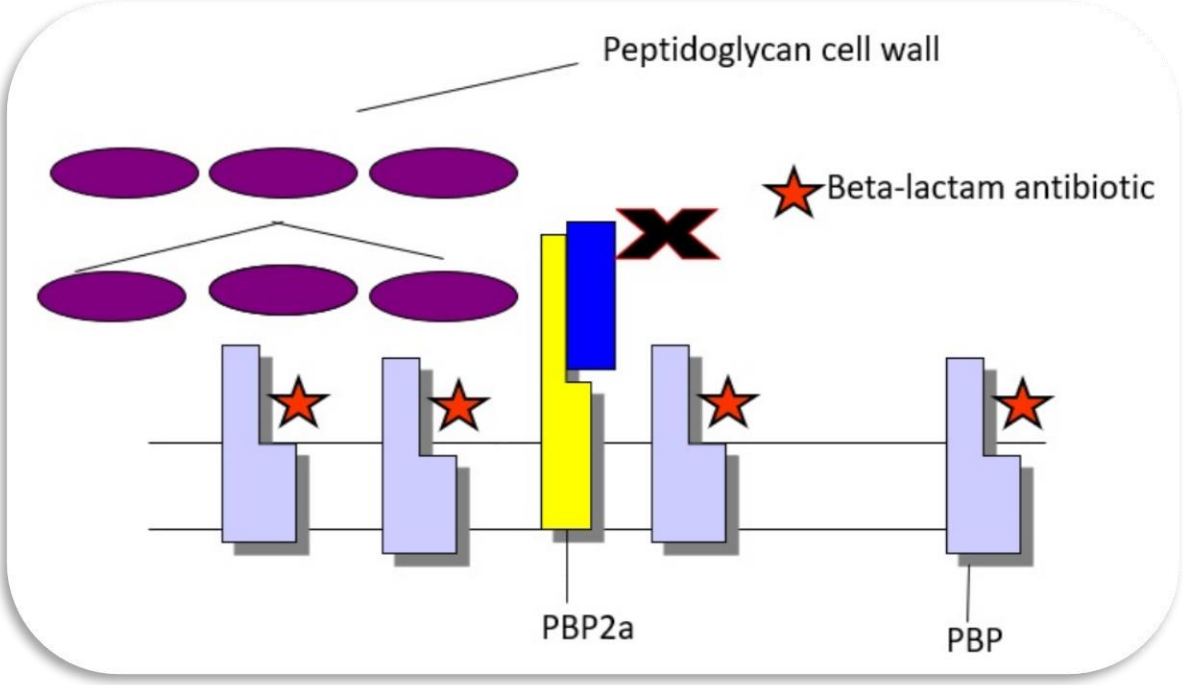

Figure 9-1. Antibiotic resistance by the target change mechanism. The *mec*A causes resistance to beta-lactams by encoding PBP2a. The beta-lactam antibiotics cannot bind to PBP2a methicillin, penicillin, and other penicillin-like antibiotics.

4-1-5-1. Efflux pumps systems

The efflux pump systems are membrane brane-associated pumps that can cause resistance to different antibiotics agents. This mechanism causes a reduction in antibiotics accumulation in the bacteria cell (Figure 8-1). Efflux pump systems have been classified into five superfamilies, based on the energy source required to drive export, substrate specificities of the different pumps, primarily on amino acid sequence identity. This type of resistance mechanism is seen in gram-negative and gram-positive bacteria (2).

2-5-1. Resistance mechanisms to ciprofloxacin

The two main mechanisms of resistance to fluoroquinolones, such as ciprofloxacin, are changes in drug target and efflux pumps over activity (Figure 8-1). Both mechanisms chromosomally are mediated in many bacteria (1).

3-5-1. Resistance mechanisms to gentamicin

Mechanisms of resistance to aminoglycosides include the following (1):

1. Mutation of the ribosomal binding site

2. Decreased uptake of the antibiotic (Anaerobic bacteria)

3. Increased expulsion of the antibiotic from the cell

4. Enzymatic modification by:

- Phosphotransferases (APHs)
- Adenyltransferases (ANTs)
- Acetyltransferases (AACs)

Enzymatic modification is the most common mechanism of resistance to aminoglycosides such as gentamicin. In this type of resistance mechanism, these enzymes inactivate aminoglycosides by the addition of chemical groups including phosphor, adenyl, and acetyl to them.

6-1. Resistance to antibacterial agents by biofilm formation

Biofilm production is one of the important mechanisms to increase resistance to antibiotics and other antimicrobial agents.

7-1. The Antibiotic Resistance Crisis

Since the discovery of antibiotics, these agents have been used to treat and prevent infections**.** Over time, with the increasing use of antibiotics in medicine and agriculture, resistance to them is increasing. According to the World Health Organization (WHO) investigations and recommendation (3, 6):

- "Antibiotic resistance is one of the biggest threats to global health, food security, and development today.
- Antibiotic resistance can affect anyone, of any age, in any country.
- Antibiotic resistance occurs naturally, but misuse of antibiotics in humans and animals is accelerating the process.
- A growing number of infections – such as pneumonia, tuberculosis, gonorrhoea, and salmonellosis – are becoming harder to treat as the antibiotics used to treat them become less effective.
- Antibiotic resistance leads to longer hospital stays, higher medical costs and increased mortality ".

Therefore, it is necessary to develop research to identify new antimicrobial agents and their mechanisms, mechanisms of antibiotic resistance, and how to prevent the spread of antibiotic resistance.

8-1. Anti-inflammatory drugs

Anti-inflammatory drugs have an important role in reducing inflammation in many infectious diseases caused by various microorganisms such as bacteria, viruses, and fungi (7, 8). Typically, two types of anti-inflammatory drugs, including corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs), are used to

control inflammation (7, 8). The most important steroidal anti-inflammatory drugs include dexamethasone, betamethasone, etc., and aspirin, ibuprofen, celecoxib, and naproxen are the most common non-steroidal anti-inflammatory drugs used in the clinic (Figure 10-1) (7, 8, 9, 10). NSAIDs reduce inflammation and pain by inhibiting the production of prostaglandins. Glucocorticoids and mineralocorticoids, are the two main groups of corticosteroids and are involved in different physiological processes, including immune response, and regulation of inflammation, stress response, protein catabolism, carbohydrate metabolism, and levels of electrolytes in blood (7, 8, 9, 10, 11). Bacteria are one of the most important infectious agents in humans and cause a wide range of infections in humans (12). Today, with the increase of immunosuppressive and immunocompromised patients, the rates of bacterial infections especially in hospitalized patients are increasing (12, 13). Therefore, the use of anti-inflammatory drugs to reduce inflammation caused by infection has increased.

Currently, several studies show the antimicrobial effects of anti-inflammatory drugs, as well as their effect on the formation of biofilms and changes in drug susceptibility of bacteria (14).

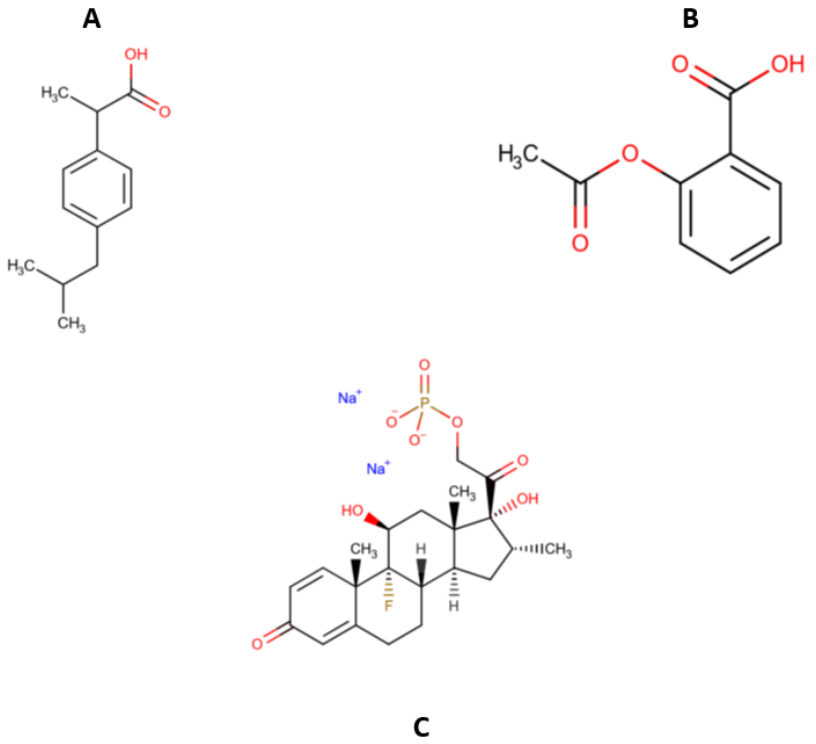

Figure 10-1. Molecular structure of ibuprofen and aspirin as NSAIDs and dexamethasone sodium phosphate as corticosteroid.

9-1. Biofilm: formation and importance

In living organisms, a biofilm refers to a complex community of bacteria within an exopolysaccharide matrix that attaches to surfaces (15). Evidence shows that the presence of biofilms on earth dates back millions of years, but its importance in causing infections was not known until the 1980s (13). Biofilm formation is very important in nature, industry, and medicine. In many natural settings, biofilm formation often allows mutualistic symbioses. For example, *Actinobacteria* often grows on ants and allows ants to live in pathogen-free fungal gardens. In industries,

especially the dairy industry, biofilm formation causes severe health problems and economic losses because of food spoilage and equipment impairment. On the other hand, biofilm formation in clinics is associated with chronic infections, nosocomial infections, and medical device-related infections (14, 15).

Infections related to implanted medical devices are very common and cause serious illness and death. These implanted medical devices include urinary catheters, pacemakers, heart valves, stents, intravascular catheters, and orthopedic implants that are normally used to save patients' lives but cause severe problems when colonized by bacterial biofilms (18). %80 of all microbial infections in humans and %65 of nosocomial infections are biofilm-related and it has been estimated that treatment of these biofilm-based infections costs >$1 billion annually (17,18).

Biofilm formation is beneficial for bacteria in many ways. For example, biofilm protects bacteria against osmolality, pH changes, immune system responses, and nutrient scarcity. On the other hand, biofilm can cause bacteria resistant to antibiotics and disinfectants (19).

10-1. Biofilm Structure

A biofilm is a complex set that includes exopolysaccharides, lipids, proteins, amyloidogenic proteins, and extracellular DNA (e-DNA), in an extracellular matrix (20) .

1-10-1. The extracellular polymeric substance (EPS): Exopolysaccharides

The extracellular matrix, also called EPC, is a complex combination of lipids, proteins, extracellular DNA (eDNA), and polysaccharides. Exopolysaccharide acts as scaffolds for other carbohydrates, lipids, proteins, and nucleic acids. The composition, structures, and properties of exopolysaccharides vary from species to species (21). These constituents aid in the attachment of biofilm to the surface, also involved in trapping food, escaping the immune system, and resistance to antimicrobial therapies. In addition to the above roles, EPS is responsible for the close attachment of biofilm cells and cell-to-cell communication through quorum sensing (QS) and facilitating the exchange of genetic coworkers on the analysis of compounds and the association of EPS matrix in *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, P. aeruginosa*, and *Enterococcus species* showed that the most abundant carbohydrates in EPS structure are: Mannose, galactose, and glucose followed by acetyl glucose amine and galacturonic acid and arabinose, fructose, rhamnose, and xylose (22) . Among the bacteria causing biofilm-related infections, the gram-negative bacteria *P. aeruginosa* and the gram-positive bacteria *S. aureus* and *Staphylococcus epidermidis* are the most important etiological agents of biofilm-associated infections (23, 24). *P. aeruginosa* causes chronic cystic fibrosis and *S. aureus*

and *Staphylococcus epidermidis* is the most common causes of nosocomial infections on indwelling medical devices (23, 24).

In *Pseudomonas aeruginosa*, 3 exopolysaccharides are associated with biofilm, PEL, PSL, and alginate. Alginate is a combination of d-mannuronic acid residues. Alginate is not necessary for the onset of biofilm initiation, but it is an important factor in chronic infections. Also, alginate protects *P. aeruginosa* cells against antibiotics such as ciprofloxacin, gentamicin, ticarcillin, and ceftazidime and it also inhibits the host immune response (25, 26). The enzyme AlgC is essential for the synthesis of lipopolysaccharides and alginates. This enzyme, encoded by *algC* gene that has two functions, phosphomannomutase and phosphoglucomutase activity. This enzyme converts Man-6-P into Man-1-P and glucose-6-P into glucose-1-P that Man-1-P essential for the synthesis pathway of alginate and LPS (25, 26, 27, 28).

In addition to alginate, Pel and Psl are both important, in the maturation of *P. aeruginosa* biofilm. Psl polysaccharides are a combination of a repeating pentamer consisting of D-mannose, L-rhamnose, and D-glucose residues. It plays a significant role in promoting the initial surface attachment process in *P. aeruginosa* biofilm. The AlgC enzyme is essential for the synthesis of Pel and Psl. Mannose 1 phosphate is a key intermediate compound for the synthesis of Psl, alginate, and lipopolysaccharide (LPS). A total of 24 genes are involved in the production and

secretion of alginate in *P. aeruginosa*. Eight of the genes are responsible for regulating the excretion of allergenic acid, twelve are responsible for regulating polysaccharide biosynthesis, and the remaining four are responsible for regulating synthesis (28, 29).

In *Staphylococcus* spp, biofilm formation has demonstrated that bacteria cell aggregation and biofilm accumulation are mediated by the products of the *ica* operon [*icaR*; regulatory and *icaADBC*; biosynthetic genes], which comprises four genes *icaA*, *icaB*, *icaC* and *icaD*, and a regulator gene *ica*R, which seems to function as a repressor (30, 31). The production of *ica* genes is polysaccharide intercellular adhesin (PIA). The PIA has a different role such as intercellular adhesion, resistance to antibiotic agents, and resistance to phagocytosis or immune response (32). PIA is composed of b-1,6-linked N-acetylglucosamine residues (80–85%) and an anionic fraction with a lower content of non-N-acetylated D-glucosaminyl residues that contains phosphate and ester-linked succinate (15–20%) (30) (33). Some studies have been shown *ica* operon is necessary for biofilm formation and virulence and response to anaerobic growth (33, 33). The accessory gene regulatory (*agr*) operon including *agrA*, *agrB*, *agrC*, and *agrD* genes is one of the major regulatory systems for expression and control of virulence genes and pathogenicity in *S. aureus* and some studies have shown the

relation between type of *agr* with the presence of virulence genes and biofilm formation (30, 33).

2-10-1. Extracellular proteins

Other important components of the exopolysaccharide matrix are extracellular proteins. An important example is glucan-binding proteins (GBPS) in *Streptococcus mutans* biofilm (34). GBPS protein plays a key role in the structure of *S. mutans* biofilm and binds to bacteria and exopolysaccharides (35).

Amyloids are insoluble fibrous proteins (FP) that play a key role in biofilm structure. Overexpression of this protein leads to cell accumulation and increased biofilm formation in *Pseudomonas* spp. (36). Another example for amyloid proteins is TasA that is one of the main components of *B. subtilis* biofilms (37). This protein forms a strong fibrous layer around the biofilm that holds the biofilm cells together and protects them from harsh conditions (38).

Another example is Bap protein and Esp protein in *S. aureus* and *E. faecalis* respectively that both of them belong to biofilm-associated protein (bap family). This protein involved biofilm formation and infection processes (39). Other examples include outer-membrane lectins of *Azospirillum brasiliense* (40), the galactophilic lectin lecA, and l-fucose binding lectin lecB of *P. aeruginosa* (39, 40) (41).

3-10-1. Extracellular DNAs (eDNAs)

Extracellular DNAs (eDNAs), play a key role in biofilm formation. Its negative charge helps to bind biofilm in the initial attachment. Also, eDNA in *P. aeruginosa* involves the twitching motility (42). Due to negative charge, eDNA can chelate metal ions such as magnesium ions, which activate the PhoPQ/PmrAB two-component system and cause drug resistance in bacteria like that *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium (43, 44). Another example is resistance to vancomycin in *S. epidermidis* that due to inhibiting the transportation within the biofilm (45).

11-1. Stages of biofilm formation

Briefly, the stages of biofilm formation can be divided into 3 stages: attachment, maturation, and detachment. The attachment step itself is divided into two stages, the initial reversible attachment and the irreversible attachment (46).

In most bacteria, the key mediators in the initial attachment are the flagellum and the type IV pili. Flagella are essential for the interaction between cells and surface Pili type IV through twitching motilities causes the cells to aggregate and attach and form microcolonies (46). In step two biofilm starts to mature by developing microorganisms and increasing layer. Finally, after full maturation biofilm releases microcolonies that migrate to a new surface (46).

12-1. Antibiotic resistance and biofilm

In general, biofilm resists antibiotics and disinfectants in two general ways: intrinsic resistance and inductive resistance. These mechanisms are briefly discussed below.

1-12-1. Intrinsic mechanisms

- **Limited drug penetration:**

  This mechanism is one of the most common methods of biofilm resistance to antibiotics, however, it is not always effective. For example, ciprofloxacin and ampicillin can penetrate and diffuse through *Klebsiella pneumoniae* biofilms (47). Also, ciprofloxacin can penetrate and diffuse into the *P. aeruginosa* biofilms. But despite the examples mentioned, many antibiotics are not able to overcome the biofilm (48).

- **Decreased growth rate and metabolism**

  It has been shown that reducing oxygen and nutrients down-regulate the growth of bacteria. Bacteria that enter the dormant phase within a biofilm are not affected by antibiotics and thus exhibit high levels of antibiotic tolerance, because most antibiotics active against replicating bacterial cells (49).

- **Persister Cells**

  Presister cells are a small subpopulation of bacterial cells that exist in dormant form and show high antimicrobial resistance. The presence of these cells in *S. aureus* was first demonstrated. Hobby et al (1942) Found that about one percent of *S. aureus* cells were not killed by penicillin (50).

2-12-1-. Induced mechanisms

The mechanisms of induced resistance in biofilms seem to be more complex than the mechanisms of intrinsic resistance. In a small number of studies, including inductive resistance in biofilms has been investigated (51, 52, 53) (54, 55). An interesting example of the induction of drug resistance is presented by Ziebuhr *et al.,* in this mechanism, the inhibitory concentration of common antibiotics on *Staphylococcus epidermidis* induces the expression of the *ica* gene cluster, which mediates the production of polysaccharide intercellular adhesin (PIA), as a key factor in biofilm production (56).

