

Activity of siderophores against drug-resistant Gram-positive and Gram-negative bacteria

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Abstract: Infections by drug-resistant bacteria are life-threatening. As iron is a vital element for the growth of bacteria, iron-chelating agents (siderophores) can be used to arrest their multiplication. Exogenous siderophores – exochelin-MS and deferoxamine-B – were evaluated for their inhibitory activity against methicillin-resistant *Staphylococcus aureus* and metallo- β -lactamase producers – *Pseudomonas aeruginosa* and *Acinetobacter baumannii* – by disc diffusion, micro-broth dilution, and turbidimetric growth assays. The drug-resistant isolates were inhibited by the synergistic activity of siderophores and antibiotics. Minimum inhibitory concentration of exochelin-MS+ampicillin for different isolates was between 0.05 and 0.5 mg/mL. Minimum inhibitory concentration of deferoxamine-B+ampicillin was 1.0 mg/mL and greater. Iron-chelation therapy could provide a complementary approach to overcome drug resistance in pathogenic bacteria.

Keywords: iron-chelation, xenosiderophores, exochelin MS, deferoxamine B

Introduction

Most nosocomial infections are caused by Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-negative metallo- β -lactamase (MBL)-producing bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Emergence of drug resistance in such pathogens has made treatment difficult, making exploration of new therapeutic options imperative. As iron is an essential cofactor of many important biochemical pathways for all cells, one possible strategy would be to impose an iron-deficient environment on these pathogens.

Iron is acquired by microorganisms from the environment with the help of siderophores that are taken up via species-specific receptors. Xenosiderophores (“Xeno” – not “self”) are exogenous siderophores, and when Fe^{3+} ion is complexed with these, iron uptake may be affected because of the absence of receptors. Exochelin-MS (Exo-MS) is a siderophore produced by *Mycobacterium smegmatis* and deferoxamine-B (DFO-B) is a siderophore made by *Streptomyces pilosus* (now available commercially). These two are xenosiderophores for MRSA and for MBL-producing bacteria, and therefore, the effect of Exo-MS and DFO-B on these pathogens was determined by disc diffusion, micro-broth dilution, and turbidimetric growth assays.

The isolates of *S. aureus*, *P. aeruginosa*, and *A. baumannii* confirmed as drug resistant were used to assess the susceptibility to siderophores. Twenty isolates of MRSA, 15 isolates of MBL-producing *P. aeruginosa*, and 15 isolates of MBL-producing *A. baumannii* (a total of 50 isolates) were used in this study.

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Materials and methods

Identification of MRSA and MBL-producing *P. aeruginosa* and *A. baumannii* (MBL producers)

A large number of clinical isolates of *S. aureus*, *P. aeruginosa* and *A. baumannii* were procured from the Microbiology Laboratory of Sir H.N. Hospital, Mumbai. Identification of the isolates based on the morphological and biochemical parameters was carried out at Sir H.N. Medical Research Centre, Mumbai.

S. aureus isolates were identified as methicillin-resistant by the following tests:

Oxacillin disc susceptibility test: Mueller–Hinton agar (MHA) plates supplemented with 4% NaCl were inoculated with the isolates whose turbidity was adjusted to 0.5 McFarland standard. A 1.0 µg oxacillin disc and a 30 µg cefoxitin disc were placed on the seeded agar surface and incubated at 37°C for 18 h.^{1,2} The isolate resistant to either or both the antibiotics was selected for further tests (Table S1).

Commercially available oxacillin E test strip (Biomérieux) on MHA plates supplemented with 2% NaCl was used to determine the minimum inhibitory concentration (MIC) for the isolates.³

The latex agglutination test was carried out for the detection of *mecA* gene product PBP2A (Biomérieux Slidex MRSA agglutination kit) in the isolates.¹

P. aeruginosa and *A. baumannii* isolates were identified as MBL producers by the following EDTA-based microbiological tests:

EDTA+imipenem/ceftazidime combination assay

Extracts of each isolate were prepared by lysing cells in 50 mM Tris–HCl buffer (pH=8) by repeated cycles of freezing and thawing at –20°C and 37°C, respectively. The supernatant was used as the crude enzyme extract to detect the presence of MBLs.⁴ MBLs are distinguished from serine-carbapenemases by evaluating the effect of EDTA on the growth of the carbapenem-sensitive strain of *Escherichia coli* ATCC 25922 in the presence of a carbapenem.

Culture suspension of *E. coli* ATCC 25922 adjusted to 0.5 McFarland standard was swabbed uniformly on MHA plate. A 10 µg imipenem or a 30 µg ceftazidime disc (Himedia, India) was placed on the center of the seeded agar plate.

Four sterile filter paper discs containing different solutions as given below were placed at the periphery of the central disc at 10 mm edge-to-edge distance:

- Disc 1: 20 µL of the crude enzyme extract;

- Disc 2: 20 µL of the same extract and 0.1 mM ZnSO₄;
- Disc 3: 20 µL of the same extract and 20 mM EDTA (pH=8);
- Disc 4: 20 µL 50 mM Tris HCl buffer (pH=8).

After incubation at 37°C for 18 h, the zone of inhibition around each disc was measured.

EDTA-double disc synergy test

Culture suspensions of *E. coli* ATCC 25922 (control) and the *P. aeruginosa* and *A. baumannii* isolates adjusted to 0.5 McFarland standard were swabbed individually on MHA plates. A 10 µg imipenem disc was placed on the agar surface. A blank disc with 10 µL of sterile 0.5 mM EDTA (750 µg) was placed at a 10 mm edge-to-edge distance from the imipenem disc. Similarly, a 30 µg ceftazidime and an EDTA disc (750 µg) were placed on the same plate.⁵ After incubation at 37°C for 18 h, the zone of inhibition around each disc was measured.

Modified Hodge test for carbapenemase detection

Here, carbapenem inactivation by a carbapenemase-producing strain was tested along with a carbapenem-sensitive *E. coli* strain ATCC 25922. A culture suspension of *E. coli* ATCC 25922 adjusted to 0.5 McFarland standard was swabbed on MHA plates. A 10 µg imipenem disc was placed at the center of the plate and an isolate of *P. aeruginosa* or *A. baumannii* was streaked from the edge of the disc to the periphery of the plate in four different directions.⁵ The zone of inhibition was recorded after incubation at 37°C for 18 h.

Determination of in vitro effect of Exo-MS and DFO-B on MRSA and MBL-producing bacteria

Confirmed isolates of MRSA and MBL-producing *P. aeruginosa* and *A. baumannii* were used for determining the effect of siderophores.

Exo-MS was extracted, purified, and analyzed in our laboratory.⁶ The concentration of Exo-MS was 19 mg/mL. DFO-B, which is commercially available (Desferal, Novartis), was procured in powder form and reconstituted in sterile distilled water at a concentration of 250 mg/mL. Both the siderophores tested were in desferri (non-Fe³⁺ bound) forms. The in vitro effect of Exo-MS and DFO-B on drug-resistant pathogenic bacteria was determined by the following methods:

Disc diffusion method

The antibacterial susceptibility testing was performed as per the Clinical and Laboratory Standards Institute (CLSI)

recommendations (Kirby–Bauer method),⁷ using standard antibiotic discs (HiMedia, India) in combination with siderophores. The standard antibiotic discs contained 10 µg of ampicillin, imipenem, meropenem, and 5 µg of cefdinir. For MRSA, ampicillin and cefdinir discs were used. For MBL producers, ampicillin, imipenem, and meropenem discs were used. On each antibiotic disc, 10 µL of 5 mg/mL Exo-MS solution or 10 µL of 10 mg/mL DFO-B solution was loaded. As a control, 10 µL of sterile distilled water was loaded on the antibiotic discs.

Culture suspensions of MRSA and MBL-producing *P. aeruginosa* and *A. baumannii* isolates adjusted to 0.5 McFarland standard were swabbed uniformly on MHA plate. The sterile antibiotic discs were placed on the surface of the seeded agar plate. After incubation at 37°C for 18 h, the zone of inhibition around each disc was measured.

