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

Short Title: Antibiofilm and Antibacterial Effect of Deferoxamine

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Abstract

Objectives: Antivirulence therapy approaches have emerged as remarkable strategies in the fight against antibiotic resistance. Metal ions, particularly iron, play a crucial role in the metabolic activities and virulence of bacteria. Loading iron into siderophore molecules offers a potential avenue to circumvent antimicrobial resistance. This study aimed to evaluate the antibiofilm and antimicrobial effect of deferoxamine (DFO) 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 (30μg), amoxicillin (25μg), colistin (10μg), and imipenem (10μg), was investigated against MRSA and CRAB isolates using the disk diffusion method. The spectrophotometric microplate method was performed to detect the in vitro antibiofilm effect of DFO.

Results: The results showed that DFO exhibited a synergistic effect with vancomycin, amoxicillin, and colistin, and also significantly disrupted mature biofilms of MRSA and CRAB isolates. Notably, the antibiofilm effect of DFO was found to be 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 susceptibility of certain microorganism species to antibiotics.

Keywords: Deferoxamine, iron chelator, non-antibiotics, antibiofilm, synergism

INTRODUCTION

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

The ability of these pathogens to form biofilms is one of the key reasons for their antimicrobial resistance. Bacterial biofilm, the adherence of microbial cells to biotic or abiotic surfaces, represents a target for multidrug-resistant pathogens. The role of iron in biofilm formation, crucial for the survival of both host and pathogen, has garnered significant attention. Iron chelation has been proposed as a strategy to enhance the antimicrobial activity of antibiotics by disrupting bacterial biofilms. 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 utilized. Deferoxamine (DFO), an iron chelator and natural siderophore, is used in the treatment of iron overload and intoxication. Originally discovered in Streptomyces pilosus, DFO is also produced by various terrestrial and marine actinomycetes species. Siderophores enhance permeability by depleting iron and may facilitate the entry of antibiotics into cells.

The urgent need for new antibiotics has prioritized the development of novel medications. However, the process of developing new drugs is both time-consuming and expensive. The repurposing of approved medications has...
gained attention as an accelerated approach to overcome antibiotic resistance. Additionally, combining antibiotics with non-antibiotic drugs may exhibit synergistic effects and hold promise against antibiotic resistance. Therefore, our objective is to investigate the potential synergistic effect of DFO with antibiotics against carbapenem-resistant Acinetobacter baumannii (CRAB) and methicillin-resistant Staphylococcus aureus (MRSA), as well as explore the antibiofilm effect of DFO on mature biofilm.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

The clinical methicillin-resistant S. aureus (n=5) and carbapenem-resistant A. baumannii isolates (n=4), which 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 and colistin), even though DFO alone shows 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 the biofilm assays. All the bacterial isolates were stored in brain–heart infusion broth with 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 utilized for the 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 test and antibiofilm experiments.

**Iron Chelator and Antimicrobials**

Deferoxamine mesylate (DFO) which is commercially available (Desferal®, Novartis, Switzerland), was procured in powder form. The preparation of DFO solutions was done as described in the package insert, 500 mg DFO in each vial was reconstituted in 2 mL sterile distilled water at 380 mM concentration. These freshly prepared DFO solutions whose concentration after reconstitution was 213 mg/mL (the indicated concentration for 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 included vancomycin (VAN-30μg), amoxicillin (AX-25μg), colistin (COL-10μg), and imipenem (IMP-10μg) from Bioanalyse ®, Türkiye.

**Determination of in vitro antimicrobial effect of deferoxamine**

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 (EUCAST). 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 CFU/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 discs and each antibiotic disc. Following inoculation, the standard antibiotic discs (VAN, AX, COL, IMP), DFO discs, and antibiotic+DFO discs were placed on the MHA plates. The plates were incubated at 37°C for 18 ± 2 h, then the inhibition zones surrounding each disc were measured.

**Detection of biofilm forming capacities of bacterial strains**

The biofilm-forming capacities of bacterial isolates were quantified with spectrophotometric microplate method with crystal violet (CV) staining. Initially, 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 sterile 96-well flat-bottom microplates. 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 bacterial cells. 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 incubated for 15 minutes at room temperature. Subsequently, the wells were aspirated and gently rinsed with tap water until the water became colorless. After drying at room temperature, each well was destained with 200 μL of 95% ethanol for 10 minutes.