13-1. Effect of non-steroidal anti-inflammatory drugs (NSAID) on microorganisms and biofilm formation

Ibuprofen, aspirin, diclofenac, celecoxib, and naproxen are the most common non-steroidal anti-inflammatory drugs used in the clinic (7, 8, 9, 10). NSAIDs reduce inflammation and pain by inhibiting the production of prostaglandins. The

antimicrobial and antifungal activities of ibuprofen were firstly described by Hersh and colleagues in 1991. The antimicrobial activity of ibuprofen, an extensively used non-steroidal anti-inflammatory drug (NSAID), has been previously reported against many Gram-negative and Gram-positive bacteria, fungi, and viruses by unclear mechanisms (57). It seems that ibuprofen is involved in the inhibition of biofilm development and quorum sensing (QS) in *P. aeruginosa* (58). Another study showed that ibuprofen inhibited pulmonary vasoconstriction and bronchiolar constriction in pigs infected with *P. aeruginosa* (59). Moreover, ibuprofen declined the recruitment of granulocytes to airways and repressed lung inflammation in a murine *P. aeruginosa* acute pulmonary infection model (60). The inflammatory factor leukotriene B4 (LTB4) production was also inhibited by ibuprofen descending in lung inflammation in a chronic pulmonary infection rat model (61). A randomized controlled trial study showed that two-thirds of female patients with UTIs convalesced by a single dose of ibuprofen without an antibiotic regimen (62). However, several studies reported that ibuprofen is a good treatment substitute for antibiotics and/or therapeutic potential in combination with the antibiotics (62, 63, 64, 65). In contrast, more studies have been shown that ibuprofen cannot be suggested as a stand-alone treatment for UTI patients (66). Further studies have informed that ibuprofen inhibits the growth of *P. aeruginosa* in a dose-dependent

mode and exerts antibacterial properties in high concentrations which surpass levels perceived in normal human blood (67, 68). The attachment activity for *P. aeruginosa* biofilm formation was inhibited by ibuprofen (69). The maximum effect of ibuprofen on biofilm inhibition in *P. aeruginosa* has been reported in a concentration of 100 μg/mL. The production of virulence factors, such as elastase, protease, pyocyanin, and rhamnolipids were also reduced with 100 μg/mL ibuprofen (58). Ibuprofen can prevent the development of lung decadence in cystic fibrosis patients (69). Ibuprofen able to prevent N-acyl-homoserine lactone (AHLs) production, the key mediates in QS in the initial attachment of biofilms, and stops the responses to nucleotides in cystic fibrosis airway epithelium (70). Furthermore, the synthesis of N-butanoyl-L-homoserine lactone (C4-HSL) was repressed by ibuprofen in a concentration and time-dependent manner. Nevertheless, ibuprofen does not reduce the secretion of N-3-oxododecanoyl-homoserine lactone (3-oxo-C12-HSL). Thus, ibuprofen exerts an anti-QS effect by the decrement of C4-HSL levels rather than the direct cell death properties (58). The LuxR-type QS system of *P. aeruginosa* is mediated by Las and Rhl genes (71). The results of a study showed that the expression of genes encoding QS proteins (*lasI*, *lasR*, *rhlI*, *rhlR*, *pasA*, and *pqsR*) in *P. aeruginosa* was significantly reduced following ibuprofen therapy within 18 hours (58).

Aspirin, an additional NSAID, decreases lasR gene expression by challenging with 3-oxo-C12-HSL as described by Somaia *et al*. (72). The simulations and molecular docking studies of ibuprofen with QS proteins revealed that ibuprofen can bind to LuxR, LasR, LasI, and RhlR proteins with a high affinity to dimerization, receptor activity, and inactivation of the QS proteins. The proteins associated with the QS system seem to be a good candidate for anti-QS and anti-biofilm of ibuprofen activities (58).

Diclofenac is a 2-(2,6-dichloranilino) phenylacetic acid. Diclofenac is available in both sodium and potassium salts and dissolves well in solvents such as methanol and DMSO. Most studies have been performed on the antimicrobial effect of diclofenac on its sodium form (73).

Alqahtani *et al.,* (2018) studied chitosan nanoparticles loaded with diclofenac and demonstrated high effectiveness against *S. aureus* and *B. subtilis* which depended on the molecular weight of chitosan and pH (74).

The mechanism of action of diclofenac appears to be inhibition of DNA synthesis (75). A good example is *E. coli*; it seems some of the NSAIDs cause inhibition of the DNA polymerase III β subunit. Inhibition of this subunit as a consequence of the binding of the NSAID molecule results in inhibition of DNA replication and repair (70). The effects of diclofenac bactericides on both gram-positive and gram-

negative and also *Mycobacteria* have been reported (75, 76, 77, 78, 79). However, Perilli (2000) showed that the action of this drug on the growth of *S. epidermidis* was bacteriostatic (80).

The combination of diclofenac with antibiotics can increase or decreases of antibiotic susceptibility and in some cases does not affect antibiotic efficacy. For example, the combination of diclofenac with ciprofloxacin, ofloxacin, and norfloxacin increased susceptibility of bacteria to these antibiotic agents, but combination of diclofenac with oxacillin and vancomycin decreased susceptibility of bacteria to these antibiotic agents. However, the combination of diclofenac with drugs such as tetracycline and chloramphenicol does not affect the effectiveness of these drugs (73). Hegazy (2016) reported that sub-inhibitory concentrations of diclofenac can cause inhibition of biofilm formation produced by *P. mirabilis* (81). In some research like Baldiris *et al.,* and Reslinski *et al.,* the focus was on the combination of NSAIDs with each other or associated with other substances affect biofilm formation. For example, Reslinski *et al.,* (2015), showed that diclofenac and ibuprofen reduce the formation of biofilms in *S. aureus* and *E. coli*. Electron microscopic study showed that the mechanism of action of these drugs is by reducing the binding of bacteria to each other (31). In another study conduct by Baldiris *et al.,* (2016), reported that the combination of diclofenac and ibuprofen in

sub-inhibitory concentrations produced a significant reduction in biofilm formation in clinical isolates of *K. pneumoniae* and *E. coli* (82). Also, Mohsen *et al.,* in 2015 reported a combination of diclofenac (and also ibuprofen) with N-acetyl cysteine resulted in disruption of biofilm in *S. aureus* and some gram-negative bacteria(83). A study by Ashraf *et al.,* on the effect of some nonsteroidal anti-inflammatory drugs on biofilm formation in *Candida* species, showed that sodium diclofenac had the lowest inhibitory concentrations against *Candida albicans* and *Candida glabrata* while ibuprofen had the lowest inhibitory concentrations against *Candida krusei*. Electron microscopy study shows the mechanism of action of these drugs through damage to membranes (84). A study conducts by El-Baky and El-Gendy (2016) on the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) and dexamethasone on biofilm formation and gene expression of some biofilm-associated adhesins in *Candida albicans* and *S. aureus* showed that sodium diclofenac had the highest antimicrobial effect and followed by meloxicam. Also, dexamethasone and ketoconazole down-regulated the expression of biofilm-related genes in *C. albicans*, while ketoprofen up-regulated *HWP1* gene expression. NSAIDs and levofloxacin reduce the expression of the *icaA* gene, but dexamethasone did not affect the *icaA* gene. The results of study by El-Baky and El-

Gendy showed that although dexamethasone had no antimicrobial effect, it had a good anti-biofilm effect on *S. aureus* and *C. albicans* (85).

A study conduct by Yang *et al.,* (2016) on interaction between some three NSAIDs (aspirin, ibuprofen, and diclofenac sodium) and some common antifungal agents (fluconazole, itraconazole, voriconazole, caspofungin, and amphotericin B) against planktonic and biofilm cells of *Trichosporon asahii* showed that combination of amphotericinB/ibuprofen had highest effective against planktonic cells (86.67%) and biofilm cells (73.33%) and followed by caspofungin/ibuprofen (73.33% for planktonic form and 60 % for biofilm) as well. The results of this study show that the combination of amphotericinB/ibuprofen and caspofungin/ibuprofen are effective against *T. asahii* (86)**.** Alem and Douglas (2004) studied the effect of nine NSAIDs drugs on biofilm and planktonic cells of *C. albicans*. This study showed that seven of nine drugs tested at a concentration of 1 mM inhibited biofilm formation. Aspirin and diclofenac produced the greatest effects, with aspirin causing up to 95% inhibition. The drugs celecoxib, nimesulide, ibuprofen, and meloxicam also inhibited biofilm formation, but to a lesser extent. Aspirin was the most effective drug against growing and fully mature (48h) biofilms. The mechanism of this drug is dose-dependent (87). Zamanian *et al.,* (2017) reported that a combination of

aspirin with antifungal drugs can cause induce the anti-fungal effect of fluconazole at high concentrations conferring a synergistic effect and fungicidal activity (88).

Stepanovic *et al.,* (2004) reported that minimal inhibitory concentrations of aspirin obtained ranged from 2.17 to 8.67 mM and minimal fungicidal concentration between 0.43 to 1.73 mM depending on the tested yeast strain (89).

Al-Bakri *et al.,* (2008) reported that aspirin used at the minimal biofilm eradication concentration values (MBEC) for 24 h was successful in eradicating *P. aeruginosa*, *E. coli,* and *C. albicans* biofilms established on abiotic surfaces. Aspirin and EDTA are 'non-antibiotic drugs', the combination of which can be used successfully to treat and eradicate biofilms established on abiotic surfaces (90).

14-1. Effect of corticosteroids anti-inflammatory drugs on microorganism and biofilm formation

Corticosteroid drugs such as cortisone, hydrocortisone, and prednisone are anti-inflammatory drugs and suppress inflammation (7, 9). These drugs mimic the effects of hormones that produce naturally in adrenal glands and closely resemble cortisol. There are different types of anti-inflammatory drugs including non-steroidal (NSAIDs; aspirin, ibuprofen, diclofenac, naproxen, etc.) and corticosteroidal anti-inflammatory drugs, such as dexamethasone, betamethasone, and hydrocortisone that are commonly used for ameliorate fever

and other symptoms of acute and chronic infections (7, 9). The use of a combination of anti-inflammatory drugs with antibiotics in the treatment of infections can have different effects on biofilms (91).

Many reports showed that the steroid hormones increase the expression level of virulence-associated genes, efflux pumps associated with multidrug-resistant bacteria, and increased rate of replication and adherence to surfaces in bacteria (14, 92). The virulent mucoid biofilm phenotype in *P. aeruginosa* enhancement in presence of estradiol and maybe steroid hormones can function as quorum signaling molecules (92). Estradiol downregulates some genes involved in nucleotide metabolism, fatty acid biosynthesis, and upregulates genes associated with stress response, and other genes including, *cydB*, *omcB*, *cydA*, *trpB*, *yggV*, and *pyk* genes that may be associated with enhanced survival and persistence in *Chlamydia trachomatis* (92).

On the other hand, a study by Esposito *et al.,* were shown the glucocorticoid DFZ and its synthetic precursors were not any activity against standard and clinical isolates of *Stenotrophomonas maltophilia*, but the sub-inhibitory concentrations of glucocorticoid PYED-1 were able to reduce biofilm formation in *S. maltophilia* (93). Also, Esposito *et al.,* were shown the expression level of biofilm and virulence-

associated genes including *StmPr1*, *StmPr3*, *sphB*, *smeZ*, *bfmA*, *fsnR* was significantly downregulation in *S. maltophilia* after PYED-1 treatment of them (93). In 2014, Fteita *et al.,* were shown the estradiol compounds increase the planktonic growth and ability to co-aggregate of *F. nucleatum* and they were shown polysaccharide production, and biofilm formation of *P. intermedia* enhanced by estradiol *in vitro* (14).

It was demonstrated that some anti-inflammatory drugs are effective on biofilm formation in bacteria alone and with some antibiotic agents (84, 91). In a study in 2018, dexamethasone (1-dehydro-16a-methyl-9a-fluorohydrocortisone; DEXA) as a corticosteroidal anti-inflammatory abrogates the activity of different antimicrobial drugs when combined in vitro against microbial biofilms of *S. aureus* and *P. aeruginosa* (91). Abd El-Baky *et al.,* were shown that dexamethasone and ketoconazole can down-regulate *C. albicans* adhesion-related genes. Also, Abd El-Baky *et al.,* were reported, NSAIDs and levofloxacin down-regulated the expression *icaA* gene but dexamethasone showed no effect on *icaA* gene expression and shown that dexamethasone had no antimicrobial activity, but has anti-biofilm activity against *S. aureus* and *C. albicans* (84). Unlike the study by Abd El-Baky et al, Rodrigues *et al.* were shown dexamethasone abrogates the antimicrobial and

antibiofilm activities of different drugs against clinical isolates of *S. aureus* and *P. aeruginosa.*

Table 2-1 shows the antimicrobial effects, ability of biofilm production among various microorganisms in in presence of anti-inflammatory drugs and targets of them.

Table 2-1. The antimicrobial effects and ability of biofilm production in presence of anti-inflammatory drugs.

Anti-inflammatory drugs and estradiol compounds	Antibacterial Activity	Biofilm Formation	Target(s)	Type of Bacteria
Ibuprofen	+	↓	Antibacterial, Antibiofilm, AntiQS, Antivirulent,	Gram-negative and Gram-positive
Diclofenac	+	↓	Inhibition of DNA synthesis (DNA replication and repair), Inhibition the DNA polymerase, Damage to membranes,	Gram-negative and Gram-positive
Aspirin	+	↓	Antibacterial, Antibiofilm	Gram-negative and Gram-positive
Celecoxib	+	↓	Antibiofilm	Gram-negative and Gram-positive
Nimesulide	+	↓	Antibiofilm	Gram-negative and Gram-positive
Meloxicam	+	↓	Antibiofilm	Gram-negative and Gram-positive
Dexamethasone	-	↑	-	Gram-negative and Gram-positive
PYED-1	-	↓	-	Gram-negative
Glucocorticoid DFZ	-	↑	-	Gram-negative

QS: Quorum sensing; ↑: Increase (Up); ↓: Decrease (Down).

Chapter 2:

Materials and Methods

1-2. Devices and Materials

Table 1-2. Devices used in this Study.	
Number	**Device name**
1	Incubator
2	Autoclave
3	Micro centrifuge
4	Electrophoresis Tank
5	Thermal cycler
6	Gel Documentation
7	Sampler
8	Refrigerated Microcentrifuge
9	Nanodrop spectrophotometer
10	Real-time system
11	Microplate reader

Table 2-2. Materials used in this Study.	
Number	**Materials**
1	Mueller Hinton Agar medium
2	Tryptic Soy Broth (TSB) medium
3	Blood Agar medium
4	Tip (10-100 µl)
5	Tip (100-1000 µl)
6	Tip (10-10 µl)
7	Micro tube 1.5 ml
8	Micro tube 0.2ml
9	Cotton swab
10	Sterile Petri dish (8 and 10 cm)
11	Sterile microplate (96 well)
12	Dimethylsulfoxide (DMSO)
13	Syringe filter
14	Ethylenediaminetetraacetic acid (EDTA)
15	Boric acid
16	Tris base
17	Agarose
18	Sodium Chloride
19	Barium chloride
20	2X RED Master mix
21	DNA ladder 100bp+3k
22	DNA green viewer
23	Glucose
24	Glycerol
25	Chloroform
26	Isopropyl alcohol
27	cDNA Synthesis Kit
28	qPCR5x Mix plus Master Mix
29	Chloroform
30	Ethanol
31	Crystal violet dye
32	Safranin dye

Table 3-2. Antibiotics and anti-inflammatory drugs used in this Study.				
Drug	**Abbrevia-tion**	**Initial Solvent**	**Diluent Solvent**	**Company**
Gentamicin	GEN	Water	Water or medium culture	Sigma-Aldrich, Inc, St. Louis, MO 68178, USA
Ciprofloxacin	CIP	Water	Water or medium culture	
Cefepime	FEP	Phosphate buffer pH 6.0, 0.1 mol/L	Phosphate buffer pH 6.0, 0.1 mol/L	
Imipenem	IPM	Phosphate buffer, pH 7.2, 0.01 mol/L	Phosphate buffer pH 6.0, 0.1 mol/L	
Meropenem	MEM	Water	Water and medium culture	
Aspirin (acetylsalicylic acid)	ASA	DMSO	Water and medium culture	Temad Pharmaceutical, Co, Tehran, Iran
Ibuprofen	IBP	DMSO	Water and medium culture	
Dexamethasone sodium phosphate	DXP	DMSO	Water and medium culture	Sinadarou Pharmaceu-tical, Co, Tehran, Iran

Table 4-2. Antibiotic Discs.			
Antimicrobial agent	**Disc (µg)**	**Abbreviation**	**Manufacturer**
Vancomycin	30	VAN	Mast Group Ltd, Liverpool, UK
Tetracycline	30	TE	
Ciprofloxacin	5	CIP	
Gentamicin	10	CEN	
Amikacin	30	AM	
Erythromycin	15	E	
Clindamycin	2	CL	
Trimethoprim/sulfamethoxazole	1.25/23.75	SXT	
Linezolid	30	LIN	
Meropenem	10	MEM	
Imipenem	10	IPM	
doripenem	10	DOR	
Piperacillin/tazobactam	100/10	PTZ	
Cefepime	30	FEP	
Aztreonam	30	AZT	
Ceftazidime	30	CAZ	

2-2. Solutions

1-2-2. McFarland Standard

McFarland turbidity standard was used to standardize the inoculum for performing antimicrobial susceptibility test.

- 0.5 McFarland turbidity standard consists of the following steps:
    - 0.05 ml of $BaCl_2$ was Added (1.175% w/v $BaCl_2 \times 2H2O$) to 9.95 mL of 0.18 mol/L H2SO4 (1% v/v).
    - The correct density of the turbidity standard was verified using a spectrophotometer with a 2-cm light path, afterward it was matched cuvette to determine the absorbance. The absorbance at 600 nm should be 0.08 to 0.10 for the 0.5 McFarland standard.
    - 4 to 6 mL of the above solution was distributed into screw-cap tubes of the same size as those were used in growing or diluting the broth culture inoculum.
    - It was tightly sealed the tubes and was stored in dark at room temperature.
    - It was vigorously agitated by this turbidity standard on a mechanical vortex mixer just before use.
    - The Standards were replaced or rechecked after preparation.

2-2-2. TBE Buffer

- A 5X stock solution was prepared in 1 L of H_2O:
    - Tris base: 54.072 g

- Boric acid: 27.83 g
- EDTA: 3.72 g

TBE was made and stored as a 5X stock solution. The pH of the concentrated stock buffer should be ~8.3. Dilute the concentrated stock buffer was diluted just before use. To be used for electrophoresis, it was diluted 10 times with distilled water to make 0.5 X TBE buffer.

3-2. Bacterial isolates and identification

This study was performed on 10 clinical isolates of *S. aureus* and 10 clinical isolates of *P. aeruginosa*. All samples were identified by biochemical tests. Standard microbial tests including gram staining, catalase reaction, coagulase production, DNase reaction, beta-hemolysis on blood agar, and mannitol fermentation tests were used to identify *S. aureus* isolates and all of them were confirmed by detection of *nuc* gene using PCR methods (Table 5-3). *P. aeruginosa isolates* were identified using standard microbial tests including gram staining, colony morphology, non-lactose fermentation on MacConkey agar, oxidase and catalase tests, triple sugar iron agar (TSI), and oxidative-fermentative (OF) tests.

4-2. Standards strains

E. coli ATCC 25922, P. aeruginosa ATCC 27853, and *Staphylococcus aureus* ATCC 25923 were used as control strains in antibacterial susceptibility test and *P. aeruginosa* PAO1, *S. aureus* ATCC 25923, and *Staphylococcus epidermidis* Rp62A used as standard strains for biofilm formation and molecular experiments including PCR.

5-2. Antibacterial susceptibility tests

According to the Clinical & Laboratory Standards Institute (CLSI) recommendations, the disk diffusion method was used to evaluate the susceptibility of bacterial isolates to different antibiotic agents (94) . The following antibiotic disks, including penicillin (P, 10 units), gentamicin (GEN, 10 µg), amikacin (AM, 30 µg), erythromycin (E, 15µg), ciprofloxacin (CIP, 5µg), tetracycline (TE, 30µg), clindamycin (CL, 2µg) trimethoprim/sulfamethoxazole (SXT, 1.25/23.75µg), and linezolid (LIN, 30µg) (MAST, Co, U.K.), were used for S. aureus isolates. Meropenem (MEM, 10 µg), doripenem (DOR, 10 µg), imipenem (IPM, 10 µg), ciprofloxacin (CIP, 5 µg), piperacillin/tazobactam (PTZ, 100/10 µg), gentamicin (GEN, 10 µg), aztreonam (AZT, 30 µg), cefepime (FEP, 10 µg), and ceftazidime (CAZ, 30 µg) disks were used for *P. aeruginosa* isolates. Minimum inhibitory concentration (MIC) of the bacterial

isolates to GEN, CIP, CPM, MEM, and IPM were determined using broth microdilution method (94).

6-2. Determination of methicillin-resistant *S. aureus* (MRSA)

The MRSA isolates were determined using cefoxitin (FOX; 30µg) disk according to CLSI recommendation (94).

