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ORIGINAL ARTICLE

A Preliminary Study on the Effect of Deferoxamine on the Disruption of Bacterial Biofilms and Antimicrobial Resistance

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ABSTRACT

Objectives: Antiviral therapy approaches have become significant strategies to combat antibiotic resistance. Metal ions, particularly iron, play crucial roles in metabolic activities and virulence of bacteria. Loading iron into siderophore molecules could potentially circumvent antimicrobial resistance. This study aimed to evaluate the antibiofilm and antimicrobial effects of deferoxamine (DFO), an iron chelator and natural siderophore, on antibiotic susceptibility in clinical methicillin-resistant *Staphylococcus aureus* (MRSA) and carbapenem-resistant *Acinetobacter baumannii* (CRAB) isolates.

Materials and Methods: The *in vitro* antibacterial activity of DFO alone and in combination with vancomycin [VAN (30 µg)], amoxicillin (25 µg), colistin (10 µg), and imipenem (10 µg), was investigated against MRSA and CRAB isolates using the disk diffusion method. The spectrophotometric microplate method was used to detect the *in vitro* antibiofilm effect of DFO.

Results: DFO exhibited a synergistic effect with VAN, amoxicillin, and colistin and significantly disrupted mature biofilm formation in MRSA and CRAB isolates. Notably, the antibiofilm effect of DFO was more pronounced in CRAB strains.

Conclusion: These findings highlight the potential of DFO as an antibiofilm agent candidate and suggest that it can enhance the antibiotic susceptibility of certain microorganism species.

Keywords: Antibiofilm, deferoxamine, iron chelator, non-antibiotics, synergism

INTRODUCTION

Bacterial antimicrobial resistance poses a significant global public health challenge[1] and renders various antibiotics ineffective. The World Health Organization has identified several microorganisms, including *Staphylococcus aureus* and *Acinetobacter baumannii*, as antibiotic-resistant "priority pathogens".[2] Methicillin-resistant *Staphylococcus aureus* (MRSA) is classified as a high-priority pathogen,[2] with vancomycin (VAN) and daptomycin suggested as first-line treatments.[2,3] Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is listed as a critical priority pathogen[2] and has limited treatment options because of higher resistance rates. Polymyxins [polymyxin B and colistin (COL)] and tetracycline derivatives (minocycline, doxycycline, and tigecycline) are used to treat drug-resistant *Acinetobacter* infections.[4]

The ability of these pathogens to form biofilms is one of the key reasons for their antimicrobial resistance.[5,6] Bacterial biofilms, the adherence of microbial cells to biotic or abiotic surfaces, represent a target for multidrug-resistant pathogens.[5,6] The role of iron in biofilm formation, crucial for the survival of both host and pathogen, has garnered significant attention.[7] Iron chelation has been proposed as a strategy to enhance the antimicrobial activity of antibiotics by disrupting bacterial biofilms.[8,9] Considering the potential effects of iron chelators on infections, it is argued that iron chelators may be of benefit in combination with antibiotics, but pathogen-specific chelators should be

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utilized.[10] Deferoxamine (DFO), an iron chelator and natural siderophore, is used to treat iron overload and intoxication. Originally discovered in *Streptomyces pilosus*, DFO is also produced by various terrestrial and marine actinomycetes species.[11] Siderophores enhance permeability by depleting iron and may facilitate the entry of antibiotics into cells.[12]