MIC determination

For each isolate, MIC was determined by micro-broth dilution method in triplicates in 96-well microtiter plates using Mueller–Hinton broth (MHB) No. 2 with control cations (HiMedia, Mumbai, India). The total volume in each well was 200 µL and the plates were incubated at 37°C for 24 h.

To determine MIC of siderophores, 0.05, 0.125, 0.25, and 0.5 mg/mL of Exo-MS was used individually and in combination with ampicillin for MRSA and MBL-producing isolates. Similarly, 0.5, 1, 2.5, 5, 7.5, and 10 mg/mL of DFO-B was used individually and in combination with ampicillin for MRSA and MBL-producing isolates. The concentration of ampicillin was kept constant at 0.01 mg/mL as recommended by CLSI.

Medium control contained 200 µL MHB without any isolate, growth control contained 200 µL MHB with each isolate, and ampicillin control contained 200 µL MHB+ampicillin with each isolate. Ferric ammonium citrate at a final concentration of 0.5 mg/mL was used for each isolate as “Fe” control. Each experiment mentioned above was carried out three times.

The turbidity of each well at the end of 24 h at 37°C was measured on a microplate reader at 620 nm. The lowest concentration of the Exo-MS or DFO-B that did not allow growth of the isolate was the MIC of the siderophore.

To determine whether the siderophore concentration had a bacteriostatic or a bactericidal effect, a loopful of broth from each well showing no growth after 24 h was streaked onto MHA plates and incubated at 37°C for 24 h.

Turbidimetric assay

The growth curves of two isolates of MRSA and six MBL producers were determined in the presence of Exo-MS or

DFO-B. Here too, the concentration of ampicillin was kept constant at 0.01 mg/mL. The growth assay was carried out in 96-well microtiter plates, each well having a total volume of 200 µL for each isolate. The different concentrations of siderophores and their antibiotic combinations were used as described for MIC determination, in triplicate. Each plate was incubated at 37°C with continuous shaking in Epoch 2 Microplate Spectrophotometer (Biotek, Winooski, VT, USA). Optical density (OD) of each well was monitored at 620 nm every hour for 24 h.

Results

In vitro inhibitory effect of Exo-MS and DFO-B on MRSA and MBL producers

Disc diffusion method

Figure 1 shows representative examples of zones of inhibition of MRSA by Exo-MS, DFO-B, and their combinations with antibiotics. No zone of inhibition with standard antibiotic disc was observed when 10 µL of distilled water was loaded instead of siderophores (Figure 1, second row, left plate). The same plate also shows that the zone of inhibition was negligible when ferric ammonium citrate was added to the ampicillin disc along with DFO-B.

Figure 2 shows representative examples of zones of inhibition for MBL-producing *P. aeruginosa* by Exo-MS, DFO-B, and their combinations with antibiotics.

Figure 3 shows representative examples of zones of inhibition for MBL-producing *A. baumannii* by Exo-MS, DFO-B, and their combinations with antibiotics.

Zones of inhibition with Exo-MS, DFO-B, and their combination with antibiotics for all the isolates tested are given in Tables S2–S4.

Table 1 shows the percentage of isolates that were susceptible to the combined effect of siderophores+antibiotics by the disc diffusion method.

MIC of Exo-MS and DFO-B

For each isolate, growth was observed in growth control and controls containing only ampicillin. For MRSA, MICs of Exo-MS alone and in combination with ampicillin were between 0.05 and 0.5 mg/mL. For *P. aeruginosa*, MIC of Exo-MS alone was 0.125 mg/mL and MICs of Exo-MS+ampicillin combination were between 0.05 and 0.125 mg/mL. For *A. baumannii*, MICs of Exo-MS alone and in combination with ampicillin were between 0.05 and 0.25 mg/mL (Table 2).

For MRSA, MICs of DFO-B alone were between 7.5 and 10 mg/mL. MICs of DFO-B+ampicillin combination

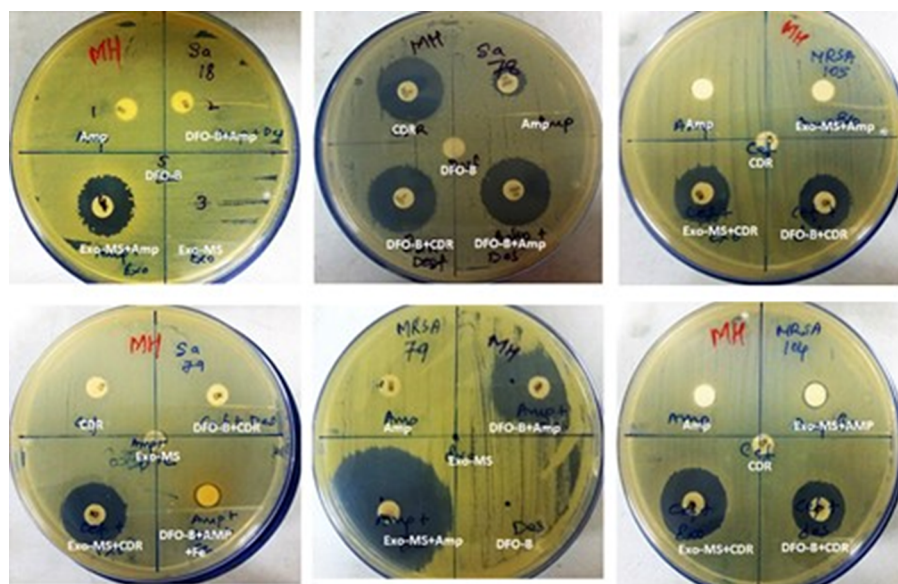


Figure 1 Susceptibility of MRSA to siderophores and antibiotics.

Notes: MRSA isolates were swabbed on the surface of MHA plates and antibiotic discs were placed. Then, either Exo-MS or DFO-B (10 μ L each) was loaded onto the antibiotic discs. The antibiotics used were Amp and CDR. On each plate, a different isolate was seeded. Zones of inhibition were observed with the siderophore+antibiotic combinations, indicating synergism. For example, in the second row, the left plate showed no zones of inhibition with cefdinir. The cefdinir disc was loaded with 10 μ L of distilled water. The siderophore+cefдинir combination showed a zone of inhibition. Exo-MS and DFO-B individually showed no zones of inhibition. The zone of inhibition was negligible when the disc was loaded with ferric ammonium citrate along with DFO-B on the Amp disc.

Abbreviations: Amp, ampicillin; CDR, cefдинir; DFO-B, deferroxamine-B; Exo-MS, exochelin-MS; MRSA, methicillin-resistant *Staphylococcus aureus*; MHA, Mueller–Hinton agar.

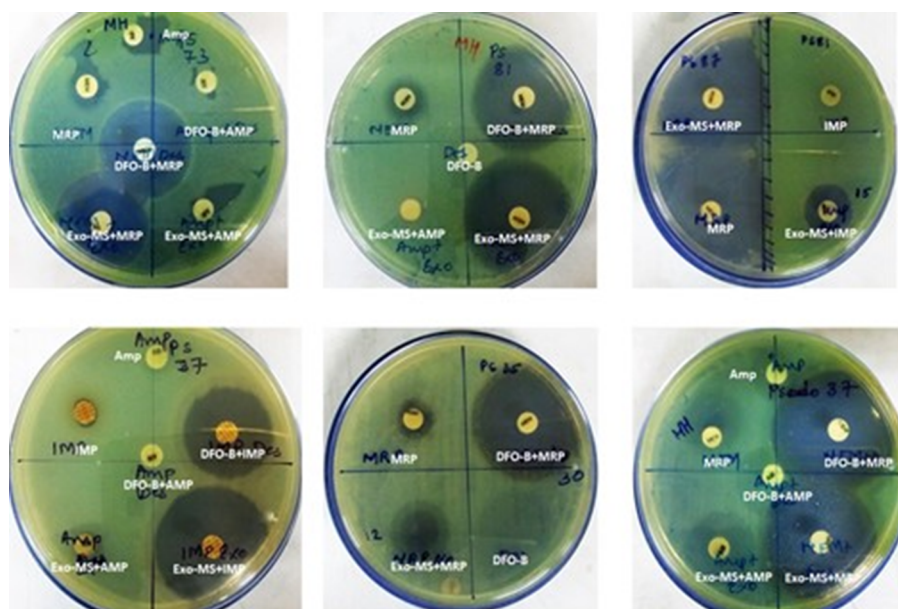


Figure 2 Susceptibility of metallo- β -lactamase-producing *Pseudomonas aeruginosa* to siderophores and antibiotics.