7-2. Detection *mecA* and *icaABCD* operon genes using PCR method in *S. aureus* isolates

The genomic DNA of isolates was extracted using DNA extraction kit (GeneAll Biotechnology Co., South Korea) following manufacturer's instructions. The quality and quantity of the extracted DNA were measured by determination of absorbency at the wavelength A260 nm and 280nm. Polymerase chain reaction (PCR) technique in volume 25µL was used for detection of *nuc*, *mecA*, and *icaABCD* operon. Each 25µl PCR mixture was containing of 1µL of bacterial DNA, 0.5 µl(10pM) of each oligodeoxynucleotide primer, 12.5 µL of 2X Master Mix Red (Ampliqon, Denmark) and 10.5 µL water (DNase and RNase free). The sequences of primers used in this study and PCR condition are presented in Table 5-2. The PCR products were electrophoresed on a 1.5% agarose with DNA Green Viewer safe dye and visualized in a gel documentation system.

8-2. Detection of *algD* gene among *P. aeruginosa* isolates

Among clinical isolates of *P. aeruginosa* we detected *algD* gene by PCR methods. First, clinical isolates of *P. aeruginosa* isolates cultured on LB (Luria-Bertani) liquid medium and after overnight genomic DNA of isolates was extracted using DNA extraction kit (GeneAll Biotechnology Co., South Korea) following manufacturer's instructions. The quality and quantity of the extracted DNA were measured by determination of absorbency at the wavelength A260 nm and 280nm. The sequences of primers used in this study and PCR condition are presented in Table 3. Each 25µl PCR mixture was containing of 1µL of bacterial DNA, 0.5 µl (10pM) of each primer, 12.5 µL of 2X Master Mix Red (Ampliqon, Co, Denmark) and 10.5µL DNase and RNase free water. The sequences of primers used in this study and PCR condition are presented in Table 5-2. PCR products were electrophoresed on a 1.5% agarose with DNA Green Viewer safe dye and visualized in a gel documentation system.

Table 5-2. List of primer sequences, PCR, and qPCR conditions in this study.					
Target genes	**Sequnce of primer (5'-3')**	**PCR conditions**	**Product (bp)**	**Use**	**Ref**
nuc	F-GCGATTGATGGTGATACGGTT R-AGCCAAGCCTTGACGAACTAAAGC	5 min in 95˚C for 1 cycle, 45 sec 60˚C, 1min 72˚C for 30 cycles.	279	PCR	(95)
mecA	F-TCCAGATTACAACTTCACCAGG R-CCACTTCATATCTTGTAACG	5 min in 95˚C for 1 cycle, 45 sec 56˚C, 1min 72˚C for 30 cycles.	162		
***ica*A**	F-TCTCTTGCAGGAGCAATCAA R-TCAGGCACTAACATCCAGCA	5 min in 95˚C for 1 cycle; 1 min 95˚C, 45 sec 60˚C, 1min 72˚C for 30 cycles.	188		
icaB	F-ATGGCTTAAAGCACACGACGC R-TATCGGCATCTGGTGTGACAG	5 min in 95˚C for 1 cycle; 1 min 95˚C, 45 sec 61˚C, 1min 72˚C for 30 cycles.	526		
icaC	R-CTCTCTTAACATCATTCCGACGCC F-ATCATCGTGACACACTTACTAACG	5 min in 95˚C for 1 cycle, 45 sec 63˚C, 1min 72˚C for 30 cycles.	1013		
icaD	F-GAACCGCTTGCCATGTGTTG R-GCTTGACCATGTTGCGTAACC	5 min in 95˚C for 1 cycle, 45 sec 61˚C, 1min 72˚C for 30 cycles.	483		
algD	F-GCGACCTGGACCTGGGCT R-TTGTGGTCCTGGCAGA	5 min in 95˚C for 1 cycle, 45 sec 56˚C, 1min 72˚C for 30 cycles.	457		
rpoD	F-GGGCGAAGAAGGAAATGGTC R-CAGGTGGCCTAGGTGGAGAA	15 min in 95˚C for 1 cycle, 30 sec 60˚C, 30 sec 72˚C for 30 cycles and melt curve analysis at 95-60˚C*.	178	qPCR for *P. aeruginosa*	
algD	F-CGCCGAGATGATCAAGTACA R-TGTAGTAGCGCGACAGGTTG		157		(96)
icaA	F-AGTTGTCGACGTTGGCTAC R-CCAAAGACCTCCCAATGT	15 min in 95˚C for 1 cycle, 30 sec 61˚C, 30 sec 72˚C for 30 cycles and melt curve analysis at 95-60 ˚C*.	148	qPCR for *S. aureus*	(97)
gyrB	F-AGGTCTTGGAGAAATGAATG R-CAAATGTTTGGTCCGCTT		113		
*** We used the melt-curve analysis function of real-time instruments for distinguish specific products from nonspecific products in qPCR experiment.**					

9-2. Biofilm formation assay

We used microtiter assay method for determination of biofilm formation in bacterial isolates according to Table 6-2 (98). Briefly, bacterial isolates cultured on trypticase soy agar (TSA; CONDA, Co, Spain) at 37ºC for 24h. Few grown colonies suspended in sterile physiological saline with turbidity equal to 0.5 McFarland. In the 96 well microtiter plates (Cell and Tissue Culture plates, flat well bottom,) we added 180 µL trypticase soy broth (TSB; CONDA, Co, Spain) supplemented with 1% glucose and then 20µL of bacterial suspension added to each well. After incubation for 24h at 37ºC, broth carefully drawn off, and the plates were gently washed three times with sterile phosphate-buffered saline (PBS) and microtiter plates dried for 1 h at room temperature. For biofilm quantification, 200µL of 1% crystal violet dye solution in water added to each well and the microtiter plates stranded for 40 min at room temperature. The wells were subsequently washed three times with sterile PBS. The crystal violet dye that bounded to the biofilm extracted with 200mL ethanol (95%; Merck, Co, German), and the absorbance of the extracted crystal violet measured at 530 nm in an ELISA reader (BioTek, Co, USA). Each assay is done in triplicate. As a negative control, TSB+1% glucose medium is used to determine background optical density (OD) and cut-off ODc for biofilm formation determined as average OD of negative control +3×standard deviation (SD) of negative control.

Table 6-2. OD Cut off = mean OD negative control + 3SD.	
Cut-off value calculation	**Biofilm formation abilities**
OD > 4×ODc	**Strong**
2×ODc < OD ≤4×ODc	**Moderate**
ODc < OD ≤ 2×ODc	**Weak**
OD ≤ ODc	**Non-biofilm**

10-2. Anti-inflammatory drugs interference experiments

To evaluate the possible effect of anti-inflammatory drugs, including ASA, IBP, and DXP, on the antibacterial and anti-biofilm activity of antibiotic agents, we determined MIC, minimum biofilm inhibitory concentration (MBIC), and minimum biofilm eradication concentration (MBEC) of each antibiotic agent including GEN, CIP, FEP, IPM, and MEM with/without sub-MIC ASA, IBP, and DXP. Dimethyl sulfoxide (DMSO; Merck, Co, Darmstadt, Germany) was used as a solvent for anti-inflammatory drugs, and 10 mg/mL stock solutions of IBP, ASA, and DXP in DMSO were used to prepare working solutions of ASA (200 µg/mL), IBP (200 µg/mL), and DXP (500 µg/mL) in an Mueller Hinton Broth (MH Broth; Condalab, Co, Madrid, Spain) containing DMSO (5%, vol/vol). MIC, MBIC, and MBEC experiments were performed in triplicate in MH Broth, and uninoculated MH broth and MH broth with 5% DMSO without IBP, ASA, DXP, and antibiotics were used as controls for the experiment (69). *Staphylococcus epidermidis* RP62A (ATCC 35984) and *Pseudomonas aeruginosa* PAO1 were used as standard controls in biofilm

formation assays and anti-inflammatory drug interference experiments.

1-10-2. MIC of isolates to antibiotics agents with/without ASA, IBP, and DXP

The MIC of isolates to GEN, CIP, FEP, IPM, and MEM was determined by the microbroth dilution method according to CLSI recommendations with/without 200, 200, and 500 µg/mL of ASA, IBP, and DXP, respectively (94).

2-10-2. MBIC of isolates to antibiotics agents with/without ASA, IBP, and DXP

MBIC assays were performed by the broth microdilution method in 96-well polystyrene sterile plates flat-bottom microplate format according to CLSI recommendation (94). Briefly, bacterial isolate suspension with an inoculum of 1×10^6 CFU/mL was diluted in MH Broth plus 1% glucose with serial dilution of antibiotic agents including GEN, CIP, FEP, IPM, and MEM, with/without constant concentration one of the sub-MIC of ASA (200 µg/mL), IBP (200 µg/mL), and DXP (500 µg/mL), and then incubated for 24 h at 37 °C. After incubation, MBIC was determined by crystal violet staining as the lowest concentration of antibiotic agents with/without anti-inflammatory drugs that resulted in an OD_{600} difference at or below 10% of the mean of two positive growth-control well readings (99, 100).

3-10-2. MBEC of isolates to antibiotics agents with/without ASA, IBP, and DXP

In this step, the effects of each anti-inflammatory drug along with GEN, CIP, FEP, IPM, and MEM on biofilm eradication were investigated. MBEC assay was

performed as previously described (91, 99, 100). Biofilms in clinical isolates of *S. aureus* and *P. aeruginosa* were formed overnight in TSB plus 1% glucose at 37°C in non-treated 96-well polystyrene sterile flat-bottom plates. Biofilm was washed three times and exposed to different serial dilutions of antibacterial agents, including GEN, CIP, FEP, IPM, and MEM with/without sub-MIC of ASA, IBP, and DXP in fresh MH Broth. Briefly, the antimicrobial drugs were diluted in fresh MH Broth to reach the MBEC, and 50 µL was dispensed in each biofilm. Stock solutions of ASA, IBP, and DXP were prepared in DMSO (Co,) and diluted in sterile MH Broth to reach concentrations of 200, 200, and 500 µg/mL, respectively, and then 50µL of one of the anti-inflammatory drugs was added to each well with/without one of each serial dilution of antibiotic agents and then plates incubated for 18 h at 37 °C. The concentration of antibiotic agents with/without IBP, ASA, and DXP that eradicated the mature biofilm was considered as MBEC.

11-2. Determination expression level of biofilm-related genes with/without sub-MIC of anti-inflammatory drugs by using quantitative Real time-PCR (qPCR)

Quantitative real-time PCR (qPCR) was used to determine the transcription levels of *ica*A in *S. aureus* and *algD* in *P. aeruginosa* (97, 96). The expression levels of *gyrB* in *S. aureus* and *rpoD* in *P. aeruginosa* were assessed in parallel with normalized transcriptional levels of biofilm-associated genes (97, 96).

1-11-2. Bacterial growth conditions

Briefly, clinical isolates were grown in MH Broth with/without anti-inflammatory (ASA= 200 µg/mL, IBP= 200 µg/mL, and DXP= 500 µg/mL) by using a shaker incubator at 37 °C and 180 rpm to the log phase (optical density at 600 nm [OD 600] = 0.8–1) and then bacterial cells were collected by centrifugation at 12,000 rpm in 5 min.

2-11-2. RNA extraction and cDNA synthesis

Total RNA of bacterial isolates was extracted with RNX-Plus (SINACLON, Co, Iran) according to the manufacturer's recommendations. Then RNase-Free DNase I enzyme (SINACLON, Co, Iran) was used to eliminate DNA contaminations. Total RNA concentration was determined by spectrophotometer (PCRmax Lambda spectrophotometer, Co, UK), and cDNA synthesis was performed by the Easy cDNA Synthesis Kit (Parstous, Co, Iran), according to the manufacturer's recommendations.

3-11-2. qPCR

Transcription levels of *icaA* and *algD* were determined by relative qPCR as described using the standard curve method using the RealQ Plus 2x Master Mix Green Kit with ROX (Ampliqon, Co, Danmark) in StepOnePlus Real-Time PCR System (Applied Biosystems, Co, USA). The list of primer sequences used for qPCR are

presented in Table 5-2. For Validation of SYBR Green I reactions, we used the melt-curve analysis function of real-time instruments to distinguish specific products from nonspecific products in qPCR experiment. Transcript levels of *icaA* and *algD* were determined relative to the reference genes, and results are expressed as mean values ± standard deviation using a two-sided Student's t-test and ANOVA tests by GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA 92108, USA)

12-2. Statistical analysis

GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA 92108, USA) was used for the statistical analysis of data and figure production. All data were first assessed for normality using a Kolmogorov–Smirnov test. The results were found to be normally distributed ($p > 0.05$ in the K-S test) and were analyzed using a one-way ANOVA test and expressed as mean values ± standard (mean ± SEM). Pairwise comparisons between groups were then made using Tukey's post hoc tests, where the main effect was seen in ANOVA tests. Data that were not normally distributed ($p < 0.05$ in the K-S test) were analyzed using a Kruskal–Wallis test. Where the main effect was seen in Kruskal–Wallis tests, pairwise comparisons between groups were made using Dunn's multiple comparisons test. In each case, $p < 0.05$ was considered statistically significant.

Chapter 3:

Results

1.3. Antibacterial susceptibility tests and PCR results

Clinical isolates of *S. aureus* were resistant to penicillin, gentamicin, amikacin, erythromycin, ciprofloxacin, tetracycline, clindamycin, trimethoprim/sulfamethoxazole, and were sensitive to linezolid and vancomycin. *S. aureus* isolates were MRSA and were positive for *mecA* and the *icaADBC* operon (Figures 1-3 to 3-3).

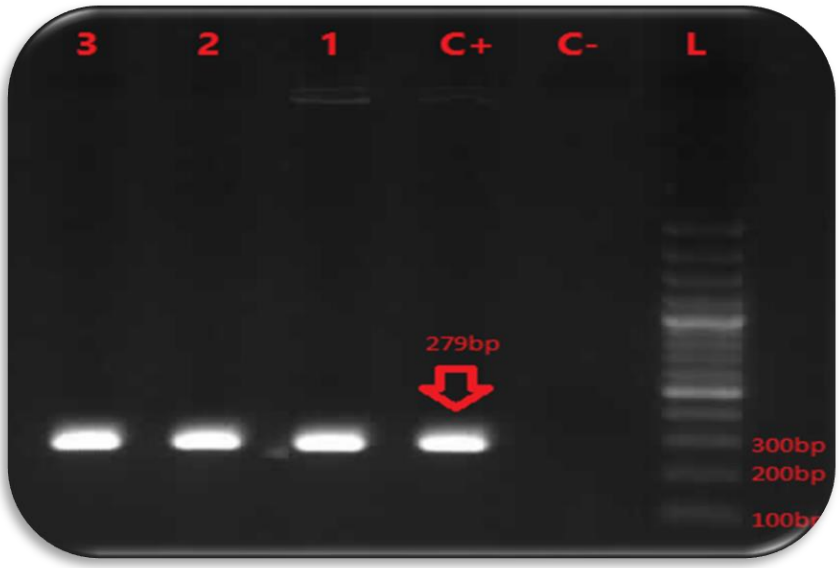

Figure 1-3. Electrophoresis of PCR products for *nuc* gene (PCR product: 162bp) in *S. aureus*; L: DNA marker, C- and C+: Negative and positive control, 1-3 positive samples.

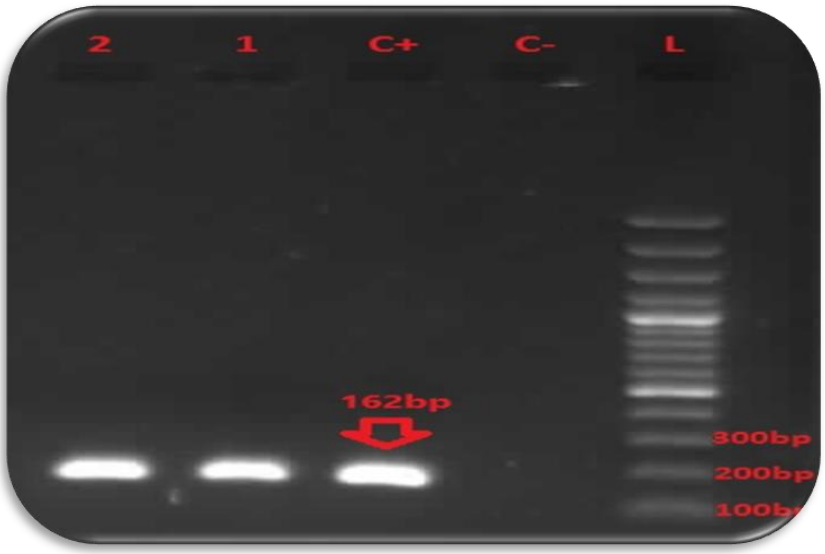

Figure 2-3. Electrophoresis of PCR products for *mecA* gene (PCR product: 162bp) in *S. aureus*; L: DNA marker, C- and C+: Negative and positive control, 1 and 2 positive samples.

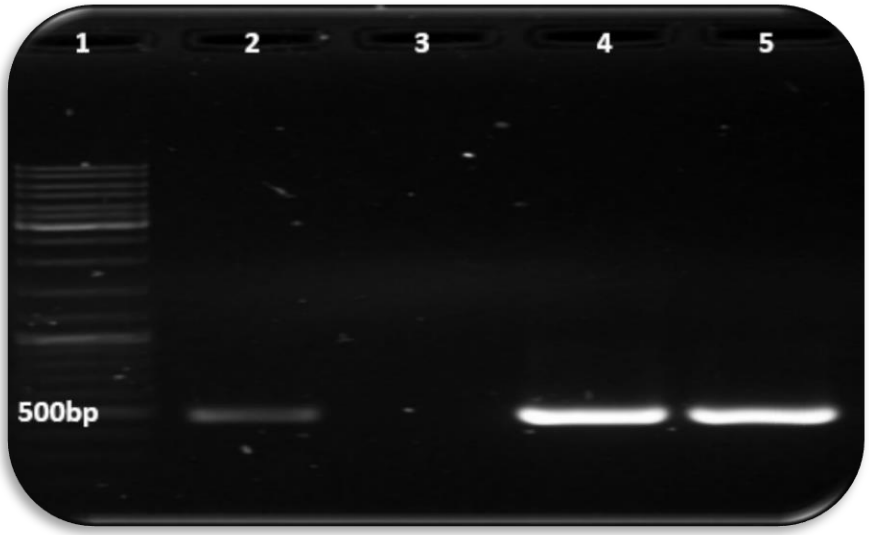

Figure 3-3. Electrophoresis of PCR products for *icaD* gene (PCR product: 483bp) in *S. aureus*; 1: DNA marker, 2: Negative control, 3 and 4 positive samples.

All clinical isolates of *P. aeruginosa* were resistant to meropenem, doripenem, imipenem, ciprofloxacin, piperacillin/tazobactam, gentamicin, aztreonam, cefepime, and ceftazidime. Both clinical isolates were considered biofilm producers according to the microtiter method (Figure 4-3).

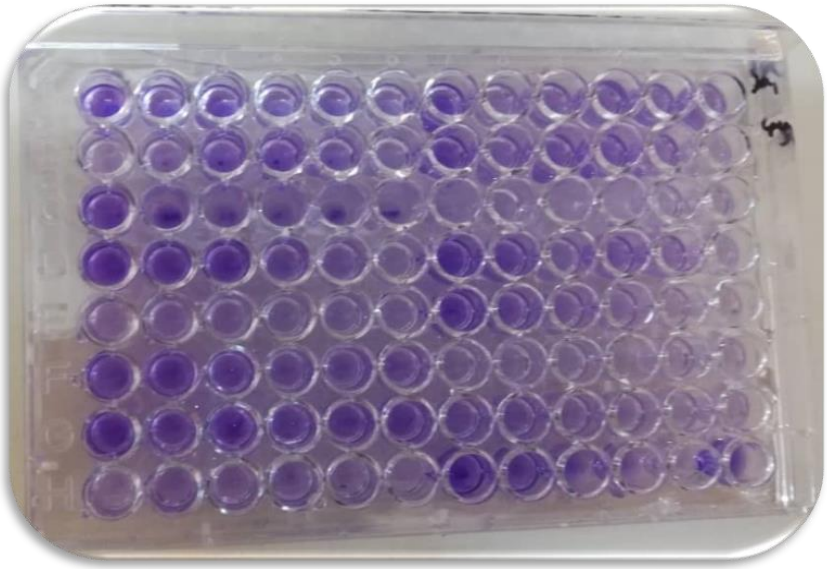

Figure 4-3. Biofilm formation assay with microtiter assay method in 96 well microplate using by staining.