The urgent need for new antibiotics has prioritized the development of novel medications. However, developing new drugs is both time-consuming and expensive. Repurposing approved medications has gained attention as an accelerated approach to overcoming antibiotic resistance. Additionally, combining antibiotics with non-antibiotic drugs may exhibit synergistic effects against antibiotic resistance. Therefore, our objective was to investigate the potential synergistic effect of DFO and antibiotics against CRAB and MRSA and to explore the antibiofilm effect of DFO on mature biofilm.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Clinical methicillin-resistant *S.aureus* (n= 5) and carbapenem-resistant *A. baumannii* isolates (n= 4), are part of the collection of our laboratory. The main reason for choosing methicillin- and carbapenem-resistant bacterial isolates in this study was to investigate the interactions of DFO with commonly used antibiotics against drug-resistant isolates [such as imipenem (IMP) and COL], even though DFO alone has low antibacterial activity. MRSA and CRAB isolates were selected from those previously identified using the automated *VITEK® 2* Compact system (bioMérieux). *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as internal quality control strains, and *Enterococcus faecalis* ATCC 29212 served as a positive control for biofilm assays. All bacterial isolates were stored in brain-heart infusion broth containing 10% glycerin (Merck, Darmstadt, Germany) at 20 °C. Mueller-Hinton Agar (MHA) (Merck, Darmstadt, Germany) and tryptic soy broth with 2.5% glucose (TSBG) medium (Oxoid, UK) were used for antimicrobial activity tests and biofilm experiments, respectively. As a result of the biofilm production assays, two MRSA and one CRAB isolates that were found not to be strong biofilm producers were excluded from the study. The antibacterial and antibiofilm effects of DFO were evaluated against six isolates (MRSA3, MRSA6, MRSA21, CRAB35, CRAB50, CRAB89) in the disc diffusion and antibiofilm experiments.

Iron chelators and antimicrobials

DFO mesylate, commercially available (Desferal®, Novartis, Switzerland), was procured in powder form. The preparation of DFO solutions was performed as described in the package insert. Briefly, 500 mg DFO in each vial was reconstituted in 2 mL sterile distilled water at a concentration of 380 mM. These freshly prepared DFO solutions whose concentration after reconstitution was 213 mg/mL (the indicated concentration for the intramuscular route) were used in the experiments. The commercial antibiotic discs were utilized for the antimicrobial susceptibility and synergy testing in this study. The antibiotics used were VAN-30 µg, amoxicillin (AX-25 µg), COL-10 µg, and IMP-10 µg from Bioanalyse®, Türkiye.

Determination of the in vitro antimicrobial effect of DFO

The *in vitro* antimicrobial effect of DFO against MRSA and CRAB isolates was assessed using the disk diffusion method, following the criteria outlined by the European Committee on Antimicrobial Susceptibility Testing.[13] The bacterial strains were cultured on MHA and incubated overnight. Subsequently, bacterial suspensions in sterile physiological saline were adjusted to 0.5 McFarland turbidity standard (approximately 1-2 x 10^8 colony-forming units/mL) using a densitometer device (Biosan, DEN-1). The suspensions were then evenly spread on MHA plates using sterile swab sticks. 10 µL of the DFO solution was loaded onto both blank and antibiotic disks. In the following inoculation, the standard antibiotic disks (VAN, AX, COL, and IMP), DFO disks, and antibiotic and DFO disks were placed on the MHA plates. The plates were incubated at 37 °C for 18 ± 2 hours, and the inhibition zones surrounding each disk were measured.[12,13]

Detection of the biofilm-forming capacities of bacterial strains

The biofilm-forming capacities of bacterial isolates were quantified using the spectrophotometric microplate method with crystal violet (CV) staining.[14,15] The bacterial strains were cultured on MHA and incubated at 37 °C overnight. Following incubation, bacterial suspensions adjusted to a 0.5 McFarland turbidity standard were prepared in TSBG medium (3 mL) using the direct colony suspension method. Then, 180 µL of TSBG medium and 20 µL of the bacterial suspension were added to each well of a sterile 96-well flat-bottom microplate. As controls, TSBG (200 µL) medium without bacterial suspension was added to designated wells. The microplates were incubated at 37 °C for 24 hours to allow biofilm formation. After incubation, the contents of the wells were aspirated and washed with sterile phosphate-buffered saline (200 µL) (Oxoid, UK) to remove nonadherent bacteria. Following the washing steps, the microplates were allowed to dry at 25 °C. The remaining attached microorganisms were fixed by adding 200 µL of methanol and waiting for 15 minutes. After discarding the methanol, 200 µL of 0.1% CV solution was added to each well, and the mixture was incubated for 15 minutes at room temperature. Subsequently, the wells were aspirated and gently rinsed with tap water until colorless. After drying at room temperature, each well was destained with 200 µL of 95% ethanol for 10 minutes.[14,15]

Spectrophotometric measurements were performed at a wavelength of 570 nm using a microplate reader (*CLARIOstar* Plus Microplate Reader, BMG LabTech, Cary NC). The optical density (OD) of the wells containing only the TSBG medium was used as the negative control. *E. faecalis* ATCC 29212 was used as a positive control for biofilm production. The cut-off ODc was defined as three standard deviations above the mean OD of the negative controls.