Notes: *P. aeruginosa* isolates were swabbed on the surface of MHA plates and antibiotic discs were placed. Then, either Exo-MS or DFO-B (10 μ L each) was loaded onto the antibiotic disc. The antibiotics used were Amp, IMP, and MRP. On each plate, a different isolate was seeded. Larger zones of inhibition were observed with the siderophore+antibiotic combinations, indicating synergism. Such an effect is seen in the first row, middle plate for siderophore+meropenem combinations, where meropenem alone shows a small zone of inhibition; Exo-MS and DFO-B individually showed no zones of inhibition.

Abbreviations: Amp, ampicillin; DFO-B, deferroxamine-B; Exo-MS, exochelin-MS; IMP, imipenem; MHA, Mueller–Hinton agar; MRP, meropenem.

were between 5 and 10 mg/mL. For *P. aeruginosa*, MICs of DFO-B alone were between 5 and 10 mg/mL. MICs of DFO-B+ampicillin combination were between 2.5 and 10 mg/mL. For *A. baumannii*, MICs of DFO-B alone were between 2.5

and 10 mg/mL. MICs of DFO-B+ampicillin combination were between 1.0 and 10 mg/mL (Table 3).

When a loopful of broth from wells showing no growth was re-streaked on MHA plates and incubated at 37°C for

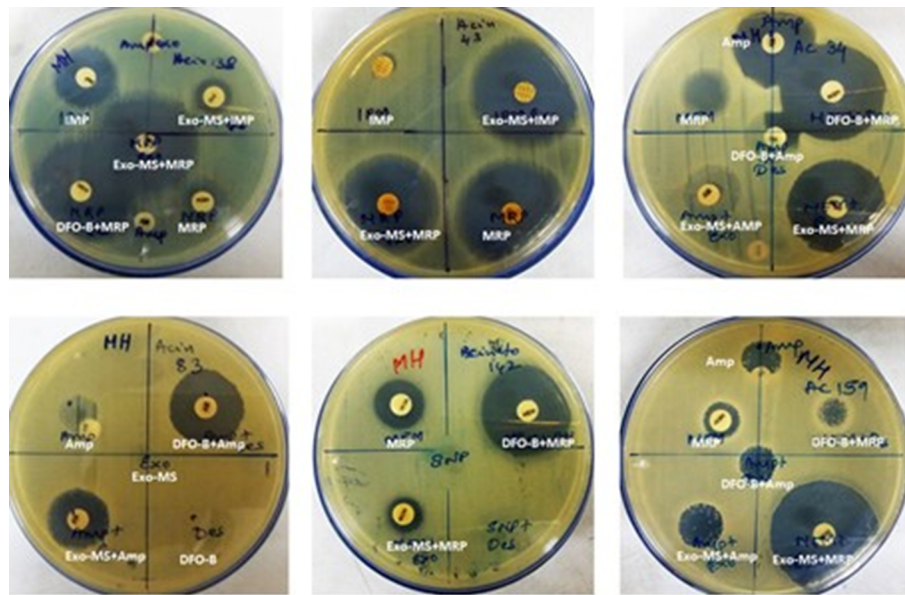


Figure 3 Susceptibility of metallo- β -lactamase-producing *Acinetobacter baumannii* to siderophores and antibiotics.

Notes: *A. baumannii* isolates were swabbed on the surface of MHA plates and antibiotic discs were placed. Then, either Exo-MS or DFO-B (10 μ L each) were loaded onto the antibiotic discs. The antibiotics used were Amp, IMP, and MRP. On each plate, a different isolate was seeded. Zones of inhibition were observed with the siderophore+antibiotic combinations, indicating synergism. For example, in the second row, the left plate showed no zones of inhibition with ampicillin and siderophores individually. The siderophore+ampicillin combination showed zones of inhibition. Exo-MS and DFO-B individually showed no zones of inhibition.

Abbreviations: Amp, ampicillin; DFO-B, deferoxamine-B; Exo-MS, exochelin-MS; IMP, imipenem; MHA, Mueller–Hinton agar; MRP, meropenem.

Table 1 Percent susceptibility of drug-resistant Gram-positive MRSA and Gram-negative MBL producers to combinations of Exo-MS and DFO-B with antibiotics by the disc diffusion method

Pathogen	Standard antibiotics	Isolates susceptible to combination of Exo-MS + antibiotic (%)	Isolates susceptible to combination of DFO-B + antibiotic (%)
MRSA (n=20)	Ampicillin (0.01 mg)	15 (75)	10 (50)
	Cefdinir (0.005 mg)	5 (33)	1 (05)
MBL-producing <i>Pseudomonas aeruginosa</i> (n=15)	Ampicillin (0.01 mg)	4 (27)	5 (33)
	Imipenem (0.01 mg)	7 (47)	8 (53)
	Meropenem (0.01 mg)	9 (60)	6 (40)
MBL-producing <i>Acinetobacter baumannii</i> (n=15)	Ampicillin (0.01 mg)	7 (47)	6 (40)
	Imipenem (0.01 mg)	7 (47)	8 (53)
	Meropenem (0.01 mg)	10 (67)	10 (67)

Abbreviations: DFO-B, deferoxamine-B; Exo-MS, exochelin-MS; MBL, metallo- β -lactamase; MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 2 MIC of Exo-MS for MRSA and MBL-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

Isolates	Number of isolates inhibited at Exo-MS (mg/mL)				Total isolates inhibited (%)	Number of isolates inhibited at Exo-MS (mg/mL) + ampicillin 0.01 mg/mL				Total isolates inhibited (%)
	0.05	0.125	0.25	0.5		0.05	0.125	0.25	0.5	
MRSA (n=20)	4	6	2	1	13 (65)	6	6	2	1	15 (75)
MBL-producing <i>P. aeruginosa</i> (n=15)	0	3	0	0	3 (20)	1	3	0	0	4 (27)
MBL-producing <i>A. baumannii</i> (n=15)	1	2	2	0	5 (33)	3	2	2	0	7 (47)

Notes: MIC was determined by adding Exo-MS at different concentrations with or without ampicillin at 0.01 mg/mL. The number of isolates inhibited at the end of 24 h at 37°C are shown. n = total number of isolates of each pathogen. Medium control showed no growth, whereas growth control, ampicillin control, and "Fe" control showed growth (data not shown).

Abbreviations: Exo-MS, exochelin-MS; MBL, metallo- β -lactamase; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 3 MIC of DFO-B for MRSA and MBL-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

Isolates	Number of isolates inhibited at DFO-B (mg/mL)						Total inhibited (%)	Number of isolates inhibited at DFO-B (mg/mL) + ampicillin 0.01 mg/mL						Total inhibited (%)
	0.5	1	2.5	5	7.5	10		0.5	1	2.5	5	7.5	10	
MRSA (n=20)	0	0	0	0	1	1	2 (10)	0	0	0	2	4	4	10 (50)
MBL-producing <i>P. aeruginosa</i> (n=15)	0	0	0	1	1	1	3 (20)	0	0	1	1	1	2	5 (33)
MBL-producing <i>A. baumannii</i> (n=15)	0	0	1	1	1	1	4 (27)	0	1	1	1	1	2	6 (40)

Notes: MIC was determined by adding DFO-B at different concentrations with or without ampicillin at 0.01 mg/mL. The number of isolates inhibited at the end of 24 h at 37°C are shown. n = total number of isolates of each pathogen. Medium control showed no growth, whereas growth control, ampicillin control, and "Fe" control showed growth. (Data not shown.)