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 TSBG medium was used as a negative control. Enterococcus faecalis ATCC 29212 was used as a positive control strain for biofilm production. The cut-off optical density (ODc) was defined as three standard deviations above the mean OD of the negative controls.
**In vitro antibiofilm effect of deferoxamine**

The *in vitro* antibiofilm effect of DFO on biofilm producing MRSA and CRAB isolates was assessed using the spectrophotometric microplate method. First, each bacterial strain was allowed to form mature biofilms on the bottom of the sterile 96-well, F-bottom 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 enable biofilm formation. Following the aspiration of well contents, 200 μL of DFO was added to each well, directly onto 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 underwent the CV staining method as described above.

Spectrophotometric measurements were performed to obtain the OD values. To determine the percentages of biofilm disruption, the OD values were used in the following formula:

\[
\text{Percentage of Biofilm Disruption (\%)} = \left( \frac{\text{OD}_A - \text{OD}_B}{\text{OD}_A} \right) \times 100
\]

(\text{OD}_A : The optical density of biofilm control well without DFO)
(\text{OD}_B : The optical density 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, Student’s t-test was applied. Statistical analysis were performed using GraphPad Prism 9 Software (San Diego, CA, USA).

The biofilm production capacities of the 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 producer
- (4 × ODc) < OD: Strong biofilm producer.

**RESULTS**

**Antibacterial activity of deferoxamine**

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. Antibacterial effect of DFO was evaluated against six isolates (MRSA3, MRSA6, MRSA21, CRAB35, CRAB50, CRAB89) in disc diffusion test. Based on the results of the disk diffusion test, the inhibitory zone diameters of DFO, AX+DFO, VAN+DFO, COL+DFO, and IMP+DFO against clinical MRSA and CRAB isolates varied between 8 mm and 22 mm. The zone diameters resulting from the exposure to DFO, antibiotics, and their combinations are listed in Table 1.

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

<table>
<thead>
<tr>
<th>Isolate No</th>
<th>DFO</th>
<th>AX</th>
<th>AX+DFO</th>
<th>VAN</th>
<th>VAN+DFO</th>
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<tr>
<td>MRSA3</td>
<td>0</td>
<td>20</td>
<td>22</td>
<td>19</td>
<td>21</td>
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<tr>
<td>MRSA6</td>
<td>0</td>
<td>15</td>
<td>17</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
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<td>15</td>
<td>17</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>CRAB35</td>
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<td>12</td>
<td>9</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>CRAB50</td>
<td>0</td>
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</tr>
<tr>
<td>CRAB89</td>
<td>0</td>
<td>11</td>
<td>10</td>
<td>13</td>
<td>14</td>
</tr>
</tbody>
</table>

DFO: deferoxamine mesylate, AX: amoxicillin (25μg), VAN: vancomycin (30μg), IMP: imipenem (10μg), COL: colistin (10μg), MRSA: methicillin-resistant *Staphylococcus aureus*, CRAB: Carbapenem-resistant *Acinetobacter baumannii*.

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

Out 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 exhibited a significant antibiofilm effect on mature biofilms of five isolates. This effect was not only observed for MRSA 21 (data not shown). The percentages of biofilm-disruption caused by DFO ranged from 38.1% to 72.3%. Interestingly, DFO demonstrated a stronger disruptive effect on the biofilms formed by CRAB isolates compared to MRSA isolates. Specifically, the percentages of biofilm-distruption by DFO were as follows: MRSA3 (38.1%), MRSA6 (42.1%), CRAB35 (62.9%), CRAB50 (72.3%), and CRAB89 (66.5%). The OD values and the corresponding percentage of biofilm-disruption in the presence of DFO for each isolate are depicted in Figure 2.

Figure 2. (a) Optical densities of mature biofilms formed by bacterial strains and mature biofilms exposed to deferoxamine. (b) Percentage of biofilm-disruption effect of deferoxamine, quantified as a percentage relative to the control using crystal violet staining. MRSA: methicillin-resistant Staphylococcus aureus, CRAB: Carbapenem-resistant Acinetobacter baumannii.