In vitro antibiofilm effect of DFO

The *in vitro* antibiofilm effect of DFO on MRSA and CRAB biofilms was assessed using the spectrophotometric microplate method. First, each bacterial strain was allowed to form mature biofilms on the bottom of the sterile F-bottom 96-well

microplates. TSBG medium (180 µL) and bacterial suspension (20 µL) were added to the wells. The microplates were then incubated at 37 °C for 24 hours to induce biofilm formation. Following aspiration of the well contents, 200 µL of DFO was added directly to each well, forming the mature bacterial biofilm layer. The microplates were further incubated for 24 hours. After the incubation period, the well contents were aspirated, and the microplates were subjected to CV staining as described above. Spectrophotometric measurements were performed to obtain the OD values. To determine the percentages of biofilm disruption, OD values were calculated using the following formula: percentage of biofilm disruption (%) = (OD_A – OD_B) / OD_A × 100 (OD_A: the OD of biofilm control well without DFO, OD_B : the OD in the presence of DFO).

Statistical analysis

All experiments were performed in triplicate to ensure reproducibility. The data obtained from the experiments were assumed to follow a normal distribution. To compare the two groups, a Student's t-test was applied. Statistical analyses were performed using GraphPad Prism 9 Software (San Diego, CA, USA).

The biofilm production capacities of MRSA and CRAB isolates were categorized based on the following criteria: OD ≤ ODc: no biofilm production, ODc < OD ≤ (2 × ODc): weak biofilm producer, (2 × ODc) < OD ≤ (4 × ODc): moderate biofilm producers, and (4 × ODc) < OD: strong biofilm producer.

RESULTS

Antibacterial activity of DFO

Considering the results of biofilm detection experiments for nine isolates, two MRSA and one CRAB isolates, which were determined not to be strong biofilm producers, were excluded from the study. The antibacterial effect of DFO was evaluated against six isolates (MRSA3, MRSA6, MRSA21, CRAB35, CRAB50, CRAB89) in a disc diffusion test. Based on the results of the disk diffusion test, the inhibitory zone diameters of DFO, AX and DFO, VA and DFO, COL and DFO, and IMP and DFO against clinical MRSA and CRAB isolates varied between 8 and 22 mm. The zone diameters after exposure to DFO, antibiotics, and their combinations are listed in Table 1.

Representative examples of inhibition zones in the presence of DFO, antibiotics, and combinations of these for MRSA6 and CRAB35 isolates are shown in Figure 1.

Antibiofilm activity of DFO

Of the nine tested strains, MRSA3, MRSA6, MRSA21, CRAB35, CRAB50, and CRAB89 were identified as strong biofilm producers using the CV method. The results indicated that DFO exerted a significant antibiofilm effect on the mature biofilms of the five isolates Figure 2. Antibiofilm activity of DFO was not observed for MRSA21 and therefore this result is not included in Figure 2. The percentage of biofilm disruption caused by DFO ranged from 38.1% to 72.3%. DFO exerted a stronger disruptive effect on the biofilms formed by CRAB isolates than MRSA isolates. The percentages of biofilm disruption by DFO were 38.1% for MRSA3, 42.1% for MRSA6, 62.9% for CRAB35, 72.3% for CRAB50, and 66.5% for CRAB89. The OD values and corresponding percentage of biofilm disruption in the presence of DFO for each isolate are presented in Figure 2.