Abbreviations: DFO-B, deferrioxamine-B; MBL, metallo- β -lactamase; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*.

24 h, growth was observed at all concentrations of siderophores. Isolates exposed to siderophores individually and to siderophore+antibiotic combinations showed growth when transferred into a fresh growth medium without the test compounds, indicating bacteriostatic activity (data not shown).

Therefore, it was concluded that all the siderophore concentrations used were bacteriostatic.

When excess iron was supplemented in the MHB, the isolates could grow without any inhibition (data not shown).

Turbidimetric assay

This micro-broth kinetic assay was used to continuously monitor growth of four isolates each of MRSA and MBL producers. In every case, normal growth was seen in "growth control" and in "ampicillin control" wells. Figure 4 shows growth curves of a representative MRSA isolate in the presence of Exo-MS and ampicillin.

Figure 5 shows growth curves of a representative isolate of *P. aeruginosa* in the presence of Exo-MS and ampicillin.

Figure 6 shows growth curves of a representative *Acinetobacter* isolate in the presence of Exo-MS and ampicillin.

Growth curves of some isolates of MRSA, *P. aeruginosa*, and *A. baumannii* in the presence of DFO-B and ampicillin are shown in Figures S1–S3.

Discussion

This is a preliminary investigation to evaluate siderophores as adjuncts with antibiotics against commonly encountered drug-resistant pathogens such as MRSA and MBL-producing bacteria. Emergence of drug resistance in bacteria is mostly due to spontaneous mutations in their genes. Resistance in MRSA is primarily due to expression of the *mecA* gene, which encodes penicillin-binding protein (PBP2a), which has a low affinity for β -lactam antibiotics.⁸ Some of the drug resistance in Gram-negative bacteria can involve spontaneous

point mutations such as loss of porins leading to impermeability of their outer membrane, or can be acquired through horizontal transfer of resistance genes. Upregulation of efflux pumps can also lead to drug resistance, for example, resistance to β -lactams including carbapenems.⁹

We hypothesize that iron deprivation caused by Exo-MS and DFO-B in bacteria may result in inhibition or inactivation of proteins and enzymes involved in vital functions. The siderophores may also act as facilitators for antibiotics across the cell membrane because of increase in permeability caused by iron deficiency. This could be the explanation why 50%–75% of MRSA isolates are inhibited when the siderophores are used in combination with antibiotics.

Another mechanism of drug resistance in Gram-negative bacteria is the production of MBLs. These are a diverse set of enzymes that are not susceptible to β -lactamase inhibitors and require zinc for their activity. When Zn^{2+} is chelated by EDTA, the MBLs are inactivated making the pathogens susceptible to β -lactam antibiotics. A similar mechanism may be responsible for the effect seen with the siderophores.

DFO-B binds iron in the Fe^{3+} form with an affinity constant of $\beta^{III} = 10^{30}$, and reduces it to Fe^{2+} with a lower affinity constant of $\beta^{II} = 10^{7.2}$, triggering the release of bound iron. The affinity of DFO-B for Zn^{2+} is $\beta^{II} = 10^{9.55}$, which is more than that for Fe^{2+} . This affinity may be sufficient for the complex formation of DFO-B and Zn^{2+} , given that the affinity constant of EDTA for Zn^{2+} is $\beta^{II} = 10^{16.5}$. Therefore, DFO-B may also deprive these MBL producers of Zn^{2+} making them susceptible to β -lactams.¹⁰

The results of MIC determination show that Exo-MS and DFO-B individually were inhibitory to some isolates, which was not observed in the disc diffusion method. In liquid cultures, siderophores probably get easier access to Fe^{3+} , leading to better sequestration of iron. This may deprive the organisms of iron and decrease the activity of critical proteins that are Fe-dependent. One such protein is

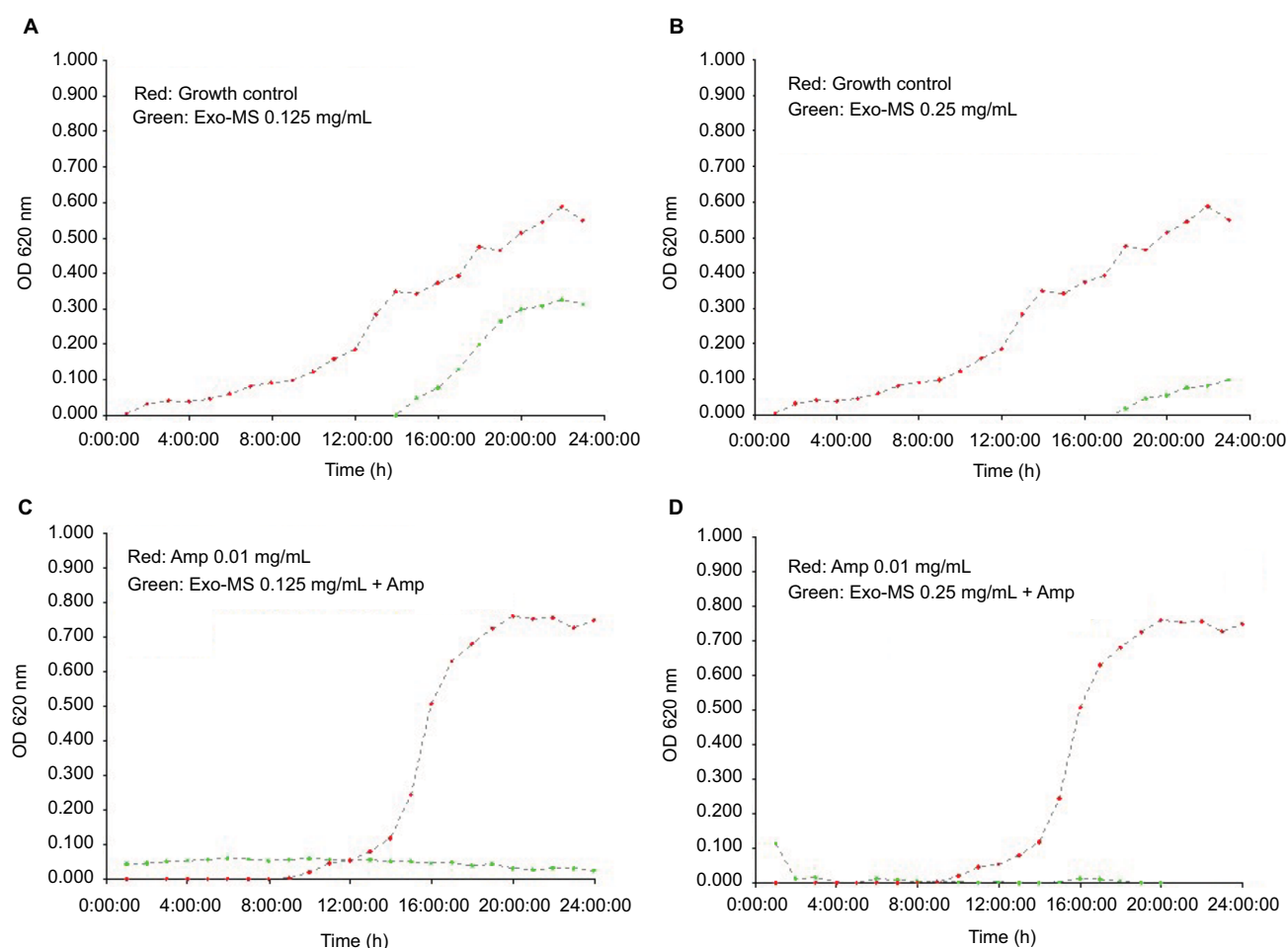


Figure 4 Growth curves of methicillin-resistant *Staphylococcus aureus* in the presence of Exo-MS.

Notes: (A) and (B) plot in red: growth curves of the isolate in medium alone and plot in green show growth curves in the presence of Exo-MS at 0.125 and 0.25 mg/mL, respectively; (C) and (D) plot in red: growth curves of the isolate in the presence of ampicillin 0.01 mg/mL and plot in green show curves in the presence of Exo-MS 0.125 and 0.25 mg/mL, respectively, along with ampicillin 0.01 mg/mL.