The range of MIC to IBP was 1024-2048 µg/mL, and the MIC range to ASA was 2048-8192 µg/mL among both clinical isolates of *S. aureus* and *P. aeruginosa* (Tables 1-3 to 5-3 and Figure 5-3). DXP had no antibacterial or anti-biofilm effect against the bacterial isolates.

Table 1-3. The MIC, MBIC, and MBEC to ciprofloxacin (µg/mL) alone and with sub-MIC of IBP, ASA, and DXP as anti-inflammatory drugs.										
Antibacterial/ anti-inflammatory drugs	**MIC/MBIC/MBEC (µg/mL)**	**Standard strain**		**Clinical isolates**						
		PAO1	**RP62A**	***S. aureus*** **(n=4)**	***S. aureus*** **(n=2)**	***S. aureus*** **(n=2)**	***S. aureus*** **(n=2)**	***P. aeruginosa*** **(n=4)**	***P. aeruginosa*** **(n=3)**	***P. aeruginosa*** **(n=3)**
IBP	**MIC**	**2048**	**1024**	**1024**	**1024**	**1024**	**1024**	**1024**	**1024**	**2048**
ASA		**8192**	**2048**	**8192**	**4096**	**4096**	**4096**	**4096**	**4096**	**8192**
DXP		-	-	-	-	-	-	-	-	-
CIP		**0.125**	**1**	**128**	**64**	**32**	**16**	**4**	**16**	**8**
CIP+IBP		≤0.03125	0.125	32	16	8	4	2	8	4
CIP+ASA		0.125	0.5	128	64	16	16	4	16	8
CIP+DXP		2	4	256	256	128	128	64	256	128
CIP	**MBIC**	**8**	**16**	**256**	**128**	**128**	**64**	**32**	**64**	**32**
IBP		4096	4096	4096	4096	4096	4096	4096	4096	8192
ASA		-	-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-	-
CIP+IBP		4	4	64	32	16	8	16	16	16
CIP+ASA		8	16	256	64	128	64	32	64	32
CIP+DXP		64	64	1024	1024	256	256	256	1024	256
CIP	**MBEC**	**512**	**512**	**4096**	**2048**	**1024**	**1024**	**256**	**512**	**512**
IBP		-	-	-	-	-	-	-	-	-
ASA		-	-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-	-
CIP+IBP		256	512	2048	1024	512	512	256	256	512
CIP+ASA		512	512	2048	2048	1024	1024	256	512	512
CIP+DXP		1024	1024	≥8192	4096	2048	2048	1024	2048	2048
MIC: Minimum Inhibitory Concentration, MBIC: Minimal Biofilm Inhibitory Concentration, MBEC: Minimal Biofilm Eradication Concentration, CIP: Ciprofloxacin, DXP: Dexamethasone sodium phosphate, ASA: Aspirin (Acetylsalicylic acid), IBP: Ibuprofen.										

Table 2-3. The MIC, MBIC, and MBEC to gentamicin (µg/mL) alone and with sub-MIC of IBP, ASA, and DXP as anti-inflammatory drugs.										
Antibacterial/ anti-inflammatory drugs	**MIC/MBIC/MBEC (µg/mL)**	**Standard strains**		**Clinical isolates**						
		PAO1	**RP62A**	***S. aureus* (n=3)**	***S. aureus* (n=3)**	***S. aureus* n=2)**	***S. aureus* (n=2)**	***P. aeruginosa* (n=3)**	***P. aeruginosa* (n=3)**	***P. aeruginosa* (n=4)**
IBP	**MIC**	**2048**	**1024**	**1024**	**1024**	**1024**	**1024**	**1024**	**1024**	**2048**
ASA		**8192**	**2048**	**4096**	**4096**	**8192**	**8192**	**4096**	**4096**	**8192**
DXP		-	-	-	-	-	-	-	-	-
GEN		**1**	**64**	**64**	**64**	**16**	**8**	**16**	**16**	**32**
GEN+IBP		0.25	32	32	32	4	2	8	8	16
GEN+ASA		1	32	32	32	8	4	16	16	32
GEN+DXP		8	256	256	256	128	64	128	128	128
GEN	**MBIC**	**8**	**256**	**256**	**256**	**64**	**64**	**64**	**64**	**128**
IBP		**4096**	**2048**	**4096**	**4096**	**2048**	**2048**	**4096**	**2048**	**4096**
ASA		-	-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-	-
GEN+IBP		4	32	64	64	8	4	32	32	64
GEN+ASA		8	128	128	256	64	64	64	64	128
GEN+DXP		32	1024	1024	1024	1024	1024	256	256	512
GEN	**MBEC**	**512**	**1024**	**2048**	**1024**	**1024**	**1024**	**512**	**1024**	**1024**
IBP		-	-	-	-	-	-	-	-	-
ASA		-	-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-	-
GEN+IBP		512	512	1024	512	512	512	512	1024	1024

Table 3-3. The MIC, MBIC, and MBEC to imipenem (µg/mL) alone and with sub-MIC of IBP, ASA, and DXP as anti-inflammatory drugs.											
Antibacterial/anti-inflammatory drugs	**MIC/MBIC/MBEC (µg/mL)**	**Standard strains**		**Clinical isolates**							
		PAO1	**RP62A**	***S. aureus* (n=4)**	***S. aureus* (n=2)**	***S. aureus* (n=2)**	***S. aureus* (n=2)**	***P. aeruginosa* (n=4)**	***P. aeruginosa* (n=2)**	***P. aeruginosa* (n=2)**	***P. aeruginosa* (n=2)**
IBP	**MIC**	**2048**	**1024**	**1024**	**1024**	**1024**	**1024**	**1024**	**2048**	**1024**	**1024**
ASA		**8192**	**2048**	**8192**	**4096**	**4096**	**4096**	**4096**	**8192**	**4096**	**4096**
DXP		-	-	-	-	-	-	-	-	-	-
IPM		**0.5**	**0.25**	**32**	**16**	**16**	**8**	**32**	**32**	**16**	**8**
IPM+IBP		0.125	0.125	2	0.5	1	1	8	16	4	2
IPM+ASA		0.25	0.125	8	4	8	4	16	16	8	4
IPM+DXP		0.5	0.25	32	16	16	16	32	32	16	16
IPM	**MBIC**	**2**	**1**	**64**	**32**	**64**	**32**	**512**	**512**	**32**	**16**
IBP		4096	2048	4096	4096	4096	4096	4096	8192	4096	4096
ASA		-	-	-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-	-	-
IPM+IBP		0.125	0.25	2	2	8	4	64	256	16	8
IPM+ASA		0.5	0.25	16	8	16	16	128	256	32	16
IPM+DXP		2	1	32	32	64	64	256	512	32	32
IPM	**MBEC**	**512**	**128**	**2048**	**1024**	**1024**	**1024**	**2048**	**2048**	**1024**	**1024**
IBP		-	-	-	-	-	-	-	-	-	-
ASA		-	-	-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-	-	-
IPM+IBP		256	64	512	512	512	512	1024	2048	1024	1024
IPM+ASA		512	64	2048	512	1024	1024	2048	2048	1024	1024
IPM+DXP		1024	128	2048	1024	2048	2048	2048	2048	1024	1024

MIC: Minimum Inhibitory Concentration, MBIC: Minimal Biofilm Inhibitory Concentration, MBEC: Minimal Biofilm Eradication Concentration, IPM: Imipenem, DXP: Dexamethasone sodium phosphate, ASA: Aspirin (Acetylsalicylic acid), IBP: Ibuprofen.

Table 4-3. The MIC, MBIC, and MBEC (µg/mL) to meropenem alone and with sub-MIC of IBP, ASA, and DXP as anti-inflammatory drugs.									
Antibacterial/anti-inflammatory drugs	**MIC/MBIC/MBEC (µg/mL)**	**Standard strains**		**Clinical isolates**					
		PAO1	**RP62A**	***S. aureus* (n=4)**	***S. aureus* (n=3)**	***S. aureus* (n=3)**	***P. aeruginosa* (n=3)**	***P. aeruginosa* (n=4)**	***P. aeruginosa* (n=3)**
IBP	**MIC**	**1024**	**1024**	**1024**	**1024**	**1024**	**1024**	**2048**	**1024**
ASA		**8192**	**2048**	**8192**	**4096**	**4096**	**4096**	**8192**	**4096**
DXP		-	-	-	-	-	-	-	-
MEM		**0.5**	**≤0.125**	**32**	**16**	**8**	**32**	**32**	**16**
MEM+IBP		0.125	0.125	2	1	1	16	16	8
MEM+ASA		0.125	0.125	16	16	4	8	8	4
MEM+DXP		2	0.125	32	16	8	64	32	32
MEM	**MBIC**	**2**	**0.5**	**128**	**64**	**32**	**64**	**128**	**64**
IBP		4096	2048	4096	4096	4096	4096	8192	4096
ASA		-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-
MEM+IBP		0.5	0.125	8	4	1	32	64	32
MEM+ASA		0.5	0.125	64	16	16	16	32	32
MEM+DXP		8	0.5	128	64	32	64	128	64
MEM	**MBEC**	**512**	**512**	**2048**	**1024**	**1024**	**1024**	**1024**	**1024**
IBP		-	-	-	-	-	-	-	-
ASA		-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-
MEM+IBP		256	256	1024	512	512	1024	1024	1024
MEM+ASA		512	512	2048	1024	1024	1024	1024	1024
MEM+DXP		1024	1024	2048	1024	1024	2048	2048	1024

MIC: Minimum Inhibitory Concentration, MBIC: Minimal Biofilm Inhibitory Concentration, MBEC: Minimal Biofilm Eradication Concentration, MEM: Meropenem, DXP: Dexamethasone sodium phosphate, ASA: Aspirin (Acetylsalicylic acid), IBP: Ibuprofen.

Table 5-3. The MIC, MBIC, and MBEC (µg/mL) to cefepime alone and with sub-MIC of IBP, ASA, and DXP as anti-inflammatory drugs.										
Antibacterial/anti-inflammatory drugs	**MIC/MBIC/MBEC (µg/mL)**	**Standard strains**		**Clinical isolates**						
		PAO1	**RP62A**	***S. aureus* (n=2)**	***S. aureus* (n=3)**	***S. aureus* (n=3)**	***S. aureus* (n=2)**	***P. aeruginosa* (n=4)**	***P. aeruginosa* (n=3)**	***P. aeruginosa* (n=3)**
IBP	**MIC**	**2048**	**1024**	**1024**	**1024**	**1024**	**1024**	**2048**	**1024**	**1024**
ASA		8192	4096	8192	8192	4096	4096	8192	4096	4096
DXP		-	-	-	-	-	-	-	-	-
FEP		1	0.5	64	32	16	16	64	32	32
FEP+IBP		0.5	0.25	4	2	2	1	32	16	8
FEP+ASA		0.25	0.25	8	8	4	2	16	8	8
FEP+DXP		2	0.25	128	128	64	64	128	128	128
FEP	**MBIC**	**4**	**0.5**	**256**	**128**	**32**	**64**	**512**	**256**	**512**
IBP		4096	4096	4096	4096	4096	4096	8192	4096	4096
ASA		-	-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-	-
FEP+IBP		2	0.125	8	8	2	4	256	128	256
FEP+ASA		2	0.25	64	64	16	32	128	64	64
FEP+DXP		4	0.5	1024	1024	64	256	1024	512	1024
FEP	**MBEC**	**512**	**256**	**2048**	**2048**	**1024**	**2048**	**4096**	**4096**	**4096**
IBP		-	-	-	-	-	-	-	-	-
ASA		-	-	-	-	-	-	-	-	-
DXP		-	-	-	-	-	-	-	-	-
FEP+IBP		256	128	512	256	512	1024	2048	2048	2048
FEP+ASA		512	256	1024	1024	1024	2048	2048	2048	2048
FEP+DXP		1024	1024	4096	4096	2048	4096	4096	4096	4096

Figure 5-3. Determination of minimum inhibitory concentration (MIC) of clinical isolates to antibiotic agents and anti-inflammatory drugs using broth microdilution method.

2.3. MIC, MBIC, and MBEC of isolates to antibiotic agents alone and with ASA, IBP, and DXP

The $MIC_{50, 90}$, $MBIC_{50, 90}$, and $MBEC_{50, 90}$ of both clinical isolates of *S. aureus* and *P. aeruginosa* to antibiotic agents alone and combined with IBP, ASA, and DXP are presented in Table 6-3. The level of MIC, MBIC, and MBEC of isolates to antibiotic agents alone and with ASA, IBP, and DXP are presented in Tables 1-3 to 5-3. At 250 µg/mL of IBP in combination with serial dilution of antibiotic agents, the MIC and MBIC levels were reduced 4-8 fold to CIP, 2-16 fold to GEN, and 8-32 fold to IPM,

MEM, and FEP, among MRSA isolates. The fold change of MIC and MBIC in the presence of ASA (250 μg/mL) were decreased by 0-2 fold to CIP and GEN, 2-16 fold to IPM, 2-4 fold to MEM, and 2-8 fold to FEP. In contrast to IBP and ASA, DXP increased the MIC and MBIC levels 2-8 fold to CIP and FEP, 4-16 fold to GEN, and 0-2 fold to IPM, and DXP had no effect on the levels of MIC and MBIC to MEM among MRSA isolates. The level of MBEC among MRSA isolates to antibiotic agents was decreased 2-fold to CIP, GEN, and MEM, 2-4 fold to IPM, and 2-8 fold to FEP. MBEC levels in the presence of ASA were reduced 0-2 fold to CIP, GEN, IPM, and FEP among clinical isolates of *S. aureus*. ASA had no effect on the levels of MBEC to MEM among MRSA isolates. In the presence of IBP (250μg/mL) in combination with serial dilution of antibiotic agents, the MIC and MBIC levels were reduced 2-4 fold to CIP, MEM, and FEP, 2 fold to GEN, and 2-8 fold to IPM, among carbapenem-resistant *P. aeruginosa*, isolates. The fold change of MIC and MBIC in the presence of ASA (250 μg/mL) were decreased by 0-4 fold to IPM, 2-4 fold to MEM, and 2-8 fold to FEP, and ASA had no effect on the levels of MIC and MBIC to CIP and GEN among carbapenem-resistant *P. aeruginosa* isolates. Similar findings for MRSA isolates, DXP increased the MIC and MBIC level 8-16 fold to CIP, 2-8 fold to GEN, 2 fold to IPM and MEM, and 2-4 fold to FEP in clinical isolates of *P. aeruginosa*. The level of MBEC to antibiotic agents was decreased 0-2 fold to CIP, IPM, and FEP. IBP

had no effect on the levels of MBEC to GEN and MEM among carbapenem-resistant *P. aeruginosa*. Also, ASA only reduced MBEC 2 fold to FEP and did not decrease levels of MBEC to CIP, GEN, IPM, and MEM in the presence of ASA among carbapenem-resistant *P. aeruginosa* isolates. MBEC levels in the presence of DXP were increased 4 fold to CIP, 0-2 fold to GEN and MEM, and we did not observe any fold change in MBEC level to IPM and FEP in the presence of DXP among clinical isolates *of P. aeruginosa*. The range and fold change in MIC, MBIC, and MBEC levels among MRSA, and carbapenem-resistant *P. aeruginosa* isolates are presented in Tables 7-3 and 8-3. For DXP in combination with CIP, GEN, and FEP, we observed a significant increase of MIC to these antibiotic agents among both clinical isolates. Contrary to the abrogated effects of DXP on the MIC level to CIP, GEN, and FEP, we did not observe a significant change in the level of MIC to IMP and MEM in combination with DXP in any of the clinical isolates of *P. aeruginosa* or *S. aureus*. Also, we observed a significant increase in MBIC to CIP and GEN in combination with DXP in both isolates and a significant increase in MBIC to FEP in combination with DXP among *S. aureus*. However, we did not observe any change in MBIC to IPM and MEM in combination with DXP in any of the clinical isolates. DXP significantly increased the level of MBEC to CIP and FEP among clinical isolates of *S. aureus*, and these changes were significant among *P. aeruginosa* isolates for GEN

and MEM. The combined use of IBP significantly reduced the MIC level to CIP, GEN, IPM, MEM, and FEP in both clinical isolates of *S. aureus* and *P. aeruginosa* (Tables 7-3 and 8-3, Figures 4-3 to 8-3). The MBIC level to CIP, GEN, IPM, and MEM among clinical isolates of *S. aureus* and *P. aeruginosa* in combination with IBP was reduced significantly. The combination of FEP and IBP significantly reduced MBIC among *S. aureus*, but these changes were not significant for *P. aeruginosa*. Also, we observed a significant decrease in MBEC in both clinical isolates of *S. aureus* and *P. aeruginosa* to FEP in combination with IBP. Among the *S. aureus* isolates, we observed a significant decrease to GEN, IMP, and MEM in MBEC, and a significant decrease in MBEC to CIP was observed among *P. aeruginosa* isolates in combination with IBP. Among both clinical isolates of *S. aureus* and *P. aeruginosa*, the fold change level of MIC to FEP and IPM in combination with ASA decreased significantly but was not significant for CIP. The fold change level of MIC for MEM in combination with ASA was significant for *P. aeruginosa* isolates but was not significant for *S. aureus* isolates. The fold change level of MIC to GEN in combination with ASA was significant for *S. aureus* isolates but not for *P. aeruginosa* isolates. The fold change level of MBIC to MEM combined with ASA was significant for both clinical isolates of *S. aureus* and *P. aeruginosa* but was not significant for CIP and GEN. Also, we observed a significant decrease in MBIC fold change to FEP and IPM

for *P. aeruginosa* and *S. aureus* in combination with ASA, respectively. Only the change in the MBEC level to FEP was significant in *P. aeruginosa* isolates combined with ASA. Our findings showed that IBP had a more effect on reducing MIC and MBIC levels to CIP, GEN, IPM, MEM, and FEP than ASA on both clinical isolates (Tables). Also, IBP had a more effect on decreasing MBIC levels to CIP, GEN, MEM, and FEP than MIC levels in MRSA isolates and had a more effect on decreasing MBIC levels to IMP and CIP than MIC among *P. aeruginosa* isolates.

IBP significantly decreased the levels of MIC and MBIC for CIP among both clinical isolate and MBEC among *S. aureus*. DXP significantly increased the MIC, MBIC, and MBEC for CIP among both clinical isolates, and ASA had no significant effects on MIC, MBIC, and MBEC for CIP (Figure 6-3). IBP significantly decreased the level of MIC, MBIC, and MBEC for GEN among clinical isolates of *S. aureus* and MIC and MBIC among *P. aeruginosa* isolates. ASA only significantly decreased the level of MIC for GEN among clinical isolates of *S. aureus*. DXP significantly increased the level of MIC and MBIC for GEN among both clinical isolates and MBEC among P. aeruginosa iso-lates (Figure 7-3). IBP significantly decreased the level of MIC, MBIC, and MBEC for IPM among both clinical isolates of *S. aureus* and *P. aeruginosa*. ASA significantly reduced the level of MIC for IPM among both clinical isolates and MBIC for IPM among *S. aureus* isolates. DXP did not significantly affect the MIC, MBC, and

MBEC for IPM among both clinical isolates (Figure 8-3). IBP significantly decreased the level of MIC, MBIC, and MBEC for MEM among clinical isolates of *S. aureus* and MIC and MBIC among *P. aeruginosa* isolates. ASA significantly decreased the level of MIC for MEM among clinical isolates of *S. aureus* and MIC, and MBIC for MEM among *P. aeruginosa* isolates. DXP only significantly increased the level of MBEC for MEM among clinical isolates of *P. aeruginosa* (Figure 9-3). IBP significantly decreased the level of MIC, MBIC, and MBEC for FEP among clinical isolates of *S. aureus* and MIC and MBEC among *P. aeruginosa* isolates. ASA significantly decreased the level of MIC for FEP among clinical isolate *S. aureus* and MIC and MBIC for FEP among *P. aeruginosa*. DXP significantly increased the levels of MIC, MBIC, and MBEC for FEP among clinical isolates of *S. aureus* and MIC among clinical isolates of *P. aeruginosa* (Figure 9-3).