DISCUSSION

The development of resistance to vancomycin and colistin, the last-resort antibiotics for MRSA and CRAB respectively, leads to the need for combination therapy. Therefore 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 amoxicillin and imipenem. 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 exhibited a synergistic effect when combined with amoxicillin, vancomycin, and colistin, but did not demonstrate antibacterial effect alone; (b) DFO significantly disrupted the mature biofilm formed by both MRSA and CRAB isolates. Pathogenesis of bacterial infections involves various virulence factors, including antimicrobial resistance gene expression, iron uptake mechanisms, and biofilm formation. Iron metabolism is closely linked to quorum sensing and biofilm formation, which influence bacterial colonisation, antibiotic susceptibility, and essential functions within the bacteria. Critical iron-dependent proteins, such as ribonucleotide reductase involved in DNA synthesis and cytochromes essential for energy metabolism, are vital for bacterial growth and
multiplication. In the absence of sufficient iron, these critical proteins are unable to perform their functions, leading to growth inhibition. Iron chelators are believed to exert their antimicrobial effects by targeting iron-dependent pathways, enzymes, and proteins in bacteria. Deferoxamine (DFO) is the first iron chelator approved for use in humans and is widely used for the treatment of iron overload. 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. In our study, we found that DFO alone did not exhibit antibacterial activity results in the disk diffusion method. However, when combined with vancomycin, amoxicillin, or colistin, DFO enhanced the inhibitory effects of these antibiotics, as evidenced by larger zone diameters (2 mm) compared to the antibiotic discs alone. This suggests a synergistic interaction between DFO and these antibiotics.

Considering the limited literature on the effect of DFO against bacteria species, there are noteworthy findings that point to a synergistic interaction between DFO and antibiotics, in line with our findings. Gokarn et al., investigated the effects of exogenous siderophores (exochelin-MS and DFO-B) in combination with antibiotics against various resistant bacterial species, including MRSA. They reported that siderophore-antibiotic (ampicillin, cefdinir, imipenem, and meropenem) combinations inhibited the growth of a significant proportion (50-75%) of MRSA isolates. Similarly, DFO-B exhibited a bacteriostatic effect on 30-50% of the tested isolates at relatively higher concentrations in parallel to our findings, these siderophores alone did not show zones of inhibition in disk diffusion method. Another study by Asbeck et al. demonstrated the synergistic interaction between DFO and antibiotics (gentamicin, chloramphenicol, cefalothin, cefotiam or cefsulodin) against Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas and Providencia. In contrast, a study investigating the effects of the iron chelators (DFO, deferiprone, Apo6619, and VK28) on the growth of nosocomial pathogens reported that DFO did not exhibit inhibitory effect (MIC ≥ 512 g/mL for all bacteria tested) whereas other chelators inhibited bacterial growth in standard mediums. These discrepancies may be attributed to variations in bacterial species, experimental conditions, and concentrations of iron chelators used.