Abbreviations: Amp, ampicillin; Exo-MS, exochelin-MS; OD, optical density.

ribonucleotide reductase that is involved in DNA synthesis. Cytochromes are examples of other iron-dependent proteins that are essential for energy metabolism. If any of these get affected, multiplication of the organism may be arrested.

In the turbidimetric growth assays, Exo-MS and DFO-B prolonged the lag time along with a reduction in the cell numbers, which was reflected in the lesser OD values at the end of 24 h. The combination of Exo-MS+ampicillin and DFO-B+ampicillin had a synergistic effect as there was total inhibition of the isolates with OD readings close to zero after 24 h. These assays validated the inhibitory effects seen in the MIC determination of Exo-MS, DFO-B, and their combination with antibiotics.

There were a few isolates that were unaffected by siderophore+antibiotic combinations. These isolates perhaps have the capability of acquiring iron from exogenous chelators because of the existence of as yet unknown and

uncharacterized iron-uptake systems. For instance, it has been shown that *P. aeruginosa* has iron-uptake ability from multiple siderophores under conditions of iron deficiency.¹¹

Earlier studies have shown that ascorbic acid is required for enhancing siderophore activity. For example, van Asbeck et al¹² have demonstrated inhibitory activity of DFO-B against Gram-positive and Gram-negative bacteria in combination with antibiotics only when ascorbic acid was present. In other studies, inhibitory effects of DFO-B and synthetic iron chelators against pathogens including *S. aureus* were tested.^{13,14} However, none of these studies evaluated the effect of iron chelators+antibiotic combinations, nor did they use drug-resistant isolates.^{12–14}

We did not observe any enhancement of siderophore activity by ascorbic acid (data not shown). Our study did not support the hypothesis of the toxic radical formation, and the inhibition was simply due to iron chelation. The

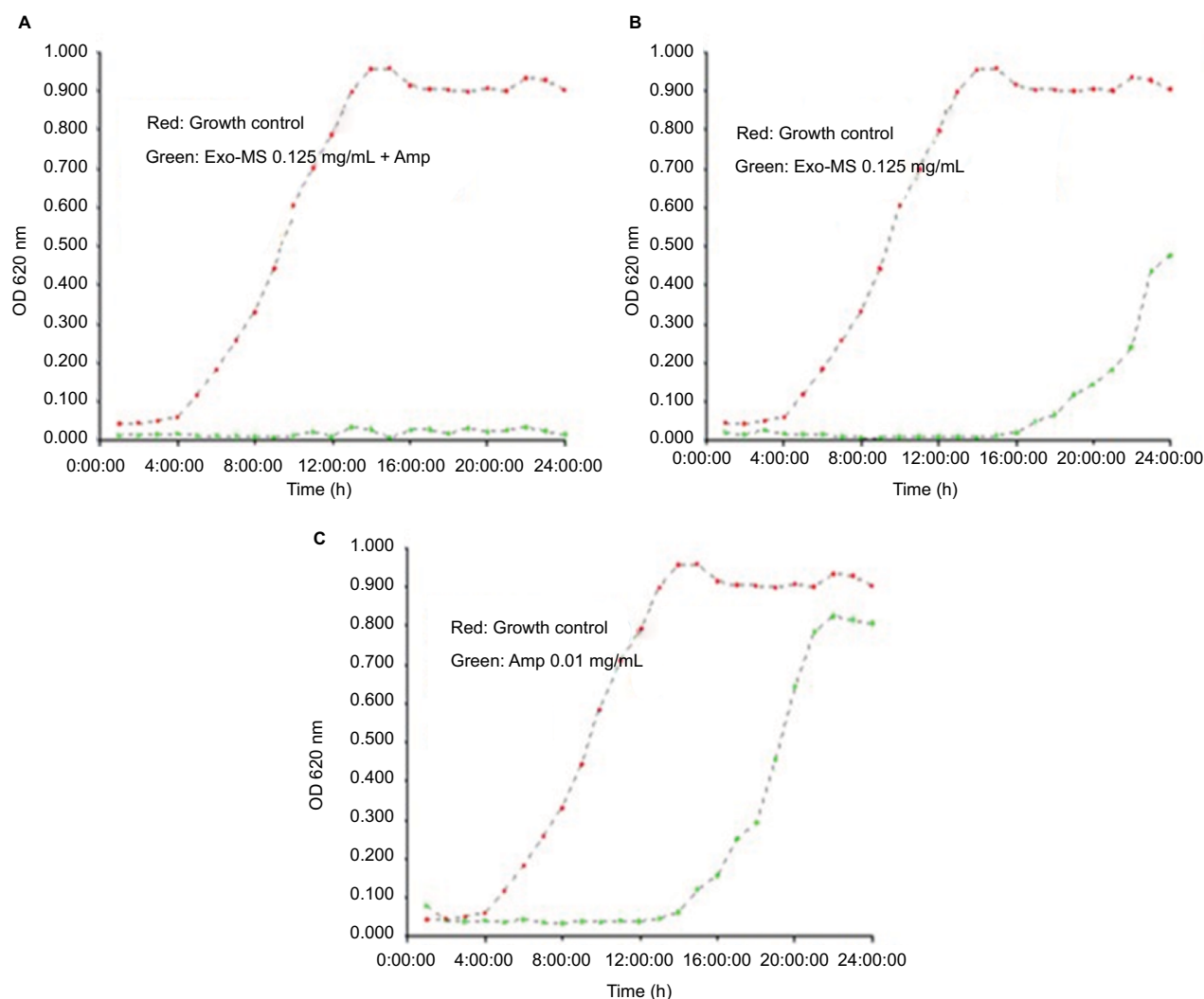


Figure 5 Growth curves of an isolate of metallo- β -lactamase-producing *Pseudomonas aeruginosa* in the presence of Exo-MS.

Notes: Plot in red: growth curves of the isolate in medium alone. Plot in green show growth curves: (A) in the presence of Exo-MS at 0.125 mg/mL + ampicillin 0.01 mg/mL, (B) only Exo-MS at 0.125 mg/mL, and (C) only ampicillin 0.01 mg/mL.

Abbreviations: Amp, ampicillin; Exo-MS, exochelin-MS; OD, optical density.

drug-resistant isolates grew normally when excess iron was added to saturate siderophores and free iron made available in the medium. This indicated that the growth inhibition was not due to toxicity caused by excess intracellular iron, as has been suggested elsewhere.¹⁵

We have reported in a pioneering study that similar concentrations of Exo-MS and DFO-B were inhibitory to multidrug-resistant *Mycobacterium tuberculosis* (*Mtb*) isolates by the Mycobacteria growth indicator tube-drug susceptibility test method.¹⁶ It has provided a “proof of concept” that exogenous siderophores could be valuable additions to fight drug-resistant *Mtb*. Importantly, in a parallel study, we have also shown that the same concentrations of siderophores did not harm normal mammalian cells in vitro.⁶

Conclusion

Siderophores can be employed effectively by themselves or with antibiotics to inhibit drug-resistant pathogenic bacteria. Exo-MS concentration up to 0.5 mg/mL hindered growth of 75% of MRSA isolates and 30%–50% of MBL-producing *P. aeruginosa* and *A. baumannii* isolates. DFO-B at relatively higher concentrations had bacteriostatic effect on 30%–50% of the isolates tested.

