

Isolation and molecular characterization of New Delhi metallo-beta-lactamase-1 producing superbug in Bangladesh

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Abstract

Introduction: New Delhi metallo-beta-lactamase-1 (NDM-1) producing superbugs create a global public health problem because of their resistance to most antibiotics. This study was conducted to determine the presence of MBL producers, including NDM-1 producers, in Bangladesh, along with the antimicrobial resistance patterns of these organisms.

Methodology: Thirty-five isolates resistant to imipenem by disk diffusion technique were investigated for MBL production. Minimum inhibitory concentration (MIC) of imipenem was determined by agar dilution method. MBL producers were phenotypically detected by double disk synergy test and combined disk assay. Gene encoding *blaIMP-1*, *blaIMP-2*, *blaVIM-1*, *blaVIM-2*, *blaNDM-1* and class 1 integron was identified by PCR.

Results: Thirty-one (88.57%) MBL producers were detected by PCR, 24 (68.57%) by double disk synergy test, and 30 (85.71%) by combined disk assay. Eight (22.86%) were positive for *blaNDM-1*, 13 (37.15%) for *blaVIM-1*, 21 (60.00%) for *blaVIM-2*, 18 (51.43%) for *blaIMP-1*, and 9 (25.71%) for *blaIMP-2*. More than one *blaMBL* was present in 23 (65.71%) of the isolates. MIC of imipenem of MBL producers ranged from ≥ 256 $\mu\text{g/ml}$ to ≤ 8 $\mu\text{g/ml}$. All the NDM-1 producing isolates carried class 1 integron. NDM-1 producers were 100% resistant to amoxicillin, cephadrine, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, aztreonam, gentamicin and piperacillin, 87.5% to amikacin, 75% to ciprofloxacin, and 62.5% to co-trimoxazole and the combination of tazobactam and piperacillin. All were sensitive to colistin.

Conclusion: The results of this study provide insight into the presence of *blaMBL*, including *blaNDM-1*, in Bangladesh. Urgent epidemiological monitoring of MBL producers in Bangladesh may combat their rapid dissemination.

Key words: metallo-beta-lactamase; New Delhi metallo-beta-lactamase-1; Bangladesh; molecular characterization; antimicrobial susceptibility patterns

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Introduction

The global dissemination of acquired metallo-beta-lactamases (MBLs) in Gram-negative bacteria is a major cause of microbial resistance to beta-lactam antibiotics. MBLs are alarming because of their ability to hydrolyze all beta-lactams except aztreonam [1]. Most of the transposable MBLs encoding genes are carried on integrons as cassettes which facilitate their rapid spread among organisms and confer resistance to both beta-lactam and other antimicrobial agents. MBL encoding genes have been detected from several Gram-negative bacilli belonging to the family *Enterobacteriaceae* and also in *Pseudomonas* species and *Acinetobacter* species [2]. Until now, multiple allelic variants of several MBLs have been described (<http://www.lahey.org/studies/>); VIMs and IMPs are the most frequent MBLs worldwide [3] and outbreaks of VIM and IMP type MBLs in the species of

Pseudomonas aeruginosa were reported from Greece, Italy, Korea and China [4-7]. Recently, a novel metallo-enzyme, New Delhi metallo-beta-lactamase-1 (NDM-1), was discovered in *K. pneumoniae* in a Swedish patient after treatment in a hospital in New Delhi, India [8] and it rapidly disseminated globally [9-12]. NDM-1 share only 32.4% amino acid sequence homology with the closely related VIM-1 / VIM-2 MBL producers and, with the exception of other MBL, transpose hastily to other bacteria by ISCR1 element via rolling circle replication [8]. Rapid worldwide expansion of NDM-1 producers now creates a public health problem because they remain extensively resistant to nearly all antibiotics except colistin and tigecycline [9-11].

Considering the public health threat the MBL producers pose and the rapid dissemination of *blaNDM-1* in bacteria, the search for the presence of

MBL producers, including NDM-1 producers, in Bangladesh seems worthy. We undertook this study to explore the incidence of multidrug resistant MBL producers, including NDM-1 producers, in Bangladesh along with antimicrobial resistant patterns of these organisms.

Methodology

Bacterial isolates

We conducted a cross-sectional study in the Department of Microbiology of Dhaka Medical College, Dhaka, Bangladesh, during 1 July 2010 to 30 June 2011. This research protocol was approved by the research review committee (RRC) and ethical review committee (ERC) of Dhaka Medical College. Thirty-five isolates resistant to imipenem by disk diffusion technique (according to Clinical and Laboratory Standards Institute [CLSI] guidelines) were investigated for MBL production in this study [13].

Identification of species among the imipenem resistant isolates

Samples collected from different sources were inoculated on MacConkey agar media and blood agar media. From the non-lactose fermenting colonies on MacConkey agar media, isolates were identified as *Pseudomonas aureuginosa* if they were oxidase positive, a triple sugar iron (TSI) agar reaction of alkaline over no change, motile, indole and urease negative in motility-indole-urea (MIU) agar media, citrate negative in simmons citrate agar media and grew at both 37° C and 42° C. Additional bacterial characteristics including its Gram stain, colony morphology, hemolytic criteria, and pigment production were also used to identify the species. Non-lactose fermenting colonies on MacConkey agar were identified as *Acinetobacter baumani* if they were Gram-negative coccobacilli, oxidase negative, non-motile, indole and urease negative, citrate positive and grew at 41° C and 44° C. *E. coli* and *K. pneumoniae* were isolated and identified on Gram stain, colony morphology, hemolytic criteria, pigment production and biochemical tests as previously described [14-15].