Table 6-3: Distribution of MIC, MBIC, and MBEC 50 and 90 in clinical isolates of *S. aureus* and *P. aeruginosa* with and without sub-MIC of anti-inflammatory drugs.

Antibiotics agents	MIC, MBIC, and MBEC (µg/mL) 50 and 90 for clinical isolates of *S. aureus* isolates						MIC, MBIC, and MBEC (µg/mL) 50 and 90 for clinical isolates of *P. aeruginosa*					
	MIC_{50}	MIC_{90}	$MBIC_{50}$	$MBIC_{90}$	$MBEC_{50}$	$MBEC_{90}$	MIC_{50}	MIC_{90}	$MBIC_{50}$	$MBIC_{90}$	$MBEC_{50}$	$MBEC_{90}$
CIP	**64**	**128**	**128**	**256**	**1024**	**2048**	**8**	**16**	**32**	**64**	**512**	**512**
CIP/IBP	16	32	32	64	1024	2048	4	8	16	16	256	512
CIP/ASA	64	128	128	256	1024	2048	8	16	32	64	512	512
CIP/DXP	256	256	256	1024	4096	≥8192	128	256	256	1024	2048	2048
GEN	**64**	**64**	**256**	**256**	**1024**	**1024**	**16**	**32**	**64**	**128**	**1024**	**1024**
GEN/IBP	32	32	64	64	512	1024	8	16	32	64	1024	1024
GEN/ASA	32	32	128	256	1024	1024	16	32	64	128	1024	1024
GEN/DXP	256	256	1024	1024	1024	2048	128	128	256	512	1024	1024
IPM	**16**	**32**	**64**	**64**	**1024**	**2048**	**32**	**32**	**512**	**512**	**2048**	**2048**
IPM/IBP	1	2	2	8	512	512	8	16	64	256	1024	2048
IPM/ASA	8	8	8	16	1024	2048	16	16	128	256	2048	2048
IPM/DXP	64	64	32	64	2048	2048	32	32	256	512	2048	2048
MEM	**16**	**32**	**64**	**128**	**1024**	**2048**	**32**	**32**	**64**	**128**	**1024**	**1024**
MEM/IBP	1	2	4	8	512	1024	16	16	32	64	1024	1024
MEM/ASA	16	16	16	64	1024	2048	8	8	16	32	1024	1024
MEM/DXP	16	32	64	128	1024	2048	32	64	64	128	1024	2048
FEP	**16**	**64**	**64**	**256**	**2048**	**2048**	**32**	**64**	**512**	**512**	**4096**	**4096**
FEP/IBP	2	4	4	8	512	512	16	32	256	256	2048	2048
FEP/ASA	8	8	32	64	1024	1024	8	16	64	128	2048	2048
FEP/DXP	64	128	256	1024	2048	4096	128	128	512	1024	4096	4096

CIP: ciprofloxacin, GEN: gentamicin, IPM: imipenem, MEM: meropenem, FEP: cefepime, IBP: ibuprofen, ASA: acetylsalicylic acid, DXP: dexamethasone sodium phosphate, MIC: minimum inhibitory concentration, MBIC: minimum biofilm inhibitory concentration, MBEC: minimum biofilm eradication concentration.

Table 7-3. The fold changes of MIC, MBIC, and MBEC to antibiotic agents with/without sub-MIC of anti-inflammatory drugs among MRSA isolates.

Drugs	MIC range	MIC fold changes range	Isolates n (%)	*p-value*	MBIC range	MBIC fold changes range	Isolates n (%)	*p-value*	MBEC range	MBEC fold changes range	Isolates n (%)	*p-value*
CIP	**16-128**	**C**	**-**	**-**	**64-256**	**C**	**-**	**-**	**1024-4096**	**C**	**-**	
CIP+IBP	4-32	4↓	10(100)	0.0058	8-64	4-8↓	10(100)	0.0051	512-2048	2↓	10(100)	0.0039
CIP+ASA	16-128	0-2↓	2(20)	>0.9999	128-256	0-2↓	2(20)	>0.9999	1024-2048	0-2↓	4(40)	0.7261
CIP+DXP	128-256	2-8↑	10(100)	0.0055	256-1024	2-8↑	10(100)	0.0058	2048-8192	2↑	10(100)	0.0141
GEN	**8-64**	**C**	**-**	**-**	**64-256**	**C**	**-**	**-**	**1024-2048**	**C**	**-**	**-**
GEN +IBP	2-32	2-4↓	10(100)	0.0012	4-64	4-16↓	10(100)	0.0013	512-1024	2↓	10(100)	0.0007
GEN +ASA	4-32	2↓	10(100)	0.0166	64-128	0-2↓	3(100)	>0.9999	1024	2↓	3(30)	0.8025
GEN +DXP	64-256	4-8↑	10(100)	0.0001	1024	4-16↑	10(100)	0.0176	1024-2048	4↑	4(40)	0.4255
IPM	**8-32**	**C**	**-**	**-**	**32-64**	**C**	**-**	**-**	**1024-2048**	**C**	**-**	**-**
IPM +IBP	0.5-2	8-32↓	10(100)	<0.0001	2-8	8-16↓	10(100)	<0.0001	512	2-4↓	10(100)	<0.0001
IPM +ASA	4-8	2-4↓	10(100)	0.0052	8-16	2-4↓	10(100)	0.0028	512-2048	0-2↓	20(20)	0.9313
IPM+DXP	16-32	2↑	2(20)	>0.9999	32-64	0-2↑	2(20)	>0.9999	1024-2048	0-2↑	4(40)	0.7792
MEM	**8-32**	**C**	**-**	**-**	**32-128**	**C**	**-**	**-**	**1024-2048**	**C**	**-**	**-**
MEM +IBP	1-2	8-16↓	10(100)	<0.0001	1-8	16-32↓	10(100)	<0.0001	512-1024	2↓	10(10)	<0.0001
MEM +ASA	4-16	0-2↓	7(70)	0.0707	16-64	2-4↓	10(100)	0.0032	1024-2048	0	-	>0.9999
MEM +DXP	8-32	0	-	>0.9999	32-128	0	-	>0.9999	1024-2048	0	-	>0.9999
FEP	**16-64**	**C**	**-**	**-**	**32-256**	**C**	**-**	**-**	**1024-2048**	**C**	**-**	**-**
FEP +IBP	1-4	8-16↓	10(100)	0.0003	2-8	16-32↓	10(100)	<0.0001	256-1024	2-8↓	10(100)	0.0023
FEP +ASA	2-8	4-8↓	10(100)	0.0468	16-64	2-4↓	10(100)	0.0934	1024-2048	2↓	5(50)	0.6736
FEP +DXP	64-128	2-4↑	10(100)	<0.0001	64-1024	2-8↑	10(100)	<0.0001	2048-4096	2↑	10(100)	0.0226

CIP: ciprofloxacin, GEN: gentamicin, IPM: imipenem, MEM: meropenem, FEP: cefepime, IBP: ibuprofen, ASA: acetylsalicylic acid, DXP: dexamethasone sodium phosphate, MIC: minimum inhibitory concentration, MBIC: minimum biofilm inhibitory concentration, MBEC: minimum biofilm eradication concentration, C: control, ↑: increase, and ↓: decrease.

Table 8-3. The fold changes of MIC, MBIC, and MBEC to antibiotic agents with/without sub-MIC of anti-inflammatory drugs among carbapenem-resistant *P. aeruginosa*.

Drugs	MIC range	MIC fold changes range	Isolates n (%)	*p-value*	MBIC range	MBIC fold changes range	Isolates n (%)	*p-value*	MBEC range	MBEC fold changes range	Isolates n (%)	*p-value*
CIP	**4-16**	**C**	**-**	**-**	**32-64**	**C**	**-**	**-**	**256-512**	**C**	**-**	**-**
CIP+IBP	2-8	2↓	10(100)	0.0031	16	2-4↓	10(100)	0.0035	256-512	0-2↓	4(40)	0.5728
CIP+ASA	4-16	0	-	>0.9999	32-64	0	0	>0.9999	256-512	0	-	>0.9999
CIP+DXP	64-256	16↑	10(100)	0.0031	256-1024	8-16↑	10(100)	0.0035	1024-2048	4↑	10(100)	<0.0001
GEN	**16-32**	**C**	**-**	**-**	**64-128**	**C**	**-**	**-**	**512-1024**	**C**	**-**	**-**
GEN +IBP	8-16	2↓	10(100)	0.0035	32-64	2↓	10(100)	0.0032	512-1024	0	-	>0.9999
GEN +ASA	16-32	0	-	>0.9999	64-128	0	0	>0.9999	512-1024	0	-	>0.9999
GEN +DXP	128	4-8↑	10(100)	0.0035	256-512	2-4↑	10(100)	0.0032	1024	0-2↑	3(30)	0.0101
IMP	**8-32**	**C**	**-**	**-**	**16-512**	**C**	**-**	**-**	**1024-2048**	**C**	**-**	**-**
IMP +IBP	2-16	2-4↓	10(100)	<0.0001	8-256	2-8↓	10(100)	0.0001	1024-2048	0-2↓	4(40)	0.0082
IMP +ASA	4-16	2↓	10(100)	0.0038	32-256	0-4↓	60(60)	0.0019 1	1024-2048	0	-	>0.9999
IMP +DXP	16-32	2↑	2(20)	>0.9999	32-512	2↑	2(20)	>0.9999	1024-2048	0	-	>0.9999
MEM	**16-32**	**C**	**-**	**-**	**64-128**	**C**	**-**	**-**	**1024**	**C**	**-**	**-**
MEM +IBP	8-16	2↓	10(100)	0.0313	32-64	2↓	10(100)	0.0012	1024	0	-	>0.9999
MEM +ASA	4-8	4↓	10(100)	<0.0001	32	2-4↓	10(100)	<0.0001	1024	0	-	>0.9999
MEM +DXP	32-64	2↑	6(60)	0.5526	64-128	0	-	>0.9999	1024-2048	2↑	10(100)	<0.0001
FEP	**32-64**	**C**	**-**	**-**	**256-512**	**C**	**-**	**-**	**4096**	**C**	**-**	**-**
FEP +IBP	8-32	2-4↓	10(100)	0.0153	128-256	2↓	10(100)	0.0724	2048	2↓	10(100)	<0.0001
FEP +ASA	8-16	4↓	10(100)	0.0003	64-128	2-8↓	10(100)	0.0001	2048	2↓	10(100)	0.0001
FEP +DXP	128	2-4↑	10(100)	0.0001	512-1024	2↑	10(100)	0.1806	4096	0↑	-	>0.9999

CIP: ciprofloxacin, GEN: gentamicin, IPM: imipenem, MEM: meropenem, FEP: cefepime, IBP: ibuprofen, ASA: acetylsalicylic acid, DXP: dexamethasone sodium phosphate, MIC: minimum inhibitory concentration, MBIC: minimum biofilm inhibitory concentration, MBEC: minimum biofilm eradication concentration, C: control, ↑: increase, and ↓: decrease.

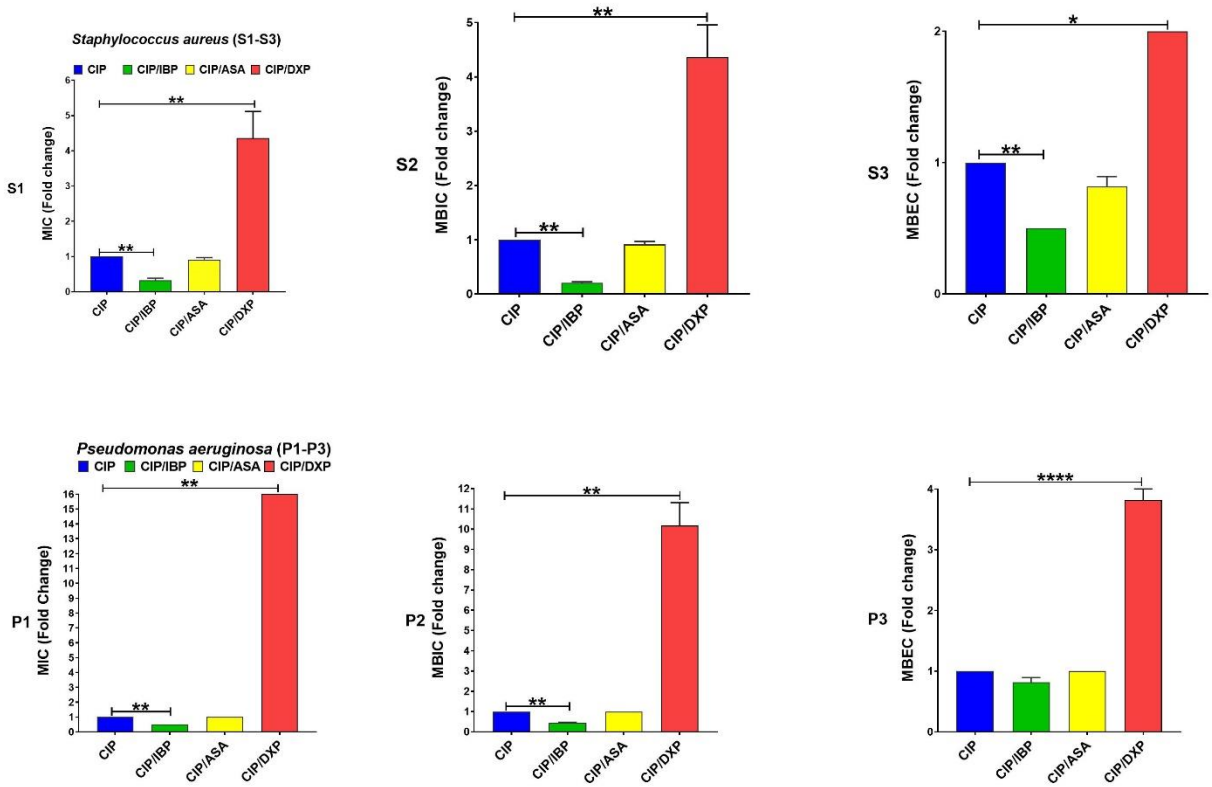

Figure 6-3. Effects sub-MIC of Ibuprofen (IBP: 200µg/mL), aspirin (ASA: 200µg/mL), and dexamethasone sodium phosphate (DXP: 500µg/mL) on the level of MIC, MBIC, and MBEC in combination with ciprofloxacin (CIP). Graphs were drawn based on fold change of MIC, MBIC, and MBEC to CIP in combination with sub-MIC of IBP, ASA, and DXP on clinical isolates of *S. aureus* (S1-S3) and *P. aeruginosa* (P1-P3). Data are displayed as the mean ± standard error of the mean and were analyzed using ANOVA and nonparametric Kruskal–Wallis test. *, statistical significance with $p \leq 0.05$; **, statistical significance with $p \leq 0.01$; ***, statistical significance with $p \leq 0.001$; and ****, statistical significance with $p \leq 0.0001$, as compared to the CIP (Control) group.

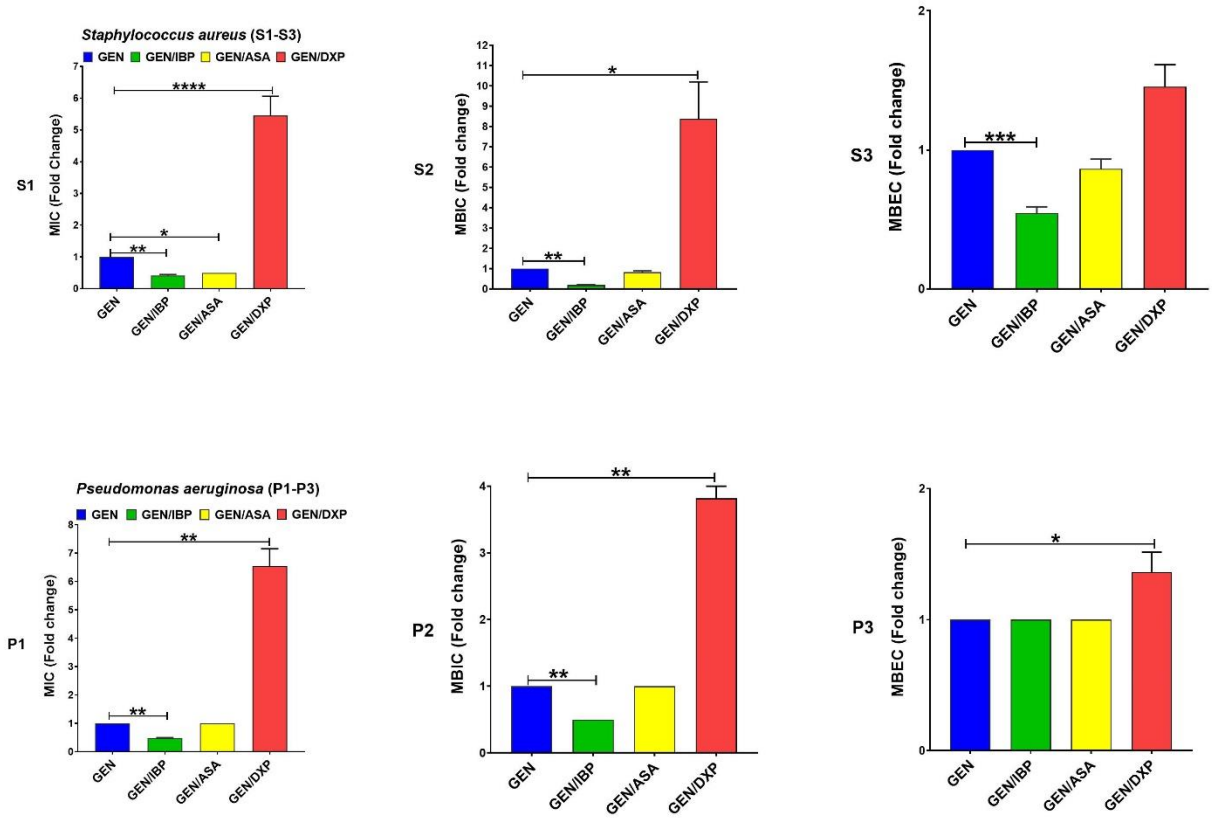

Figure 7-3. Effects sub-MIC of Ibuprofen (IBP: 200µg/mL), aspirin (ASA: 200µg/mL), and dexamethasone sodium phosphate (DXP: 500µg/mL) on the level of MIC, MBIC, and MBEC in combination with gentamicin (GEN). Graphs were drawn based on fold change of MIC, MBIC, and MBEC to GEN in combination with sub-MIC of IBP, ASA, and DXP on clinical isolates of ***S. aureus*** **(S1-S3) and** ***P. aeruginosa*** **(P1-P3).** Data are displayed as the mean ± standard error of the mean and were analyzed using ANOVA and nonparametric Kruskal–Wallis test. *, statistical significance with $p \leq 0.05$; **, statistical significance with $p \leq 0.01$; ***, statistical significance with $p \leq 0.001$; and ****, statistical significance with $p \leq 0.0001$, as compared to the GEN (Control) group.