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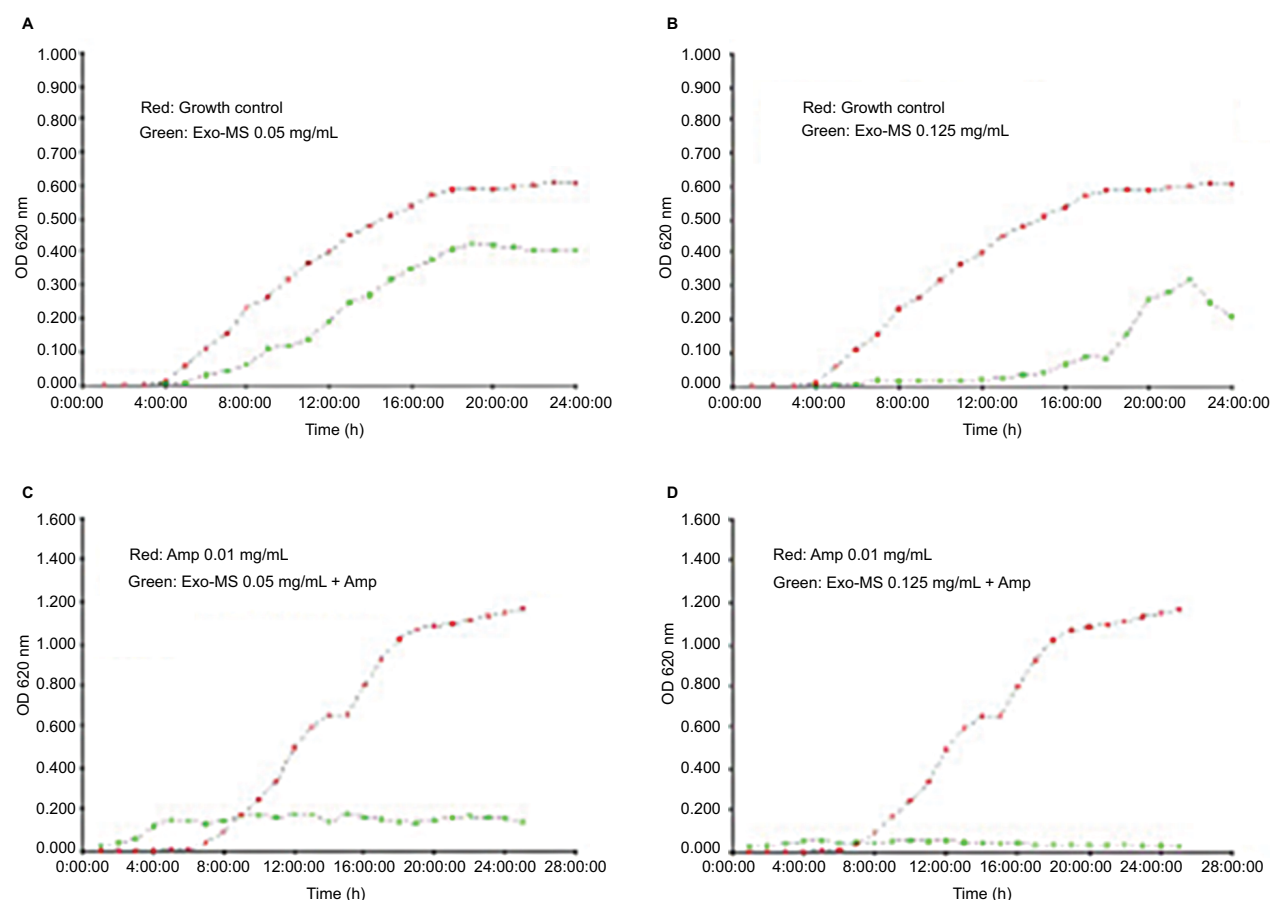


Figure 6 Growth curves of metallo- β -lactamases-producing *Acinetobacter baumannii* in the presence of Exo-MS.

Notes: (A) and (B) plot in red: growth curves of the isolate in medium alone and plot in green show curves in the presence of Exo-MS at 0.05 and 0.125 mg/mL, respectively; (C) and (D) plot in red: growth curves of the isolate in the presence of ampicillin 0.01 mg/mL and plot in green show curves in the presence of Exo-MS at 0.05 and 0.125 mg/mL, respectively, in combination with ampicillin 0.01 mg/mL.

Abbreviations: Amp, ampicillin; Exo-MS, exochelin-MS; OD, optical density.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Results

Identification of methicillin-resistant *Staphylococcus aureus* isolates:

- The Clinical & Laboratory Standards Institute guidelines for interpretation of inhibition zones for oxacillin and cefoxitin are shown in Table S1.

Identification of metallo- β -lactamase producers

In EDTA+imipenem/ceftazidime combination assay, cell extracts of the clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were used to assess the growth of indicator *Escherichia coli* strain ATCC 25922. If growth was observed around discs containing crude enzyme extract as well as ZnSO_4 + crude enzyme extract, it showed the

presence of carbapenemases in the extract of the isolates. Production of metallo- β -lactamases (MBLs) was confirmed when *E. coli* ATCC 25922 was inhibited by EDTA+crude enzyme extract of the isolates. Buffer alone showed inhibition as expected.

In EDTA-double disc synergy test, presence of expanded inhibition zone between EDTA and antibiotic discs confirmed that the isolate was an MBL producer.

In the modified Hodge test, isolate showing “cloverleaf” growth pattern of *E. coli* 25922 was considered as MBL producers.

Based on all the tests, 20 isolates of MRSA, 15 isolates of MBL-producing *P. aeruginosa*, and 15 isolates of MBL-producing *A. baumannii* were used to determine the effect of siderophores.

Table S1 Interpretive criteria (mm) for oxacillin and cefoxitin disc diffusion test

Isolate	Oxacillin			Cefoxitin		Resistant
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	
<i>Staphylococcus aureus</i>	≥ 13 mm	11–12 mm	≤ 10 mm	≥ 20 mm	–	≤ 19 mm

Note: The *S. aureus* isolates found to be resistant to these two antibiotics as per this standard chart were tested further. The isolates that showed minimum inhibitory concentrations of oxacillin E-test ≥ 4 $\mu\text{g/mL}$ and showed agglutination in the latex agglutination test were identified as methicillin-resistant *Staphylococcus aureus*.

Table S2 Susceptibility of methicillin-resistant *Staphylococcus aureus* isolates to siderophores and antibiotics

Isolate No	Zone of inhibition (diameter in mm)							
	Amp	Amp + DFO-B	Amp + Exo-MS	Cdr	Cdr + DFO-B	Cdr + Exo-MS	DFO-B	Exo-MS
1	20	20	20	–	–	15	–	–
2	–	–	13	19	19	19	–	–
3	–	–	18	17	17	17	–	–
4	–	–	20	15	15	12	–	–
5	–	10	10	18	21	20	–	–
6	–	–	–	15	15	15	–	–
7	–	–	30	–	–	23	–	–
8	–	24	20	13	13	10	–	–
9	–	–	–	–	–	–	–	–
10	–	23	20	–	–	22	–	–
11	–	20	15	16	16	16	–	–
12	–	15	15	14	14	–	–	–
13	–	26	22	17	16	15	–	–
14	–	19	15	19	19	20	–	–
15	–	11	10	–	15	16	–	–
16	–	–	23	–	–	25	–	–
17	–	14	–	18	18	19	–	–
18	–	27	–	–	–	24	–	–
19	–	–	10	15	13	23	–	–
20	–	–	10	20	20	20	–	–

Note: Zone of inhibition on Mueller–Hinton agar plates incubated at 37°C for 24 h. (–), No inhibition.

Abbreviations: Amp, ampicillin; Cdr, cefdinir; DFO-B, deferoxamine-B; Exo-MS, exochelin-MS.

Table S3 Susceptibility of metallo- β -lactamase-producing *Pseudomonas aeruginosa* isolates to siderophores and antibiotics

Isolate no.	Zone of inhibition (diameter in mm)										
	Amp	Amp + DFO-B	Amp + Exo-MS	lpm	lpm + DFO-B	lpm + Exo-MS	DFO-B	Exo-MS	Mrp	Mrp + DFO-B	Mrp + Exo-MS
1	20	20	–	10	40	34	–	–	15	22	23
2	–	23	16	15	35	15	26	–	12	15	32
3	18	19	20	–	–	28	–	–	–	30	12
4	–	19	20	–	26	13	–	–	10	40	35
5	–	–	–	15	15	20	–	–	13	13	12
6	–	–	–	–	–	15	–	–	15	15	16
7	–	–	–	–	20	20	–	–	15	15	15
8	–	–	–	10	10	10	–	–	10	30	30
9	–	–	–	–	24	–	–	–	10	30	35
10	15	10	10	–	44	34	–	–	12	12	12
11	–	22	22	22	20	20	–	–	14	14	14
12	–	14	–	–	22	–	–	–	12	16	26
13	–	–	–	–	35	–	–	–	14	20	20
14	–	–	–	13	13	13	–	–	15	12	20
15	–	20	34	26	27	33	–	–	12	10	10

Note: Zone of inhibition on Mueller–Hinton agar plates incubated at 37°C for 24 hr. (–) = No inhibition.