DISCUSSION

The development of resistance to VAN and COL, the last-resort antibiotics for MRSA and CRAB, respectively, leads to the need for combination therapy. Therefore, the primary aim of this study was to investigate the effect of DFO on the susceptibility of clinical MRSA and CRAB isolates to these last-resort antibiotics. Additionally, the study aimed to assess whether the presence of DFO could alter the susceptibility of these isolates, which were confirmed to be resistant to AX and IML. Furthermore, the secondary objective was to evaluate the antibiofilm effect of DFO against these resistant isolates. The main findings of our preliminary study are as follows: (a) DFO exerted a synergistic effect when combined with AX, VAN, and COL, but did not exert an antibacterial effect alone; (b) DFO significantly disrupted the mature biofilm formed by both MRSA and CRAB isolates.

The pathogenesis of bacterial infections involves various factors, including antimicrobial resistance gene expression,

Table 1. Susceptibility of MRSA and CRAB isolates to DFO alone and in combination with antibiotics

	Zone of inhibition (diameter in mm)				
Isolate number	**DFO**	**AX**	**AX and DFO**	**VAN**	**VAN and DFO**
MRSA3	0	20	22	19	21
MRSA6	0	15	17	20	22
MRSA21	0	15	17	19	21
Isolate number	**DFO**	**IMP**	**IMP and DFO**	**COL**	**COL and DFO**
CRAB35	0	12	9	13	15
CRAB50	0	11	8	13	15
CRAB89	0	11	10	13	14

AX: Amoxicillin (25 µg), COL: Colistin (10 µg), CRAB: Carbapenem-resistant *Acinetobacter baumannii*, DFO: Deferoxamine, IMP: Imipenem (10 µg), MRSA: Methicillin-resistant *Staphylococcus aureus*, VAN: Vancomycin (30 µg)

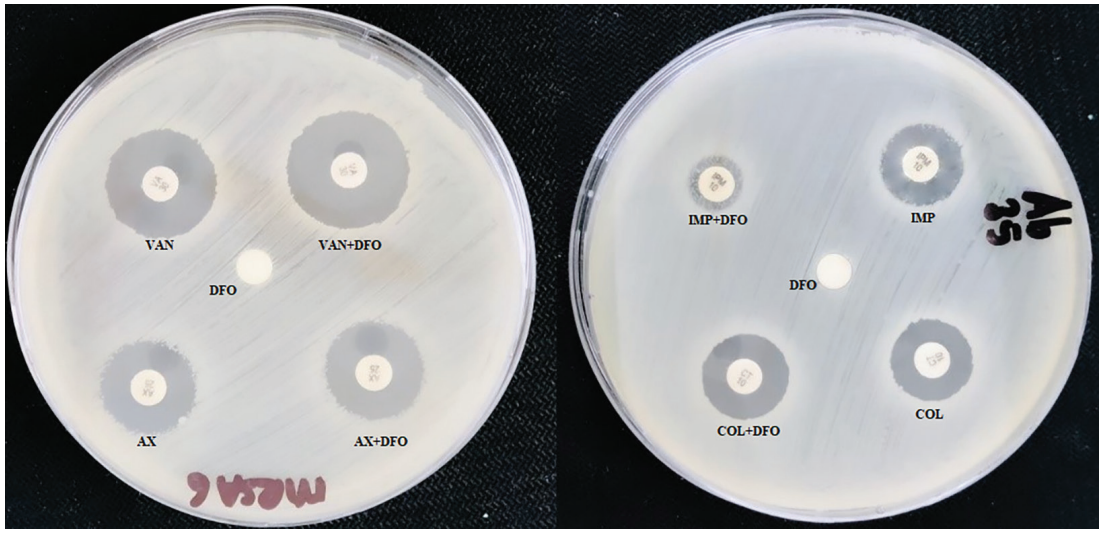

Figure 1. Inhibition zones in the presence of DFO, antibiotics, and combinations of these for MRSA6 and CRAB35

AX: Amoxicillin, COL: Colistin, CRAB: Carbapenem-resistant *Acinetobacter baumannii*, DFO: Deferoxamine, IMP: Imipenem, MRSA: Methicillin-resistant *Staphylococcus aureus*, VAN: Vancomycin