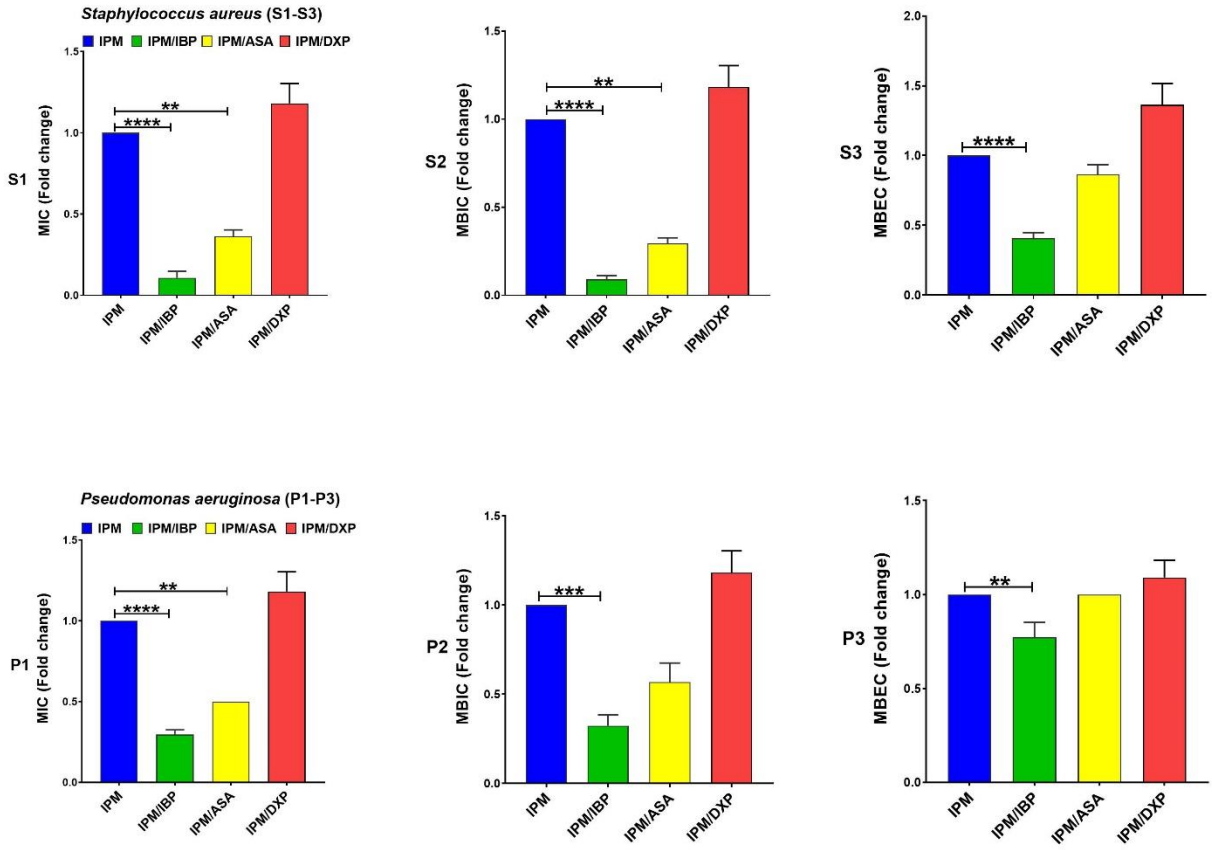

Figure 8-3. Effects sub-MIC of Ibuprofen (IBP: 200µg/mL), aspirin (ASA: 200µg/mL), and dexamethasone sodium phosphate (DXP: 500µg/mL) on the level of MIC, MBIC, and MBEC in combination with imipenem (IPM). Graphs were drawn based on fold change of MIC, MBIC, and MBEC to IPM in combination with sub-MIC of IBP, ASA, and DXP on clinical isolates of ***S.*** *aureus* (S1-S3) and *P. aeruginosa* (P1-P3). Data are displayed as the mean ± standard error of the mean and were analyzed using ANOVA and nonparametric Kruskal–Wallis test. *, statistical significance with $p \leq 0.05$; **, statistical significance with $p \leq 0.01$; ***, statistical significance with $p \leq 0.001$; and ****, statistical significance with $p \leq 0.0001$, as compared to the IPM (Control) group.

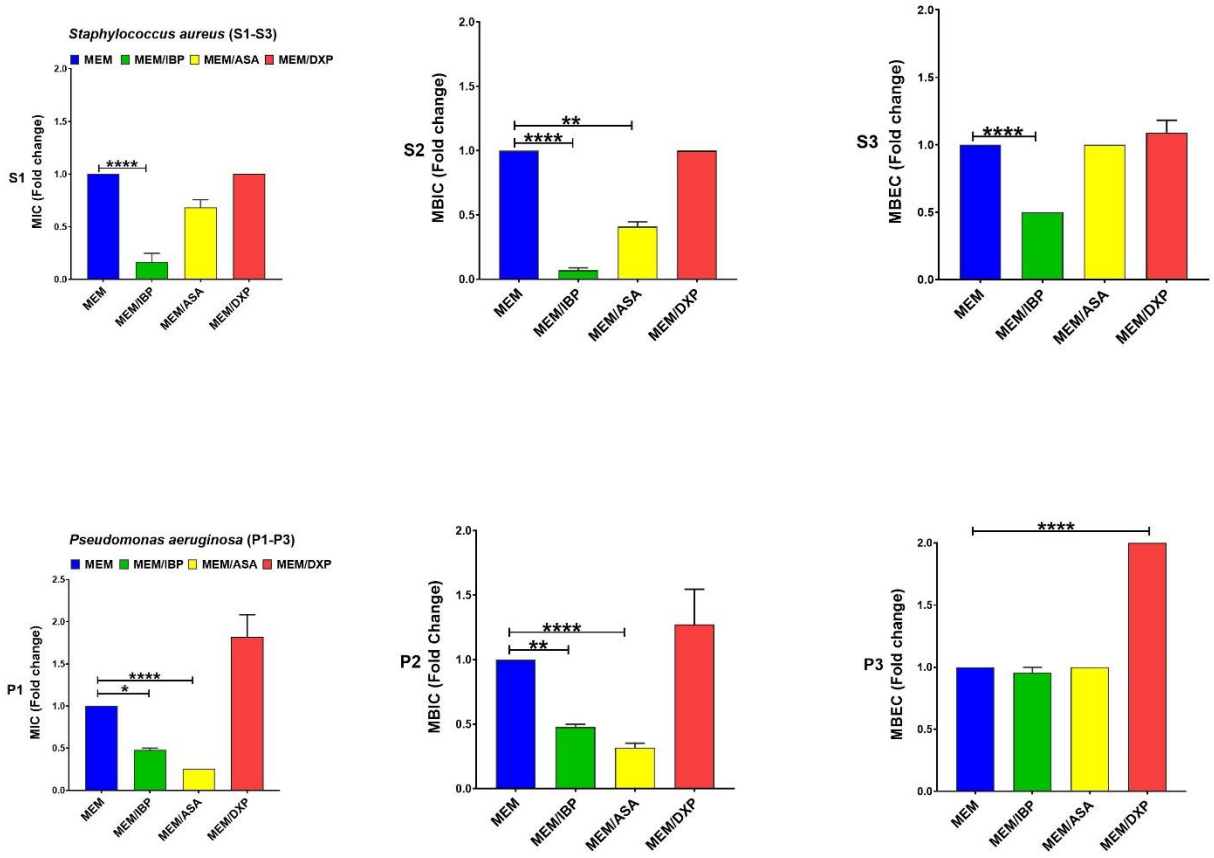

Figure 9-3. Effects sub-MIC of Ibuprofen (IBP: 200µg/mL), aspirin (ASA: 200µg/mL), and dexamethasone sodium phosphate (DXP: 500µg/mL) on the level of MIC, MBIC, and MBEC in combination with meropenem (MEM). Graphs were drawn based on fold change of MIC, MBIC, and MBEC to MEM in combination with sub-MIC of IBP, ASA, and DXP on clinical isolates of *S. aureus* (S1-S3) and *P. aeruginosa* (P1-P3). Data are displayed as the mean ± standard error of the mean and were analyzed using ANOVA and nonparametric Kruskal–Wallis test. *, statistical significance with $p \leq 0.05$; **, statistical significance with $p \leq 0.01$; ***, statistical significance with $p \leq 0.001$; and ****, statistical significance with $p \leq 0.0001$, as compared to the MEM (Control) group.

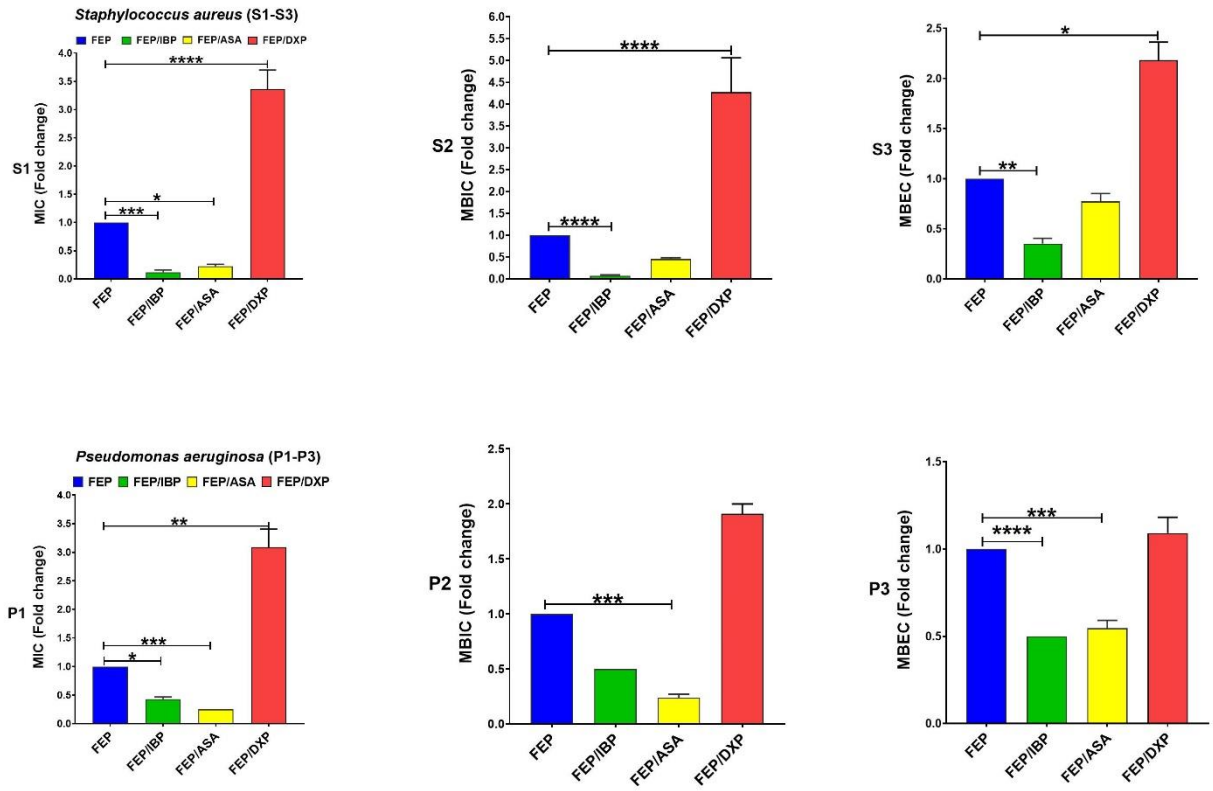

Figure 10-3. Effects sub-MIC of Ibuprofen (IBP: 200µg/mL), aspirin (ASA: 200µg/mL), and dexamethasone sodium phosphate (DXP: 500µg/mL) on the level of MIC, MBIC, and MBEC in combination with cefepime (FEP). Graphs were drawn based on fold change of MIC, MBIC, and MBEC to FEP in combination with sub-MIC of IBP, ASA, and DXP on clinical isolates of ***S. aureus*** **(S1-S3) and** ***P. aeruginosa*** **(P1-P3).** Data are displayed as the mean ± standard error of the mean and were analyzed using ANOVA and nonparametric Kruskal–Wallis test. *, statistical significance with $p \leq 0.05$; **, statistical significance with $p \leq 0.01$; ***, statistical significance with $p \leq 0.001$; and ****, statistical significance with $p \leq 0.0001$, as compared to the CIP (Control) group.

3-3. qPCR experiment

Analysis of qPCR experiment results showed changes in the transcriptional levels of *icaA* and *algD* in the presence of 200 µg/mL IBP and ASA and 500 µg/mL DXP compared to the control group. The expression level of *icaA* significantly decreased in the presence of IBP compared to the control. Although the transcriptional level of *icaA* was decreased in the presence of ASA, it was not significant. The expression level of *icaA* in clinical isolates of *S. aureus* in the

presence of DXP significantly increased compared to the control. Transcriptional levels of *algD* in *P. aeruginosa* isolates in the presence of IBP and ASA were significantly decreased compared to the control group, and expression levels of *algD* significantly increased in the presence of DXP (Figures 11-3 to 14-3).

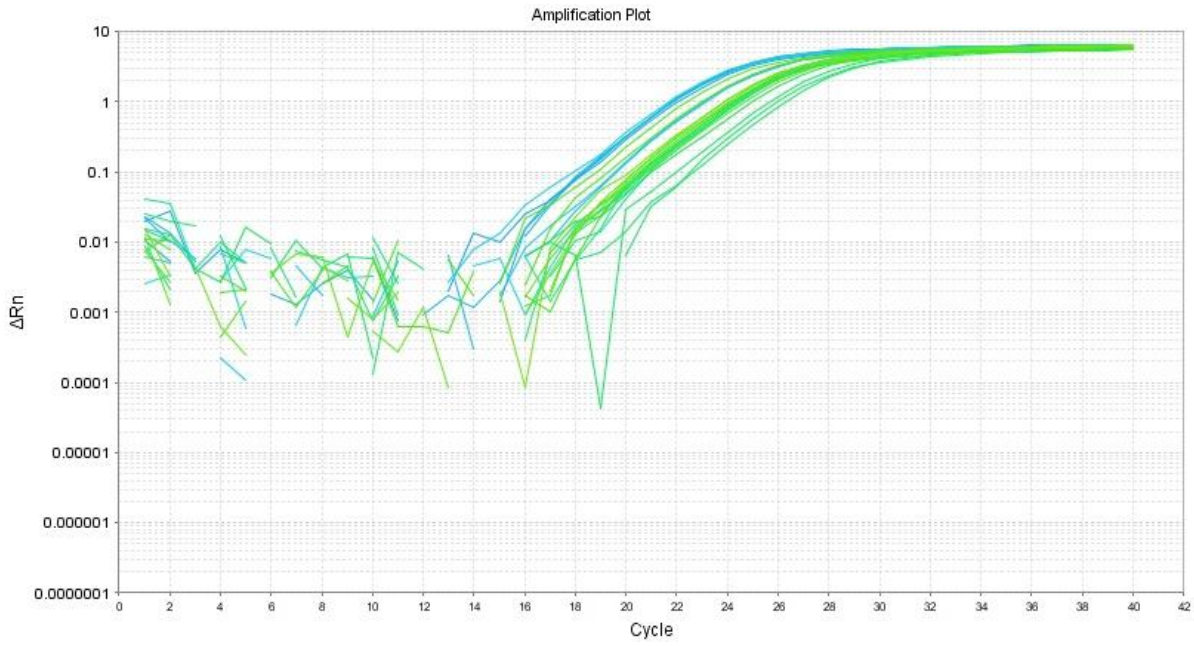

Figure 11-3. Amplification plot for *algD* gene in qPCR experiment.

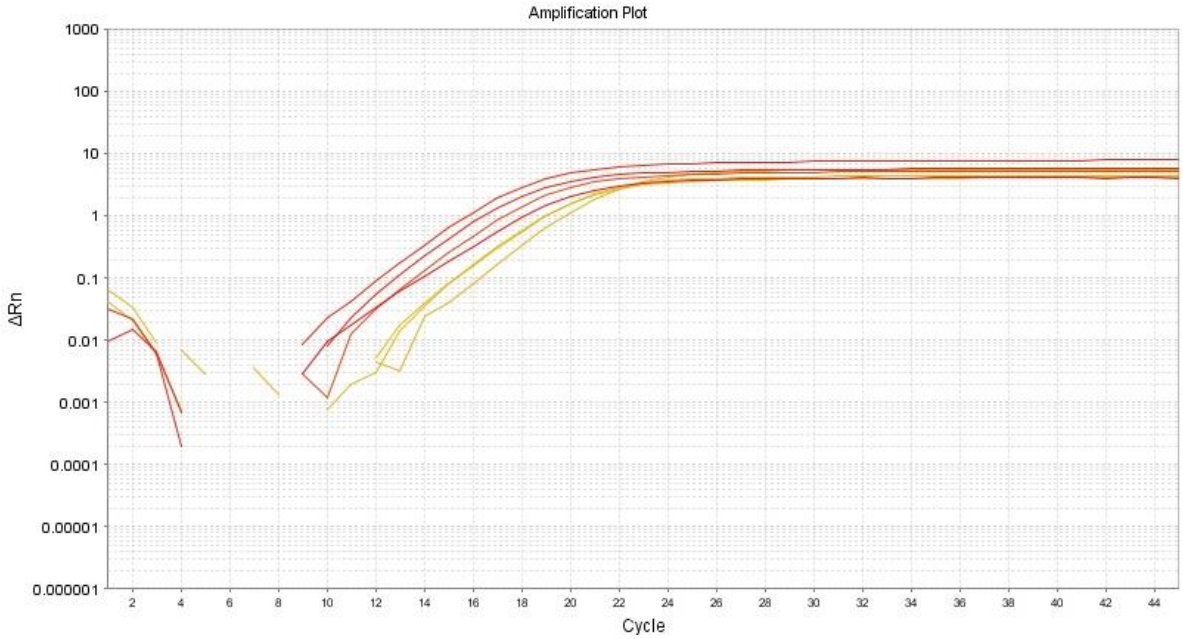

Figure 12-3. Amplification plot for *icaA* gene in qPCR experiment.

Figure 13-3. Melt-curve analysis of *icaA* and *gyrB* gene in Real-time experiment (qPCR) for distinguishing specific products from nonspecific products.

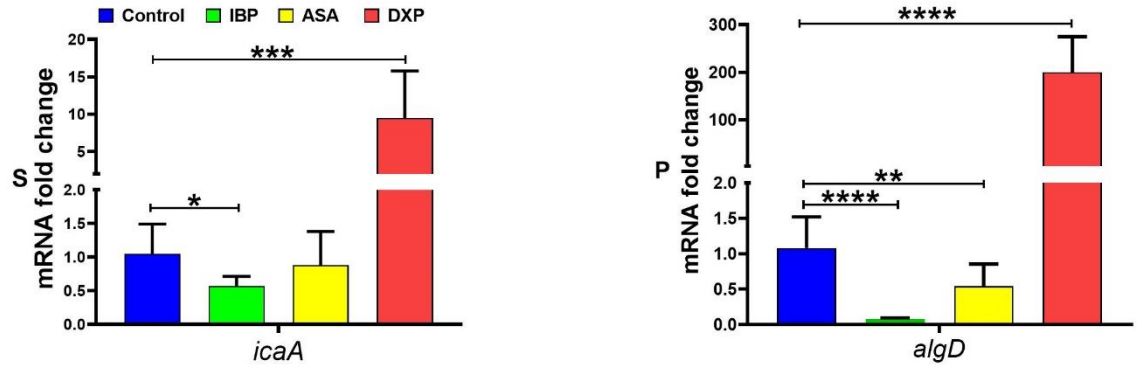

Figure 14-3. The transcriptional level of *icaA* in *S. aureus* with and without the sub-MIC level of IBP, ASA, and DXP. The transcriptional level of *algD* in *P. aeruginosa* with and without the sub-MIC level of IBP (200µg/mL), ASA (200µg/mL), and DXP (500µg/mL). Graphs were drawn based on fold change in transcriptional level of *icaA* and *algD* in clinical isolates of *S. aureus* and *P. aeruginosa*, respectively, treated with sub-MIC of IBP, ASA, and DXP. Data are displayed as the mean ± standard error of the mean from 3 replicate experiments and were analyzed using the ANOVA test. *, statistical significance with $p \leq 0.05$; **, statistical significance with $p \leq 0.01$; ***, statistical significance with $p \leq 0.001$; and ****, statistical significance with $p \leq 0.0001$.