Abbreviations: DFO-B, deferroxamine-B; Exo-MS, exochelin-MS; lpm, imipenem; Mrp, meropenem.

Table S4 Susceptibility of metallo- β -lactamase-producing *Acinetobacter baumannii* isolates to siderophores and antibiotics

Isolate no.	Zone of inhibition (diameter in mm)										
	Amp	Amp + DFO-B	Amp + Exo-MS	lpm	lpm + DFO-B	lpm + Exo-MS	DFO-B	Exo-MS	Mrp	Mrp + DFO-B	Mrp + Exo-MS
1	–	–	–	–	23	15	–	–	15	15	25
2	–	–	24	15	15	29	–	–	14	21	14
3	–	19	19	15	23	14	25	–	10	32	30
4	–	24	–	15	15	15	15	–	15	50	50
5	–	–	–	–	16	31	–	–	12	13	14
6	–	–	–	15	13	14	–	–	13	23	23
7	22	35	30	–	38	12	–	–	15	28	28
8	12	12	14	14	30	28	–	–	13	25	30
9	–	–	15	13	13	13	–	–	12	11	14
10	16	23	23	12	22	10	–	–	–	30	33
11	20	–	15	8	38	25	–	–	–	20	26
12	–	–	–	20	10	10	–	–	10	35	35
13	–	11	10	15	28	30	–	–	15	27	19
14	10	11	13	40	40	40	–	–	10	10	32
15	–	–	–	9	–	6	–	–	–	–	–

Note: Zone of inhibition on Mueller–Hinton agar plates incubated at 37°C for 24 h. (–), No inhibition.

Abbreviations: DFO-B, deferroxamine-B; Exo-MS, exochelin-MS; lpm, imipenem; Mrp, meropenem.

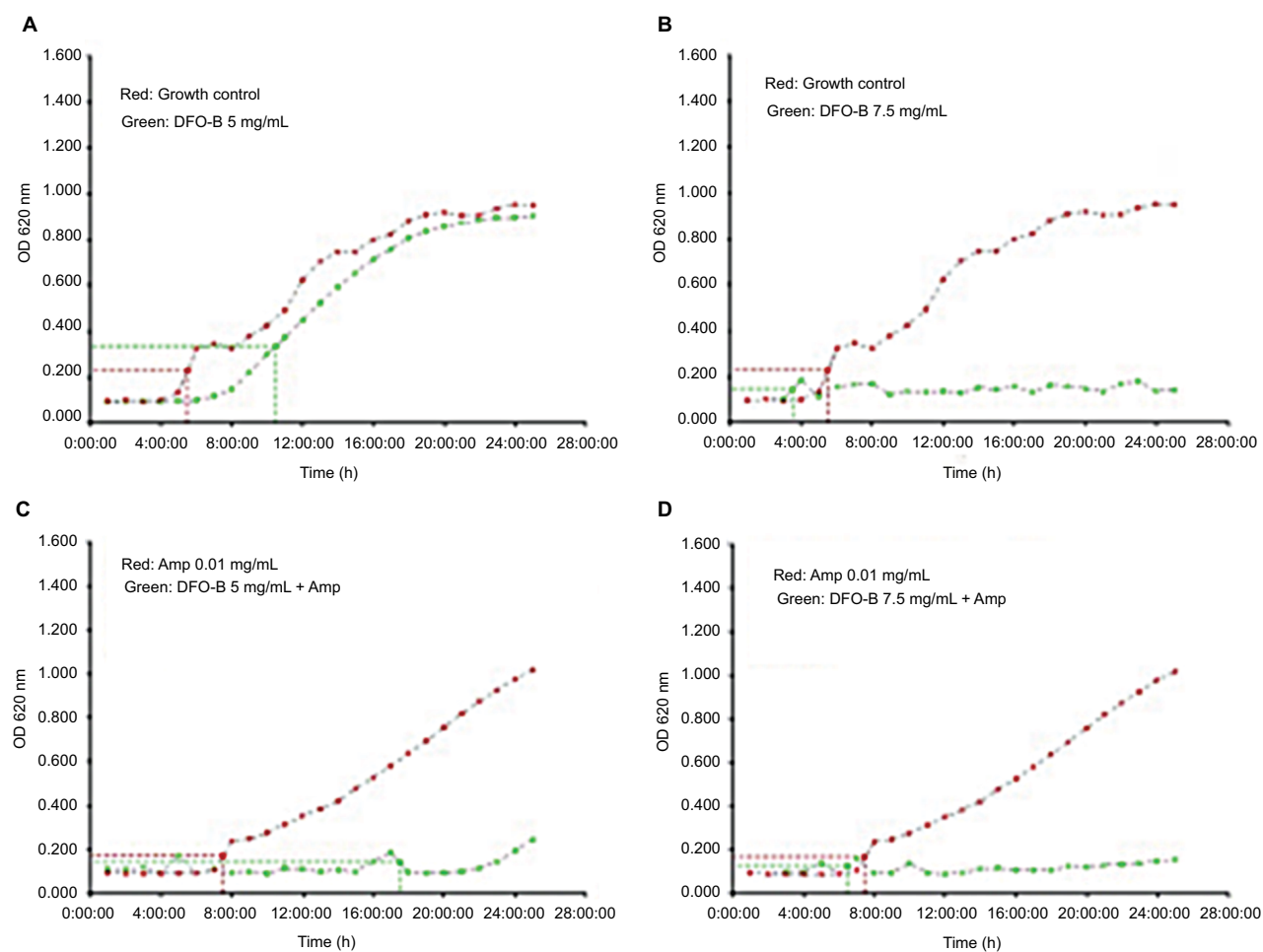


Figure S1 Growth curves of methicillin-resistant *Staphylococcus aureus* in the presence of DFO-B.

Notes: (A) and (B) plot in red: growth curves of the isolate in medium alone and plot in green show curves in the presence of DFO-B at 5 and 7.5 mg/mL, respectively. (C) and (D) plot in red: growth curves of the isolate in the presence of ampicillin 0.01 mg/mL and plot in green show curves in the presence of DFO-B at 5 and 7.5 mg/mL, respectively, along with ampicillin 0.01 mg/mL.

Abbreviations: Amp, ampicillin; DFO-B, deferrioxamine-B.