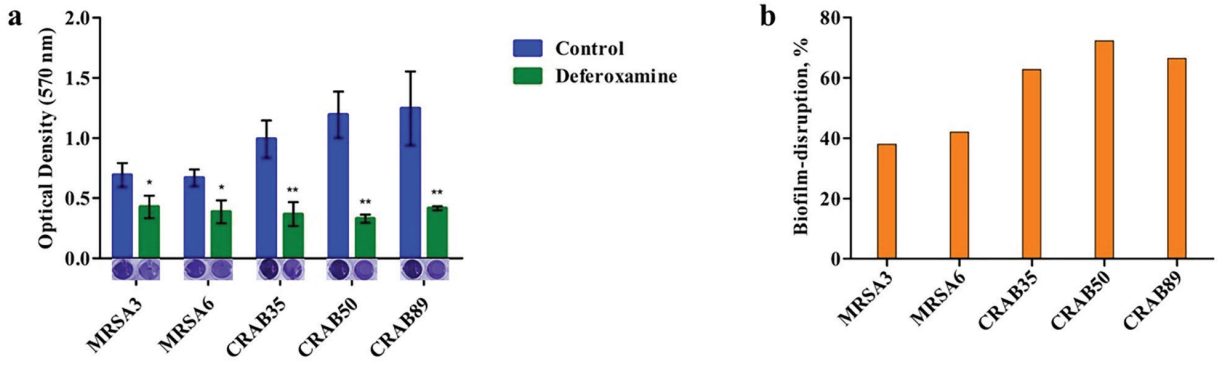

Figure 2. (A) Optical densities of mature biofilms formed by bacterial strains and mature biofilms exposed to deferoxamine. (B) Percentage of the biofilm disruption effect of deferoxamine, quantified as a percentage relative to the control using crystal violet staining

CRAB: Carbapenem-resistant *Acinetobacter baumannii*, MRSA: Methicillin-resistant *Staphylococcus aureus*

iron uptake mechanisms, and biofilm formation. Iron metabolism is closely linked to quorum sensing signaling and biofilm formation, which influence bacterial colonization, antibiotic susceptibility, and essential functions within the bacteria.[10,16] Critical iron-dependent proteins are vital for bacterial growth and multiplication, including ribonucleotide reductase, which is involved in DNA synthesis, and cytochromes, which are essential for energy metabolism.[12] In the absence of sufficient iron, these critical proteins are unable to function, leading to growth inhibition. Iron chelators are believed to exert antimicrobial effects by targeting iron-dependent pathways, enzymes, and proteins in bacteria.[11] DFO was the first iron chelator approved for use in humans and is widely used for the treatment of iron overload.[17] DFO has a higher affinity for Fe^{3+} than deferiprone and deferasirox. However, due to its siderophore nature, DFO has the potential to stimulate bacterial growth.[18] In our study, we found that DFO alone did not exhibit antibacterial activity in the disk diffusion method. However, when combined with VAN, AX, or COL, DFO enhanced the inhibitory effects of these antibiotics, as evidenced by larger zone diameters (2 mm) compared with the antibiotic discs alone. This suggests a synergistic interaction between DFO and these antibiotics.

Considering the limited literature on the effects of DFO against bacteria species, there are noteworthy findings that indicate a synergistic interaction between DFO and antibiotics, which is in line with our findings. Gokarn and Pal[12] investigated the effects of exogenous siderophore (exochelin-*Mycobacterium smegmatis* and DFO-B) in combination with antibiotics against various resistant bacteria, including MRSA. They reported that siderophore-antibiotic (ampicillin, cefdinir, IMP, and meropenem) combinations inhibited the growth of a significant proportion (50-75%) of MRSA isolates.[12] Similarly, DFO-B exhibited a bacteriostatic effect on 30-50% of the tested isolates at relatively higher concentrations.[12] In parallel to our findings, this siderophore alone did not show zones of inhibition in the disk diffusion method.[12] Another study by van Asbeck et al.[19] demonstrated the synergic interaction between DFO and antibiotics (gentamicin, chloramphenicol, cephalothin, cefotiam,

or cefsulodin) against *S. aureus, Staphylococcus epidermidis, E. coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas,* and *Providencia.*[19] In contrast, a study investigating the effects of iron chelators (DFO, deferiprone, Apo6619, and VK28) on the growth of nosocomial pathogens reported that DFO did not exhibit an inhibitory effect [minimum inhibitory concentration (MIC) ≥ 512 g/mL for all bacteria tested]. In contrast, other chelators inhibited bacterial growth in standard mediums.[20] These discrepancies may be attributed to variations in the bacterial species, experimental conditions, and concentrations of the iron chelators.