Chapter 4:

Discussion and Conclusion

The resistance of bacteria to antibiotic agents is increasing worldwide. The spread of multi-drug resistant (MDR) bacteria such as MRSA or carbapenem-resistant P. aeruginosa isolates is a global threat (101). Anti-inflammatory drugs are commonly used in combination with antibiotics to control the systemic effects of infection (91). Therefore, evaluating the combined effects of these drugs and antibiotic agents on bacteria can be important. Most studies on anti-inflammatory drugs' antimicrobial and anti-biofilm activity have focused on non-clinical bacterial isolates (102). In this study, we evaluated the effect of anti-inflammatory drugs including IBP, ASA, and DXP on the antibacterial and anti-biofilm activity of CIP, GEN, IMP, MEM, and FEP on non-duplicate clinical isolates of carbapenem-resistant *P. aeruginosa* (n=10) and MRSA (n=10). We also determined the transcriptional level of biofilm-related genes, including *icaA* and *algD* in sub-MIC of IBP (200 µg/mL), ASA (200 µg/mL), and DXP (500 µg/mL) in clinical isolates of MRSA and carbapenem-resistant *P. aeruginosa*, respectively. There are different anti-inflammatory drugs, including non-steroidal, such as aspirin, ibuprofen, diclofenac, naproxen, and corticosteroidal anti-inflammatory drugs such as dexamethasone, betamethasone, and hydrocortisone, that are commonly used to ameliorate fever and other symptoms of acute and chronic infections (103). We describe here that IBP and ASA, combined with some common antibiotics including CIP, GEN, IMP, MEM, and FEP, had decreasing

effects on MIC, MBIC, and MBEC. DXP had increasing effects on MIC, MBIC, and MBEC of some of these antibiotics' agents.

According to the reports, using a combination of anti-inflammatory drugs and antibiotics to treat infections can have different effects on the MIC, MBIC, and MBEC of antibiotic agents (91, 102). Corticosteroid drugs such as dexamethasone and betamethasone are synthetic analogs of glucocorticoids that have anti-inflammatory and immunosuppressive functions, and the effects of these drugs are through their receptors and genomic and non-genomic pathways (3). Many reports show that steroid hormones increase the expression level of virulence and biofilm-associated genes, efflux pump genes associated with MDR, and the rate of replication of bacteria (14, 92). The virulent mucoid biofilm phenotype in *P. aeruginosa* increased in the presence of estradiol (92). Moreover, estradiol can down-regulate genes involved in nucleotide metabolism and fatty acid biosynthesis and may be associated with enhanced survival and persistence in *Chlamydia trachomatis* (92). In 2014 Fteita *et al.* showed that estradiol compounds increase the planktonic growth and the ability of *Fusobacterium nucleatum* to co-aggregate, in addition to showing that polysaccharide production and biofilm formation in *Prevotella intermedia* was enhanced by estradiol *in vitro* (14). The effects of using some antimicrobial agents in combination with corticosteroid drugs *in vivo* have been described. In

a study by Sakiniene *et al*., cloxacillin combined with DXP was more effective than cloxacillin alone in treating bacterial arthritis caused by *S. aureus* in Swiss mice (104). DXP did not interfere with fluconazole in a murine model of cryptococcosis (105). Other studies show that the combined use of hydrocortisone with mupirocin and methylprednisolone with imipenem is more effective than the antibiotics alone against eczema and atopic dermatitis by *S. aureus* and severe pneumonia in children, respectively (106, 107). On the other hand, others have shown the adverse effects on the antimicrobial and anti-biofilm activity of antibiotics with corticosteroid drugs such as DXP. A study by Rodrigues *et al*. in 2017 reported that the corticosteroid anti-inflammatory drug DXP abrogates the activity of antimicrobial drugs, including gentamicin, chloramphenicol, oxacillin, ceftriaxone, and meropenem when combined *in vitro* against planktonic and microbial biofilms of *S. aureus* and *P. aeruginosa* (91). Cabellos *et al.* and Martínez-Lacasa *et al.* showed that combining DXP with ceftriaxone and vancomycin in a rabbit model of pneumococcal meningitis causes treatment failure (108, 109). These different effects of using DXP in combination with antibiotics may be due to the antibiotics' pharmacological properties, the type of bacteria (clinical or non-clinical isolates), and the possibility of DXP interference with the bacterial physiological processes. However, dexamethasone derivatives such as nitro-dexamethasone have

recently been introduced, showing acceptable antimicrobial effects. These effects of nitro-dexamethasone have been attributed to the NO group, which can cause anti-biofilm activity (10). Also, Goggin *et al.* reported that topical steroids, including fluticasone, mometasone, and budesonide, directly reduce biofilm production and MBIC in vitro in *S. aureus* ATCC 25923 (9). In this study, as in some other studies, DXP reduced the susceptibility of the isolates to CIP, GEN, and FEP. Interestingly, DXP had no significant effect on the level of MIC and MBIC in both clinical isolates of *S. aureus* and *P. aeruginosa* to IPM and MEM. These results were contrasted to reports in 2017 by Rodrigues *et al.* that showed DXP could abrogate MEM activity against clinical isolates of *S. aureus* and *P. aeruginosa* (91). These findings about IPM and MEM can be considered for their use in combination with DXP, although more studies are needed. However, the differences between our results and Rodrigues *et al*. may be due to differences in the genetic nature, antibiotic resistance mechanisms, clone type of bacteria, and growth conditions among bacterial isolates. For example, it has been shown that biofilm production in methicillin-sensitive *S. aureus* isolates (MSSA) is usually associated with polysaccharide intercellular adhesin (PIA) or poly-N-acetyl-glucosamine (PNAG), whereas in MRSA isolates formation of biofilms frequently depends more on proteinaceous matrix (110, 111, 112). Also, it has

been shown that some clones of MRSA, such as USA300, produce thicker and stronger biofilms than other MRSA clones (112).

Shah *et al.* have shown that the antimicrobial effects of IBP on PAO1 are different with other strains of *P. aeruginosa* (69). Also, they have shown that the antimicrobial effects of ibuprofen on *P. aeruginosa* are different from other Gram-negative bacteria such as *Burkholderia* spp. (69). This indicates that the pH rate and the type of medium are effective on the antimicrobial function of ibuprofen. For example, Sanyal *et al*. have reported that the MIC levels of IBP against *S. aureus* isolates are 40 to 80 µg/mL at pH 5 (113). Similarly, Elvers and Wright observed that growth suppression and almost complete growth inhibition of IBP on *S. aureus* and *Staphylococcus epidermidis* occurred at concentrations greater than 150 µg/mL to 450 µg/mL at an initial pH of 7 (114). However, these types of growth media can have effects on gene expression, metabolic functions, and other physiological growth conditions of bacteria (69). Our results, like other studies, show that ASA and IBP, in combination with some antibiotic agents, can reduce the level of MIC, MBIC, and MBEC. A few reports have shown the antibacterial and antifungal activity of IBP and its synergy with antibiotics agents. In a study by Ling Chan *et al*., ASA, IBP, and diclofenac were reported to have antibacterial activity against Gram-positive and Gram-negative pathogenic bacteria such as MRSA and *P. aeruginosa* (115). Also, Ling Chan *et al.*

reported a synergism effect between IBP/ASA in combination with cefuroxime and chloramphenicol on *S. aureus*, and they did not observe an antagonism effect between antibiotic agents and IBP/ASA in their study (115).

Sanyal *et al*. reported that IBP has antifungal activity in vitro against dermatophytes with MIC 5-40 µg/mL (113). Pina-Vaz *et al*. have demonstrated that the combination of IBP with fluconazole resulted in synergic activity against *Candida* spp., and the MICs of fluconazole among the fluconazole-resistant strains decreased 2-128-fold in combination with IBP (116). Recently it has been shown that morphogenesis and pathogenicity of fungi can be affected by cyclooxygenase (COX) inhibitors such as ASA, IBP, and indomethacin, combined with antifungal drugs (117). Rusu *et al*. have shown that the inhibitors of cyclooxygenase iso-enzymes such as ASA and diclofenac effectively decrease germ tube formation of *Candida albicans* isolates (118). Studies have shown that NSAID compounds can reduce the ability of *Candida* spp. to form biofilm when combined with fluconazole and that the combination of NSAIDs with anti-fungal drugs has synergistic effects (116, 117). These effects may be due to the inhibition of arachidonic acid synthesis of prostaglandin E2 (PGE2) due to the inhibition of COX by NSAIDs (117, 118). However, the mechanical action of NSAIDs on bacteria is not clear.

One of the important points about the antimicrobial and anti-biofilm activity of

anti-inflammatory drugs is to pay attention to the concentration of their antimicrobial function in vitro compared to their plasma concentration. This concentration for ASA is from 50 to 200 µg/mL, which are therapeutic doses in humans with antimicrobial and anti-biofilm activity against some microorganisms (87). Dai *et al.* demonstrated anti-quorum sensing and anti-biofilm of IBP against *P. aeruginosa*. Furthermore, they found that a decrease in biofilm formation is related to rising drug concentrations, and the anti-biofilm activity of IBP is concentration-dependent (58).

During infection, biofilm formation by bacteria plays an important role in bacterial colonization, resistance to antibiotics, and the immune system (119). The bacteria's production of exopolysaccharides or extracellular polymeric substances (EPS) is an important factor in biofilm formation (119). In *S. aureus,* the major exopolysaccharide produced for biofilm formation is termed polysaccharide intercellular adhesion (PIA), also known as poly-*N*-acetyl-glucosamine (PNAG) (119). The enzyme proteins that synthesize the PIA/PNAG are encoded by the *icaADBC* operon (119). Among the *ica* operon genes, *icaA* and *icaD* encode transmembrane proteins involved in oligomers synthesis for PNAG formation (119). In this study, DXP increased the transcriptional level of *icaA*, from which it can be concluded that steroid compounds such as DXP increase the production of biofilms by increasing the expression of operon *ica*

genes and thus reduce the susceptibility to some antibiotic agents in *S. aureus* in our study. However, in this study ASA as a NSAID drug did not show significant changes in *icaA* expression, although IBP significantly decreased the transcriptional level of the *icaA* in *S. aureus*.

Alginate overproduction by increasing biofilm formation can protect *P. aeruginosa* from phagocytosis and antibiotic penetration (120). *algD* is the main operon involved in biofilm formation in *P. aeruginosa* and encodes the main enzymes for alginate synthesis (5, 6). In the present study, DXP increased the transcriptional level of *algD*, from which it can be concluded that steroid compounds such as DXP increase the production of alginate or biofilm by increasing the expression of *algD* operon genes and thus reduce the susceptibility of *P. aeruginosa* isolates to antibiotic agents. On the other hand, ASA and IBP reduced the expression level of *algD* in the present study, which may explain the decrease in susceptibility of bacteria to various antibiotics in the presence of these drugs. Decreased expression of genes associated with biofilm production, including *icaA* and *algD* in the presence of IBP and ASA, may explain the reduction in MBIC to antibiotics agents among both clinical isolates, which in some cases, according to our results, this reduction in the MBIC level was more than MIC . However, some studies have reported that NSAIDs can reduce antibiotic susceptibility and bacterial pathogenicity through different

mechanisms. Shah *et al.* showed that IBP potentially uncouples oxidative phosphorylation in bacteria and causes depletion in the intracellular ATP concentration in *P. aeruginosa* PAO1 (69). Another study reported that IBP and ASA can bind to DNA gyrase and inhibit the growth of bacteria like antibiotic agents. Recently Kahlous *et al.,* through chemoinformatics and bioinformatics-based studies, have shown that IBP has a similar structure to the quinolones and fluoroquinolones classes of antimicrobials (121). In addition to the anti-biofilm activity of NSAIDs against *S. aureus*, there is evidence pointing to the activity of NSAIDs against pathogenicity of this bacterium by anti-virulence properties such as inhibition of hemolysis and staphyloxanthin production in this bacterium (122). ASA has antibacterial and anti-biofilm activity against *S. aureus* by blocking *agrA*-regulated virulence genes and down-regulating expression of biofilm-associated genes such as *icaA* and *fnbA.* However, in this study, *icaA* expression was down-regulated in the presence of ASA but was not statistically significant (102). Intercellular signaling, often known as quorum sensing (QS), is involved in biofilm development, and it has been shown that ASA can inhibit quorum-sensing in *P. aeruginosa* (102, 33). A study by Dai *et al.* demonstrated that Ibuprofen is an excellent potential inhibitor of biofilm development and quorum sensing in *P. aeruginosa*. They reported that ibuprofen inhibits biofilm formation and adherence activity, and QS attenuates the production of

virulence factors such as pyocyanin, elastase, protease, and rhamnolipids; ibuprofen can bind with LuxR, LasR, LasI, and RhlR proteins in *P. aeruginosa* (58). These proteins play an essential role in QS and biofilm formation in *P. aeruginosa*.

This study shows that the antimicrobial and anti-biofilm ability of some antibiotic agents increased in the presence of IBP and ASA as NSAIDs and decreased in the presence of DXP. This study showed that DXP may reduce the susceptibility of bacteria to ciprofloxacin, gentamicin, and cefepime by increasing biofilm production but that it has no effect on the function of imipenem and meropenem. This increase or decrease in susceptibility of bacteria to antibiotic agents in the presence of the anti-inflammatory drug, in addition to the type of anti-inflammatory drug and its concentration, may be related to the genus and species of bacteria, clone type of bacteria, physiological conditions, and antibiotics class.

5. Conclusions

With the increasing resistance to antibiotic agents among bacteria, proposing new strategies in the treatment of infections and new antibacterial compounds is an essential need. According to our results and by further investigation, ibuprofen and its derivatives can be introduced as a new anti-biofilm to treat bacterial biofilm-associated infections. Moreover, our results demonstrate the

potential of NSAIDs, especially ibuprofen, to treat and control bacterial biofilm. Therefore, the results described in this research suggest that by doing further investigations and clinical trials, NSAIDs and their derivatives may be repurposed as new antibacterial or anti-biofilm compounds to treat bacterial infections. On the other hand, the data included in this work shows that some anti-inflammatory drugs, such as dexamethasone, in interaction with antibiotic agents may have adverse effects on the function of some antibiotics, which is suggested to be considered in the treatment of infections. The results in this work highlight interactions between different antibiotic drugs and common anti-inflammatory drugs and provide important insights into the design and development of novel and current therapies and treatments to treat bacterial infections.

References

1. Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol [Internet]. 2010;8(6):423–35.

2. Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev [Internet]. 2009;22(4):582–610.

3. Stefan Riedel, Jeffery A. Hobden, Steve Miller, Stephen A. Morse, Timothy A. Mietzner, Barbara Detrick, Thomas G. Mitchell, Judy A. Sakanari, Peter Hotez RM. Jawetz, Melnick, & Adelberg's Medical Microbiology. 28th ed. Mc Graw Hill; 2019.

4. Hashemizadeh Z, Bazargani A, Kalantar-Neyestanaki D, Mohebi S, Hadi N. Determining spa-type of methicillin-resistant *Staphylococcus aureus* (MRSA) via high-resolution melting (HRM) analysis, Shiraz, Iran. BMC Res Notes. 2020;13(1).

5. Fasihi Y, Saffari F, Mansouri S, Kalantar-Neyestanaki D. The emergence of vancomycin-resistant *Staphylococcus aureus* in an intensive care unit in Kerman, Iran. Wien Med Wochenschr [Internet]. 2018;168(3–4):85–8.

6. Martens E, Demain AL. The antibiotic resistance crisis, with a focus on the United States. J Antibiot. 2017;70(5):520–6.

7. Ericson-Neilsen W, Kaye AD. Steroids: Pharmacology, complications, and practice Delivery Issues. Ochsner J. 2014;14(2):203–7.

8. Eccleston C, Cooper TE, Fisher E, Anderson B, Wilkinson NMR. Non-steroidal anti-inflammatory drugs (NSAIDs) for chronic non-cancer pain in children and adolescents. Cochrane Database Syst Rev. 2017;2017(8).

9. Goggin R, Jardeleza C, Wormald P-J, Vreugde S. Corticosteroids directly reduce Staphylococcus aureus biofilm growth: an in vitro study. Laryngoscope. 2014;124(3):602–7.

10. Yang Y, Li H, Sun H, Gong L, Guo L, Shi Y, et al. A novel nitro-dexamethasone inhibits agr system activity and improves therapeutic effects in MRSA sepsis models without antibiotics. Sci Rep. 2016 3;6:20307.

11. Wongrakpanich S, Wongrakpanich A, Melhado K, Rangaswami J. A Comprehensive Review of Non-Steroidal Anti-Inflammatory Drug Use in The Elderly. Aging Dis [Internet]. 2018;9(1):143–50.

12. Khatoon Z, McTiernan CD, Suuronen EJ, Mah T-F, Alarcon EI. Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. Heliyon [Internet]. 2018;4(12):e01067.

13. Römling U, Balsalobre C. Biofilm infections, their resilience to therapy and innovative treatment strategies. J Intern Med. 2012;272(6):541–61.

14. Fteita D, Könönen E, Söderling E, Gürsoy UK. Effect of estradiol on planktonic growth, coaggregation, and biofilm formation of the *Prevotella intermedia* group bacteria.

Anaerobe [Internet]. 2014;27:7–13.

15. Khatoon Z, Mctiernan CD, Suuronen EJ, Mah T. Bacterial bio fi lm formation on implantable devices and approaches to its treatment and prevention. Heliyon. 2018;:e01067.

16. López D, Vlamakis H, Kolter R. Biolims. Cold Spring Harb Perspect Biol. 2010;2:1–11.

17. Satpathy S, Sen SK, Pattanaik S, Raut S. Review on bacterial biofilm: An universal cause of contamination. Biocatal Agric Biotechnol. 2016;7:56–66.

18. Verderosa AD, Totsika M, Fairfull-Smith KE. Bacterial Biofilm Eradication Agents: A Current Review. Front Chem. 2019 28;7.

19. Sharma D, Misba L, Khan AU. Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrob Resist Infect Control. 2019;8:76.

20. Annous BA, Fratamico PM, Smith JL. Scientific status summary: Quorum sensing in biofilms: Why bacteria behave the way they do. J Food Sci. 2009;74(1).

21. Dawson LF, Valiente E, Faulds-Pain A, Donahue EH, Wren BW. Characterisation of *Clostridium difficile* biofilm formation, a role for Spo0A. PLoS One. 2012;7(12):e50527.

22. Prigent-Combaret C, Vidal O, Dorel C, Lejeune P. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J Bacteriol. 1999;181(19):5993–6002.

23. Høiby N, Ciofu O, Bjarnsholt T. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. Future Microbiol. 2010;5(11):1663–74.

24. Otto M. Staphylococcal biofilms. Curr Top Microbiol Immunol. 2008;322:207–28.

25. Hodges NA, Gordon CA. Protection of *Pseudomonas aeruginosa* against ciprofloxacin and β-lactams by homologous alginate. Antimicrob Agents Chemother. 1991;35(11):2450–2.

26. Simpson JA, Smith SE, Dean RT. Alginate inhibition of the uptake of *Pseudomonas aeruginosa* by macrophages. J Gen Microbiol. 1988;134(1):29–36.

27. Coyne MJ, Russell KS, Coyle CL, Goldberg JB. The *Pseudomonas aeruginosa* algC gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. J Bacteriol. 1994;176(12):3500–7.

28. Draget KI, Stokke BT, Yuguchi Y, Urakawa H, Kajiwara K. Small-Angle X-ray Scattering and Rheological Characterization of Alginate Gels. 3. Alginic Acid Gels. Biomacromolecules. 2003;4(6):1661–8.

29. Gacesa P. Bacterial alginate biosynthesis - Recent progress and future prospects. Microbiology. 1998;144(5):1133–43.

30. Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. Virulence. 2011;2(5):445–59.

31. Reśliński A, Dąbrowiecki S, Głowacka K. The impact of diclofenac and ibuprofen on

biofilm formation on the surface of polypropylene mesh. Hernia. 2015;19(2):179–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24366755

32. Cafiso V, Bertuccio T, Santagati M, Campanile F, Amicosante G, Perilli MG, et al. Presence of the ica operon in clinical isolates of *Staphylococcus epidermidis* and its role in biofilm production. Clin Microbiol Infect. 2004;10(12):1081–8.