Indeed, the lack of an inhibitory effect of DFO effect observed in the disc diffusion method, including in our study, can be attributed to several factors. Previous studies utilizing the broth microdilution method have demonstrated the inhibitory effect of DFO. The most obvious explanation is that siderophores have easier access to iron in liquid media and are better at iron sequestration. Most siderophores sequester Fe\(^{3+}\) at low concentrations under aerobic and neutral pH conditions. 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. However, the antibacterial effect of iron-bound DFO is lower compared to hydrophobic chelating agents like deferiprone, primarily due to its hydrophilic nature and limited penetration into lipid membranes. Nevertheless, the role of DFO in iron bioavailability and virulence can vary depending on the specific bacterial species and infection models. Ariffin et al. found that DFO increased iron bioavailability and enhanced virulence of bacteria in murine systemic infection model with community-associated MRSA. Similarly, DFO may have a promoting role in systemic Yersinia enterocolitica infections in humans. 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 the action of antibiotics, the diversity of mechanisms for iron uptake in bacteria, and the presence of siderophores with different iron binding capacities and chemical structures can contribute to the observability of siderophores in the antibacterial effects. The iron content of the culture media can vary, which can also influence the availability of iron and the response to iron chelators. Furthermore, it is known that DFO may facilitate the delivery of iron to bacteria through the receptors of their cognate siderophores, potentially augmenting the virulence of pathogenic bacteria. On the other hand, iron deprivation induced by iron chelators can impair essential functions and increase the effectiveness of antibiotics against bacteria. Although the precise mechanism underlying the synergistic interactions between siderophores 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. This could provide an explanation for 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. These enzymes inactivate the 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. DFO has a high affinity for both Zn\(^{2+}\) ions and Fe\(^{3+}\) ions due to its specific chemical groups. This affinity can result in the depletion of Zn\(^{2+}\) ions in the media, leading to the inactivation of the metallo-beta-lactamases and increased susceptibility of bacteria to beta-lactam antibiotics. This is believed to be responsible for the observed synergistic effect, especially in resistant bacterial isolates in the presence of DFO. In this study, while DFO showed synergy with three of the tested antibiotics, no synergy was observed with imipenem against CRAB isolates. This discrepancy may be attributed to both the chemical structure of the imipenem molecule and the expression of bacterial membrane proteins.
iron loading or restriction, the expression of proteins responsible for various metabolic functions, including cell division, antibiotic resistance, and iron acquisition, particularly membrane proteins, undergo changes in bacterial cells. A previous proteomic study has indicated that the membrane proteins and metabolism of Acinetobacter respond differently to the presence of iron, especially CRAB. Hence, the presence of multiple proteins which are also associated with carbapenem resistance, and the differentiation of their expression levels in iron-limiting conditions may be the potential reason for the different result in CRAB.

Bacterial biofilms are among the leading causes of morbidity and mortality associated with infectious diseases. Biofilms are bacterial layer that form on the surfaces of medical devices like catheters and heart valve prostheses, contributing to nosocomial infections and prevent the access of antimicrobial drugs to bacteria, resulting in reduced susceptibility to treatment. Therefore, the detection of pathogens’ biofilm-forming capacity and the discovery of antibiofilm compounds have crucial roles in effective treatment strategies. In this context, we examined the impact of iron depletion through DFO on preformed biofilms in vitro. The biofilm-forming capacities of MRSA and CRAB isolates were evaluated using the spectrophotometric microplate method, and they were found to be strong biofilm producers. DFO exhibited significant disruption on the mature biofilms, especially in CRAB strains, leading to a reduction in optical densities by over 60%). Similar studies have been conducted to explore the antibiofilm effects of DFO, deferasirox, and deferiprone against different bacterial and fungal species. In one study, combined treatment with tobramycin and iron chelators (deferoxamine or deferasirox) resulted in approximately 90% reduction in preformed P.aeruginosa biofilm biomass and 7-log units decrease in bacterial viability. Gentile et al. reported that iron starvation did not affect the biofilm-forming capacity of A. baumannii strains isolated from veterinary and clinical sources. Conversely, DFO showed lower efficacy against Protovella intermedia biofilm formation than deferasirox. Nazik et al. 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 biofilms of clinical MRSA and CRAB isolates, suggesting its potential as an antibiofilm agent.

STUDY LIMITATIONS
The present study has some limitations in a 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 to be used for MIC determination contains iron and other cations. This was considered to be an important factor that could influence the results of 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. In future studies, it is planned to determine the minimum inhibitory concentrations in iron-poor and iron-rich environments. Additionally, the antibiofilm effect of DFO at different concentrations will be investigate against various bacterial species causing biofilm-associated infections and with a larger number of isolates.

CONCLUSION
In conclusion, our findings suggest that DFO has the potential to enhance antibiotic efficacy and combat biofilm-associated infections caused by CRAB and MRSA. The prevalence of high antibiotic resistance rates and the rapid evolution of bacterial resistance to the latest antimicrobials reveals the urgent need for innovative therapeutic approaches to combat infections. In an era of limited antibiotic discovery and with antibiotic resistance posing a global health threat, the importance of drug repositioning studies has become increasingly evident. Iron chelation may be a promising antivirulence strategy for combating drug-resistant bacteria. In light of the results of previous studies and our study, it can be argued that 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 has the potential to provide clinical benefits in the treatment of resistant infections by augmenting the susceptibility of antibacterial agents.

REFERENCES