Indeed, the lack of an inhibitory effect of DFO observed in the disc diffusion method, including in our study, can be attributed to several factors. Previous studies using broth microdilution have demonstrated the inhibitory effect of DFO.[12,16] The most obvious explanation is that siderophores have easier access to iron in liquid media and are better at iron sequestration.[12,21] Most siderophore sequester Fe^{3+} at low concentrations under aerobic and neutral pH conditions.[22] DFO, with its hydroxamic functional groups surrounding the ferric ion, has a higher affinity for Fe^{3+} and forms a neutral and more stable octahedral complex.[11] However, the antibacterial effect of iron-bound DFO is lower than that of hydrophobic chelating agents like deferiprone, primarily because of its hydrophilic nature and limited penetration into lipid membranes.[21,23,24] Nevertheless, the role of DFO in iron bioavailability and virulence can vary depending on the specific bacterial species and infection models. Arifin et al.[25] found that DFO increased iron bioavailability and enhanced virulence of bacteria in a murine systemic infection model with community-associated MRSA. Similarly, DFO may play a promoting role in systemic infections of *Yersinia enterocolitica* in humans.[26] These findings highlight the complex interplay between iron chelators, bacterial pathogens, and host responses. The antibacterial effect of iron chelators alone or in combination with antibiotics can be influenced by various factors. The concentration of the iron chelator, the type and virulence characteristics of the bacteria, the mechanism of action of antibiotics, the diversity of mechanisms for iron uptake in bacteria, and the presence of siderophore with different iron binding capacities and chemical structures can contribute to the observed variability in antibacterial effects. The iron content of the culture media can vary, which can also influence the availability of iron and response to iron chelators.[27] Furthermore, DFO may facilitate the delivery of iron to bacteria through the receptors of their cognate siderophore, potentially augmenting the virulence of pathogenic bacteria.[28] On the other hand, iron deprivation induced by iron chelators can impair essential functions and increase the effectiveness of antibiotics against bacteria.[29] Although the precise mechanism underlying the synergistic interactions between siderophore and antibiotics has not been completely elucidated, it is noted that this effect may arise from the heightened permeability of the cell membrane resulting from iron deficiency.[30] This could explain the synergistic inhibition of MRSA isolates by DFO with antibiotic combinations observed in our study, as well as in previous studies with similar findings.

One of the important mechanisms contributing to antibiotic resistance is the production of metallo-beta-lactamase enzymes.[31] These enzymes inactivate beta-lactam antibiotics (such as penicillins, cephalosporins, and carbapenems) by cleaving the beta-lactam ring in their chemical structure, and they rely on the presence of Zn^{2+} ions for their enzymatic activity.[31] DFO has a high affinity for both Zn^{2+} and Fe^{3+} ions because of its specific chemical groups.[32] This affinity can result in the depletion of Zn^{2+} ions in the media, leading to the inactivation of metallo-beta-lactamases and increased susceptibility of bacteria to -lactam antibiotics. This effect is believed to be responsible for the observed synergistic effect, especially in resistant bacterial isolates in the presence of DFO. In this study, although DFO showed synergy with three of the tested antibiotics, no synergy was observed with IMP against CRAB isolates. This discrepancy may be attributed to both the chemical structure of the IMP and the expression of bacterial membrane proteins.[33] In response to *in vitro* iron loading or restriction, the expression of proteins responsible for various metabolic functions, including cell division, antibiotic resistance, and iron acquisition, particularly membrane proteins, changes in bacterial cells.[34] A previous proteomic study indicated that the membrane proteins and metabolism of *Acinetobacter* respond differently to the presence of iron, especially CRAB.[35] Hence, the presence of multiple proteins, which are also associated with carbapenem resistance, and the differentiation of their expression levels under iron-limited conditions may be the potential reasons for the different results in CRAB.