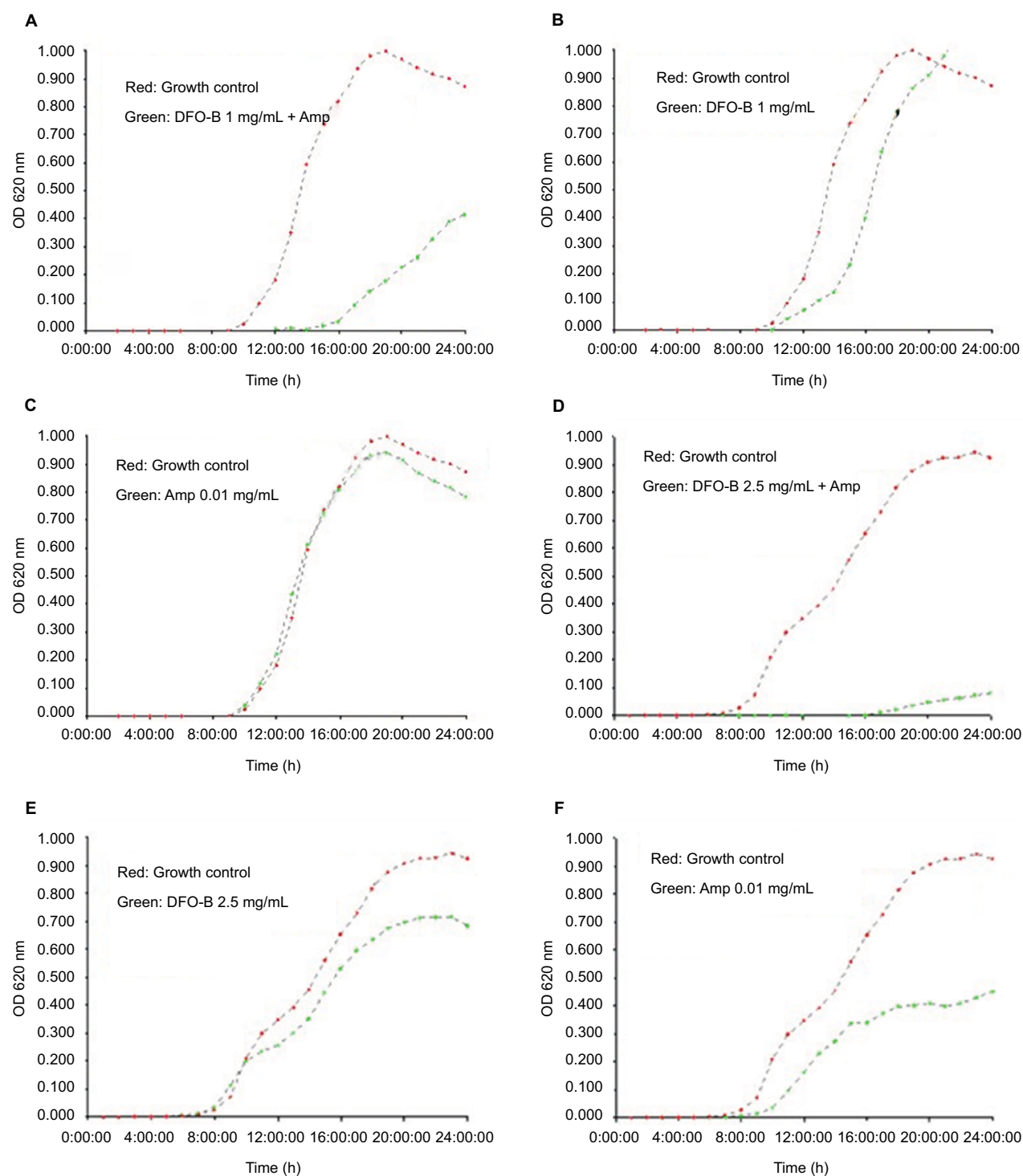


Figure S2 Growth curves of two isolates of *Pseudomonas aeruginosa* in the presence of DFO-B

Notes: Plot in red: growth curves of both the isolates in medium alone. Plot in green shows growth curves for Isolate 1: (A) green curves in the presence of DFO-B at 1 mg/mL + ampicillin 0.01 mg/mL, (B) only DFO-B at 1 mg/mL, and (C) only ampicillin 0.01 mg/mL. Plot in green shows growth curves for Isolate 2: (D) green curves in the presence of DFO-B 2.5 mg/mL + ampicillin 0.01 mg/mL, (E) only DFO-B at 2.5 mg/mL, and (F) only ampicillin 0.01 mg/mL.

Abbreviations: Amp, ampicillin; DFO-B, deferoxamine-B; OD, optical density.

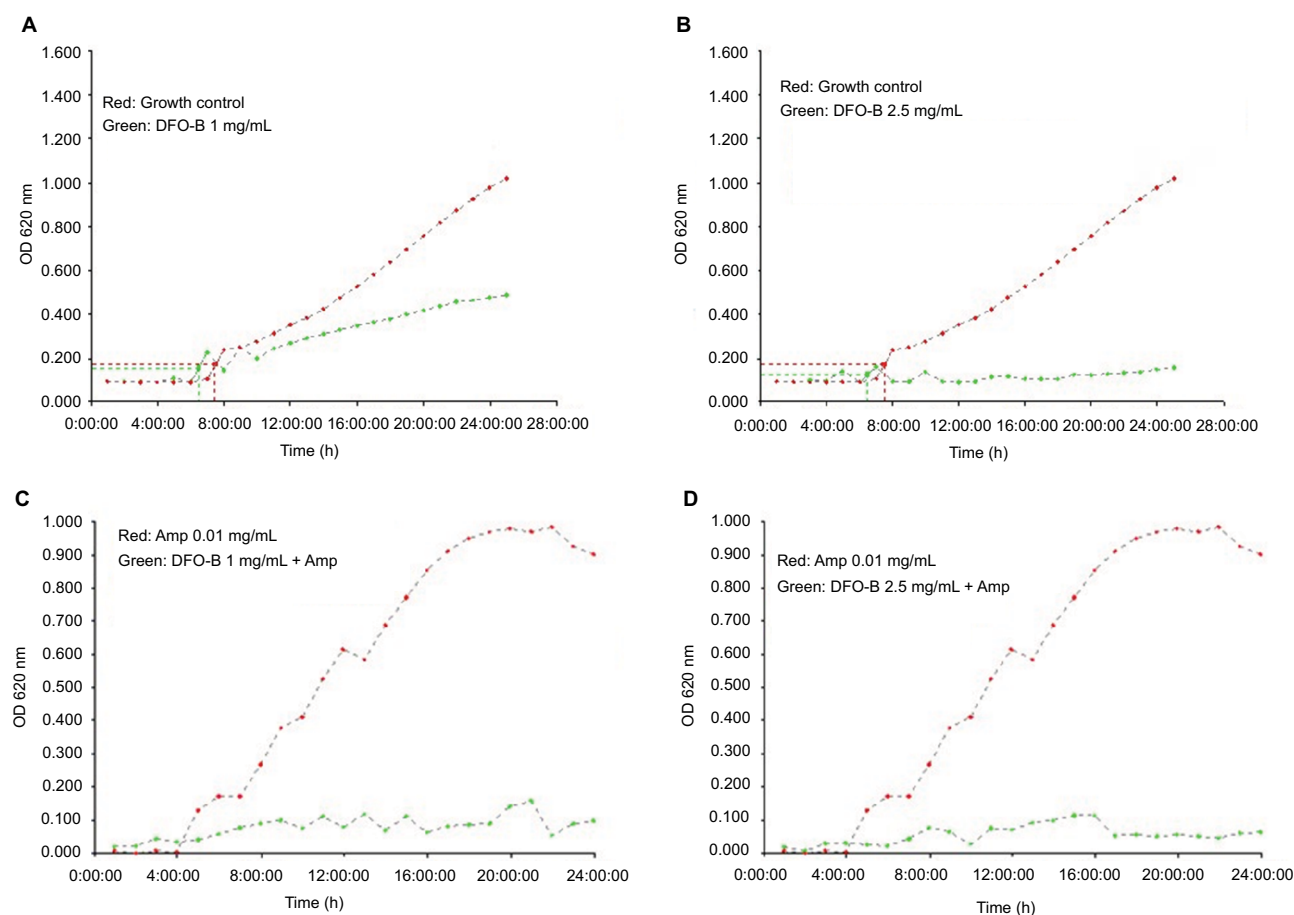


Figure S3 Growth curves of MBL-producing *Acinetobacter baumannii* in the presence of DFO-B

Notes: (A) and (B) plot in red: growth curves of the isolate in medium alone and plot in green shows curves in the presence of DFO-B at 1 and 2.5 mg/mL, respectively; (C) and (D) plot in red: growth curves of the isolate in the presence of ampicillin 0.01 mg/mL and plot in green shows curves in the presence of DFO-B at 2.5 and 1 mg/mL, respectively, in combination with ampicillin 0.01 mg/mL.

Abbreviations: Amp, ampicillin; DFO-B, deferoxamine-B; MBL, metallo- β -lactamase; OD, optical density.

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