33. Yarwood JM, Bartels DJ, Volper EM, Greenberg EP. Quorum sensing in *Staphylococcus aureus* biofilms. J Bacteriol. 2004;186(6):1838–50.

34. Frølund B, Palmgren R, Keiding K, Nielsen PH. Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Res. 1996;30(8):1749–58.

35. Lynch DJ, Fountain TL, Mazurkiewicz JE, Banas JA. Glucan-binding proteins are essential for shaping *Streptococcus mutans* biofilm architecture. FEMS Microbiol Lett. 2007;268(2):158–65.

36. Dueholm MS, Søndergaard MT, Nilsson M, Christiansen G, Stensballe A, Overgaard MT, et al. Expression of Fap amyloids in *Pseudomonas aeruginosa*, *P. fluorescens*, and *P. putida* results in aggregation and increased biofilm formation. Microbiologyopen. 2013;2(3):365–82.

37. Romero D, Aguilar C, Losick R, Kolter R. Amyloid fibers provide structural integrity to Bacillus subtilis biofilms. Proc Natl Acad Sci U S A. 2010;2;107(5):2230–4.

38. Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penadés JR. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol. 2001;183(9):2888–96.

39. Lasa I, Penadés JR. Bap: a family of surface proteins involved in biofilm formation. Res Microbiol. 2006;157(2):99–107.

40. Mora P, Rosconi F, Franco Fraguas L, Castro-Sowinski S. *Azospirillum brasilense* Sp7 produces an outer-membrane lectin that specifically binds to surface-exposed extracellular polysaccharide produced by the bacterium. Arch Microbiol. 2008;189(5):519–24.

41. Tielker D, Hacker S, Loris R, Strathmann M, Wingender J, Wilhelm S, et al. *Pseudomonas aeruginosa* lectin LecB is located in the outer membrane and is involved in biofilm formation. Microbiology. 2005;151(Pt5):1313–23.

42. Gloag ES, Turnbull L, Huang A, Vallotton P, Wang H, Nolan LM, et al. Self-organization of bacterial biofilms is facilitated by extracellular DNA. Proc Natl Acad Sci U S A. 2013;110(28):11541–6.

43. Lewenza S. Extracellular DNA-induced antimicrobial peptide resistance mechanisms in *Pseudomonas aeruginosa*. Front Microbiol. 2013;4:21.

44. Johnson L, Horsman SR, Charron-Mazenod L, Turnbull AL, Mulcahy H, Surette MG, et al. Extracellular DNA-induced antimicrobial peptide resistance in *Salmonella enterica serovar Typhimurium.* BMC Microbiol. 2013;13:115.

45. Doroshenko N, Tseng BS, Howlin RP, Deacon J, Wharton JA, Thurner PJ, et al. Extracellular DNA impedes the transport of vancomycin in *Staphylococcus epidermidis*

biofilms preexposed to subinhibitory concentrations of vancomycin. Antimicrob Agents Chemother. 2014;58(12):7273–82.

46. Renner LD, Weibel DB. Physicochemical regulation of biofilm formation. MRS Bull. 2011;36(5):347–55.

47. Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother. 2000;44(7):1818–24.

48. Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother. 2003;47(1):317–23.

49. Field TR, White A, Elborn JS, Tunney MM. Effect of oxygen limitation on the in vitro antimicrobial susceptibility of clinical isolates of *Pseudomonas aeruginosa* grown planktonically and as biofilms. Eur J Clin Microbiol Infect Dis. 2005;24(10):677–87.

50. Hobby GL, Meyer K, Chaffee E. Observations on the Mechanism of Action of Penicillin. Exp Biol Med. 1942;50(2):281–5.

51. Bagge N, Hentzer M, Andersen JB, Ciofu O, Givskov M, Høiby N. Dynamics and spatial distribution of beta-lactamase expression in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemother. 2004;48(4):1168–74.

52. Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, et al. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. Antimicrob Agents Chemother. 2004 Apr;48(4):1175–87.

53. Szomolay B, Klapper I, Dockery J, Stewart PS. Adaptive responses to antimicrobial agents in biofilms. Environ Microbiol. 2005;7(8):1186–91.

54. Redelman C V, Chakravarty S, Anderson GG. Antibiotic treatment of *Pseudomonas aeruginosa* biofilms stimulates expression of the magnesium transporter gene mgtE. Microbiology. 2014;160(Pt1):165–78.

55. Zhao J, Jiang H, Cheng W, Wu J, Zhao J, Wang J, et al. The role of quorum sensing system in antimicrobial induced ampC expression in *Pseudomonas aeruginosa* biofilm. J Basic Microbiol. 2015;55(5):671–8.

56. Ziebuhr W, Krimmer V, Rachid S, Go F, Lo I. A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis* : evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element IS256. Mol Microbiol. 1999;32:345–56.

57. Lagadinou M, Onisor MO, Rigas A, Musetescu D-V, Gkentzi D, Assimakopoulos SF, et al. Antimicrobial Properties on Non-Antibiotic Drugs in the Era of Increased Bacterial Resistance. Antibiot (Basel, Switzerland). 2020;9(3).

58. Dai L, Wu T-Q, Xiong Y-S, Ni H-B, Ding Y, Zhang W-C, et al. Ibuprofen-mediated potential inhibition of biofilm development and quorum sensing in *Pseudomonas aeruginosa*.

Life Sci. 2019;237:116947.

59. Lee CC, Sugerman HJ, Tatum JL, Wright TP, Hirsh PD, Hirsch JI. Effects of ibuprofen on a pig *Pseudomonas* ARDS model. J Surg Res. 1986;40(5):438–44.

60. Cantin AM, Hartl D, Konstan MW, Chmiel JF. Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy. J Cyst Fibros. 2015;14(4):419–30.

61. Konstan MW, Döring G, Heltshe SL, Lands LC, Hilliard KA, Koker P, et al. A randomized double blind, placebo controlled phase 2 trial of BIIL 284 BS (an LTB4 receptor antagonist) for the treatment of lung disease in children and adults with cystic fibrosis. J Cyst Fibros. 2014;13(2):148–55.

62. Gágyor I, Bleidorn J, Kochen MM, Schmiemann G, Wegscheider K, Hummers-Pradier E. Ibuprofen versus fosfomycin for uncomplicated urinary tract infection in women: randomised controlled trial. BMJ [Internet]. 2015;351:h6544.

63. Little P. Antibiotics or NSAIDs for uncomplicated urinary tract infection? BMJ. 2017;359:j5037.

64. Vik I, Bollestad M, Grude N, Bærheim A, Damsgaard E, Neumark T, et al. Ibuprofen versus pivmecillinam for uncomplicated urinary tract infection in women-A double-blind, randomized non-inferiority trial. PLoS Med. 2018;15(5):e1002569.

65. Chmiel JF, Konstan MW, Elborn JS. Antibiotic and anti-inflammatory therapies for cystic fibrosis. Cold Spring Harb Perspect Med. 2013;3(10):a009779.

66. Wawrysiuk S, Naber K, Rechberger T, Miotla P. Prevention and treatment of uncomplicated lower urinary tract infections in the era of increasing antimicrobial resistance-non-antibiotic approaches: a systemic review. Arch Gynecol Obstet. 2019;300(4):821–8.

67. Bleidorn J, Gágyor I, Kochen MM, Wegscheider K, Hummers-Pradier E. Symptomatic treatment (ibuprofen) or antibiotics (ciprofloxacin) for uncomplicated urinary tract infection?--results of a randomized controlled pilot trial. BMC Med. 2010;8:30.

68. Elvers KT, Wright SJL. Antibacterial activity of the anti-inflammatory compound ibuprofen. Lett Appl Microbiol. 1995;20(2):82–4.

69. Shah PN, Marshall-Batty KR, Smolen JA, Tagaev JA, Chen Q, Rodesney CA, et al. Antimicrobial Activity of Ibuprofen against Cystic Fibrosis-Associated Gram-Negative Pathogens. Antimicrob Agents Chemother. 2018;62(3).

70. Azam MW, Khan AU. Updates on the pathogenicity status of *Pseudomonas aeruginosa*. Drug Discov Today. 2019;24(1):350–9.

71. Fiser A, Šali A. Modeller: Generation and Refinement of Homology-Based Protein Structure Models. Methods Enzymol. 2003;374:461–91.

72. El-Mowafy SA, Abd El Galil KH, El-Messery SM, Shaaban MI. Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in *Pseudomonas aeruginosa*. Microb Pathog. 2014;74:25–32.

73. Lima e Silva AA de, Martins Silva P. Non-Antibiotic Compounds: The Activity of the

NSAID Diclofenac on Bacteria- A Review. Int J Curr Microbiol Appl Sci. 2018;7(12):340–51.

74. Alqahtani FY, Aleanizy FS, Tahir E El, Alquadeib BT, Alsarra IA, Alanazi JS, et al. Preparation, characterization, and antibacterial activity of diclofenac-loaded chitosan nanoparticles. Saudi Pharm J. 2019;27(1):82–7.

75. Dastidar SG, Ganguly K, Chaudhuri K, Chakrabarty AN. The anti-bacterial action of diclofenac shown by inhibition of DNA synthesis. Int J Antimicrob Agents. 2000;14:249–51.

76. Yin Z, Wang Y, Whittell LR, Jergic S, Liu M, Harry E, et al. DNA replication is the target for the antibacterial effects of nonsteroidal anti-inflammatory drugs. Chem Biol. 2014;21(4):481–7.

77. Dutta NK, Annadurai S, Mazumdar K, Dastidar SG, Kristiansen JE, Molnar J, et al. Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium. Int J Antimicrob Agents. 2007;30(3):242–9.

78. Dutta NK, Mazumdar K, Dastidar SG, Park J-H. Activity of diclofenac used alone and in combination with streptomycin against *Mycobacterium tuberculosis* in mice. Int J Antimicrob Agents. 2007;30(4):336–40.

79. Mazumdar K, Dastidar SG, Park JH, Dutta NK. The anti-inflammatory non-antibiotic helper compound diclofenac: an antibacterial drug target. Eur J Clin Microbiol Infect Dis]. 2009;28(8):881–91.

80. Perilli R, Marziano ML, Formisano G, Caiazza S, Scorcia G, Baldassarri L, et al. Alteration of organized structure of biofilm formed by *Staphylococcus epidermidis* on soft contact lenses. J Biomed Mater Res. 2000; 49(1):53-7

81. Wael AHH. Diclofenac inhibits virulence of *Proteus mirabilis* isolated from diabetic foot ulcer. African J Microbiol Res. 2016;10(21):733–43.

82. Rosa B, Victor T, Ricardo V-R, Alfredo M, Octavio A. Anti-biofilm activity of ibuprofen and diclofenac against some biofilm producing *Escherichia coli* and *Klebsiella pneumoniae* uropathogens. African J Microbiol Res. 2016;10(40):1675–84.

83. Mohsen A, Gomaa A, Mohamed F, Ragab R, Eid M, Ahmed A-H, et al. Antibacterial, Anti-biofilm Activity of Some Non-steroidal Anti-Inflammatory Drugs and N-acetyl Cysteine against Some Biofilm Producing Uropathogens. Am J Epidemiol Infect Dis. 2015;3(1):1–9.

84. Ashraf A, Yousri F, Taha N, Abd El-Waly O, El-Kareem Ramadan A, Ismail E, et al. Effect of Some Non steroidal Anti-Inflammatory Drugs on Growth, Adherence and Mature Biofilms of *Candida* spp. Am J Microbiol Res. 2015;3(1):1–7.

85. Rehab MAEB, Sherein GEG. Effect of non-steroidal anti-inflammatory drugs and dexamethazone on the biofilm formation and expression of some adhesion-related genes of Candida albicans and *Staphylococcus aureus*. African J Microbiol Res. 2016;10(20):694–707. A

86. Yang S, Liao Y, Cong L, Lu X, Yang R. In Vitro Interactions between Non-Steroidal Anti-Inflammatory Drugs and Antifungal Agents against Planktonic and Biofilm Forms of

Trichosporon asahii. PLoS One. 2016;11(6):e0157047.

87. Alem MAS, Douglas LJ. Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of *Candida albicans*. Antimicrob Agents Chemother. 2004;48(1):41–7.

88. Zamanian Z. Evaluation of Aspirin Effect on *Candida GlabrataIsolates* with Resistance to Azole Compounds by Real-Time PCR. MOJ Immunol. 2017;5(6).

89. Stepanović S, Vuković D, Jesić M, Ranin L. Influence of acetylsalicylic acid (aspirin) on biofilm production by *Candida* species. J Chemother. 2004;16(2):134–8.

90. Al-Bakri AG, Othman G, Bustanji Y. The assessment of the antibacterial and antifungal activities of aspirin, EDTA and aspirin-EDTA combination and their effectiveness as antibiofilm agents. J Appl Microbiol. 2009;107(1):280–6.

91. Rodrigues A, Gomes A, Marçal PHF, Dias-Souza MV. Dexamethasone abrogates the antimicrobial and antibiofilm activities of different drugs against clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. J Adv Res. 2017;8(1):55–61.

92. W Patt M, Conte L, Blaha M, J Plotkin B. Steroid hormones as interkingdom signaling molecules: Innate immune function and microbial colonization modulation. AIMS Mol Sci. 2018;5(1):117–30.

93. Esposito A, Vollaro A, Esposito EP, D'Alonzo D, Guaragna A, Zarrilli R, et al. Antibacterial and Antivirulence Activity of Glucocorticoid PYED-1 against *Stenotrophomonas maltophilia*. Antibiot (Basel, Switzerland). 2020;9(3).

94. CLSI supplement M100. Clinical and Laboratory Standards Institute; 2021.

95. Ziasistani M, Shakibaie MR, Kalantar-Neyestanaki D. Genetic characterization of two vancomycin-resistant *Staphylococcus aureus* isolates in Kerman, Iran. Infect Drug Resist. 2019;12.

96. Fothergill JL, Neill DR, Loman N, Winstanley C, Kadioglu A. *Pseudomonas aeruginosa* adaptation in the nasopharyngeal reservoir leads to migration and persistence in the lungs. Nat Commun. 2014;5(1):4780.

97. Kato F, Yabuno Y, Yamaguchi Y, Sugai M, Inouye M. Deletion of mazF increases *Staphylococcus aureus* biofilm formation in an ica-dependent manner. Pathog Dis. 2017;75(5).

98. Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Cirković 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(8):891–9.

99. Cruz CD, Shah S, Tammela P. Defining conditions for biofilm inhibition and eradication assays for Gram-positive clinical reference strains. BMC Microbiol. 2018;18(1):173.

100. Thieme L, Hartung A, Tramm K, Klinger-Strobel M, Jandt KD, Makarewicz O, et al. MBEC Versus MBIC: the Lack of Differentiation between Biofilm Reducing and Inhibitory Effects as a Current Problem in Biofilm Methodology. Biol Proced Online.

2019;21(1):18.

101. van Duin D, Paterson DL. Multidrug-Resistant Bacteria in the Community: Trends and Lessons Learned. Infect Dis Clin North Am. 2016;30(2):377–90.

102. Paes Leme RC, da Silva RB. Antimicrobial Activity of Non-steroidal Anti-inflammatory Drugs on Biofilm: Current Evidence and Potential for Drug Repurposing. Front Microbiol. 2021;12. A

103. Rainsford KD. Anti-inflammatory drugs in the 21st century. Subcell Biochem. 2007;42:3–27.

104. Sakiniene E, Bremell T, Tarkowski A. Addition of corticosteroids to antibiotic treatment ameliorates the course of experimental *Staphylococcus aureus* arthritis. Arthritis Rheum. 1996;39(9):1596–605.

105. Lortholary O, Nicolas M, Soreda S, Improvisi L, Ronin O, Petitjean O, et al. Fluconazole, with or without dexamethasone for experimental cryptococcosis: impact of treatment timing. J Antimicrob Chemother. 1999;43(6):817–24.

106. Gong JQ, Lin L, Lin T, Hao F, Zeng FQ, Bi ZG, et al. Skin colonization by *Staphylococcus aureus* in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial. Br J Dermatol. 2006;155(4):680–7.

107. Nagy B, Gaspar I, Papp A, Bene Z, Nagy B, Voko Z, et al. Efficacy of methylprednisolone in children with severe community acquired pneumonia. Pediatr Pulmonol. 2013;48(2):168–75.

108. Cabellos C, Martínez-Lacasa J, Tubau F, Fernández A, Viladrich PF, Liñares J, et al. Evaluation of combined ceftriaxone and dexamethasone therapy in experimental cephalosporin-resistant pneumococcal meningitis. J Antimicrob Chemother [Internet]. 2000;45(3):315–20.

109. Martínez-Lacasa J, Cabellos C, Martos A, Fernández A, Tubau F, Viladrich PF, et al. Experimental study of the efficacy of vancomycin, rifampicin and dexamethasone in the therapy of pneumococcal meningitis. J Antimicrob Chemother. 2002;49(3):507–13.

110. Pozzi C, Waters EM, Rudkin JK, Schaeffer CR, Lohan AJ, Tong P, et al. Methicillin resistance alters the biofilm phenotype and attenuates virulence in *Staphylococcus aureus* device-associated infections. PLoS Pathog. 2012;8(4):e1002626.

111. Lamret F, Colin M, Mongaret C, Gangloff SC, Reffuveille F. Antibiotic Tolerance of *Staphylococcus aureus* Biofilm in Periprosthetic Joint Infections and Antibiofilm Strategies. Antibiotics. 2020;9(9):547.

112. Vanhommerig E, Moons P, Pirici D, Lammens C, Hernalsteens J-P, De Greve H, et al. Comparison of Biofilm Formation between Major Clonal Lineages of Methicillin Resistant *Staphylococcus aureus*. PLoS One. 2014;9(8):e104561.

113. Sanyal AK, Roy D, Chowdhury B, Banerjee AB. Ibuprofen, a unique anti-inflammatory compound with antifungal activity against dermatophytes. Lett Appl Microbiol. 1993;17(3):109–11.

114. Elvers KT, Wright SJ. Antibacterial activity of the anti-inflammatory compound ibuprofen. Lett Appl Microbiol. 1995;20(2):82–4.

115. Chan EWL, Yee ZY, Raja I, Yap JKY. Synergistic effect of non-steroidal anti-inflammatory drugs (NSAIDs) on antibacterial activity of cefuroxime and chloramphenicol against methicillin-resistant *Staphylococcus aureus*. J Glob Antimicrob Resist. 2017 Sep;10:70–4.

116. Pina-Vaz C, Sansonetty F, Rodrigues AG, Martinez-DE-Oliveira J, Fonseca AF, Mårdh P-A. Antifungal activity of ibuprofen alone and in combination with fluconazole against *Candida* species. J Med Microbiol. 2000;49(9):831–40.

117. Liu X, Wang D, Yu C, Li T, Liu J, Sun S. Potential Antifungal Targets against a *Candida* Biofilm Based on an Enzyme in the Arachidonic Acid Cascade-A Review. Front Microbiol. 2016;7:1925.

118. Rusu E, Radu-Popescu M, Pelinescu D, Vassu T. Treatment with some anti-inflammatory drugs reduces germ tube formation in *Candida albicans* strains. Braz J Microbiol. 2014;45(4):1379–83.

119. Cue D, Lei MG, Lee CY. Genetic regulation of the intercellular adhesion locus in staphylococci. 2012;2(March):1–13.

120. Blanco-Cabra N, Paetzold B, Ferrar T, Mazzolini R, Torrents E, Serrano L, et al. Characterization of different alginate lyases for dissolving *Pseudomonas aeruginosa* biofilms. Sci Rep. 2020;10(1):9390.

121. Kahlous NA, Bawarish MAM, Sarhan MA, Küpper M, Hasaba A, Rajab M. Using Chemoinformatics, Bioinformatics, and Bioassay to Predict and Explain the Antibacterial Activity of Nonantibiotic Food and Drug Administration Drugs. Assay Drug Dev Technol. 2017;15(3):89–105.

122. Abbas HA, Atallah H, El-Sayed MA, El-Ganiny AM. Diclofenac mitigates virulence of multidrug-resistant Staphylococcus aureus. Arch Microbiol. 2020;202(10):2751–60.