Bacterial biofilms are among the leading causes of morbidity and mortality associated with infectious diseases.[36] Biofilms are bacterial layers that form on the surfaces of medical devices like catheters and heart valve prostheses, contributing to nosocomial infections and preventing access to antimicrobial drugs to bacteria, resulting in reduced susceptibility to treatment.[36] Therefore, the detection of pathogen biofilm-forming capacity and the discovery of antibiofilm compounds play crucial roles in effective treatment strategies. In this context, we examined the impact of iron depletion via DFO on preformed biofilms *in vitro*. The biofilm-forming capacities of MRSA and CRAB isolates were evaluated using the spectrophotometric microplate method, and the isolates were found to be strong biofilm producers. DFO exhibited significant disruption in the mature biofilms, especially in CRAB strains, leading to a reduction in optical densities by > 60%. Similar studies have been conducted to explore the antibiofilm effects of DFO, deferasirox, and deferiprone against different bacterial and fungal species.[20,29] In one study, combined treatment with tobramycin and iron chelators (DFO or deferasirox) resulted in an approximately 90% reduction in preformed *P. aeruginosa* biofilm biomass and a 7-log units decrease in bacterial viability.[9] Gentile et al.[37] reported that iron starvation did not affect the biofilm-forming capacity of *A. baumannii* strains isolated from veterinary and clinical sources. Conversely, DFO had lower efficacy against *Protovella intermedia* biofilm formation than deferasirox.[38] Nazik et al.[39] reported that DFO had no inhibitory or stimulant effect on planktonic growth in their study examining the effects of DFO on *Aspergillus fumigatus*. Consequently, our

findings indicate that DFO disrupted mature biofilm formation in clinical MRSA and CRAB isolates, suggesting its potential as an antibiofilm agent.

Study limitations

The present study has some limitations in terms of the comprehensive understanding of the antibacterial and synergistic effects of DFO. The reason for preferring the disc diffusion method to the broth microdilution method (BMD) is that the liquid medium used for MIC determination contains iron and other cations. This was considered to be an important factor that could influence the antibacterial potential of DFO. Although iron-rich and iron-poor media have been used in the BMD method in previous studies to investigate the effect of DFO, it is considered that this situation in the experimental design may be disadvantageous in terms of reflecting *in vivo* conditions. Future studies are planned to determine the minimum inhibitory concentrations in iron-poor and iron-rich environments. In addition, the antibiofilm effect of DFO at different concentrations will be investigated against various bacterial species causing biofilm-associated infections and with a greater number of isolates.

CONCLUSION

In conclusion, our findings suggest that DFO can enhance antibiotic efficacy and combat biofilm-associated infections caused by CRAB and MRSA. The prevalence of high rates of antibiotic resistance and the rapid evolution of resistance to the latest antimicrobials indicate the urgent need for innovative therapeutic approaches to combat infections. In an era of limited antibiotic discovery and antibiotic resistance posing global health concerns, that, the importance of drug repositioning studies has become increasingly evident. Iron chelation is a promising antiviral strategy for combating drug-resistant bacteria. In light of the results of previous studies and our study, iron chelators have significant potential for off-label use to enhance susceptibility to antibacterial drugs. Further research is warranted to explore the mechanistic aspects and clinical applications of DFO in the context of antimicrobial resistance and biofilm control. Conducting further studies on the impact of iron chelators on microorganisms and their interaction with antibiotics will contribute to the fight against infections. The combination of iron chelators with antibacterial agents can potentially provide clinical benefits in the treatment of resistant infections by augmenting the susceptibility of antibacterial agents.

Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Authorship Contributions

Surgical and Medical Practices: A.T., Z.Ş.A., Concept: A.T., Z.Ş.A., Design: A.T., Z.Ş.A., Data Collection or Processing: A.T., Z.Ş.A., Analysis or Interpretation: A.T., Z.Ş.A., Literature Search: A.T., Z.Ş.A., Writing: A.T., Z.Ş.A.

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