Antimicrobial susceptibility testing

Following CLSI guidelines, antimicrobial susceptibility pattern of MBL producers was performed by disk diffusion technique using commercially available antibiotic disks (Oxoid Ltd, Basingstoke, United Kingdom). *Escherichia coli* ATCC 25922 was used for quality control [13].

Minimum inhibitory concentration of imipenem

The minimum inhibitory concentration (MIC) of imipenem of 35 isolates was determined by agar dilution method [16]. To prepare imipenem stock solution, 500 mg imipenem powder was added to 50 ml distilled water. Next, 50 ml sterile Mueller-Hinton agar was impregnated with 40 µl, 80 µl, 160 µl, 320 µl, 640 µl and 1280 µl of imipenem stock solution to achieve concentrations of 8 µg/ml, 16 µg/ml, 32 µg/ml, 64 µg/ml, 128 µg/ml and 256 µg/ml per plate, respectively. To obtain 10⁴cfu/spot on the agar surface, one µl of 10 times diluted 0.5 McFarland turbidity of test inoculums were placed on Mueller-Hinton agar plates. After incubation at 35°C overnight, the lowest concentration of antibiotic impregnated Mueller-Hinton agar showing no visible growth on agar medium was considered the MIC of imipenem of that strain.

Detection of MBL producers by phenotypic methods

Double-disk synergy tests (DDS) [17] and combined disk (CD) assays [18] were performed to screen MBLs producers. For the DDS test, imipenem and a blank disk containing 20 µl of Tris-EDTA (1.0 M Tris – HCL, 0.1 M EDTA, pH approximately 8.0) and 20 µl of 1:320 diluted 2-mercaptopyruvic acid (MPA) were placed 10 mm apart in an inoculated Mueller-Hinton agar plate and incubated at 37°C for 24 hours. A clear extension of the edge of the inhibition zone of imipenem disk toward the Tris-EDTA-MPA disk was interpreted as MBLs production. For the CD assay, two imipenem disks were placed on an inoculated Mueller-Hinton agar plate. One imipenem disk was supplemented with 5 µl of 0.5 M EDTA and incubated at 37°C for 24 hours. An increased zone diameter of ≥ 6 mm around the disk containing imipenem supplemented with EDTA compared to the disk containing imipenem alone was interpreted as MBLs production.

Molecular characterization of MBL producers

The presence of *bla*NDM-1, *bla*IMP-1, *bla*IMP-2, *bla*VIM-1 and *bla*VIM-2 among the imipenem-resistant isolates was detected by polymerase chain reaction (PCR). Class 1 integron was also identified among the imipenem-resistant strains by PCR. To prepare bacterial pellets, a loop full of bacterial colonies was inoculated into a Falcon tube containing trypticase soy broth. After incubation overnight at 37°C, the Falcon tubes were centrifuged at 4,000 rpm for 10 minutes, after which the supernatant was discarded. A small amount of sterile trypticase soy

broth was added into the Falcon tubes with pellets and mixed evenly. Then an equal amount of bacterial suspension was placed into 2 to 3 Eppendorf tubes. The Eppendorf tubes were then centrifuged at 4,000 rpm for 10 minutes and the supernatant was discarded. The Eppendorf tubes containing bacterial pellets were kept at -20°C until DNA extraction. Bacterial DNA was extracted by the boiling method [19]. The following pairs of previously used primers were used to yield PCR products: for *bla*NDM-1- ACC GCC TGG ACC GAT GAC CA (forward), GCC AAA GTT GGG CGC GGT TG (reverse), for *bla*IMP-1- TGAGCAAGTTATCTGTATTC (forward), TTAGTTGCTTGGTTTTGATG (reverse), for *bla*IMP-2- GGCAGTCGCCCTAAAACAAA (forward), TAGTACTTGGCTGTGATGG (reverse), for *bla*VIM-1- TTATGGAGCAGCAACCGATGT (forward), CAAAAGTCCCGCTCCAACGA (reverse), for *bla*VIM-2- AAAGTTATGCCGCACTCACC (forward), TGCAACTTCATGTTATGCCG (reverse) and for class 1 integron- GGC ATC CAA GCA GCA AGC (forward), AAG CAG ACT TGA CCT GAT (reverse) [19-21]. The following cycling parameters were used: initial denaturation at 95°C for 10 minutes, then 30 cycles of denaturation at 95°C for one minute, annealing at 63°C (for *bla*NDM-1), 57°C (for *bla*IMP-1), 60°C (for *bla*IMP-2), 60°C (for *bla*VIM-1), 59°C (for *bla*VIM-2) and 58°C (for class 1 integron) for 45 seconds, extension at 72°C for one minute and 30 seconds, and a final extension at 72°C for 10 minutes. The amplified DNA were loaded into a 1.5%

agarosegel, electrophoresed at 100 volts for 35 minutes, stained with 1% ethidium bromide, and visualized under UV light.

Statistical analysis

Data were analyzed by using Microsoft Excel (2007) software (Microsoft, Redmond, WA, USA).

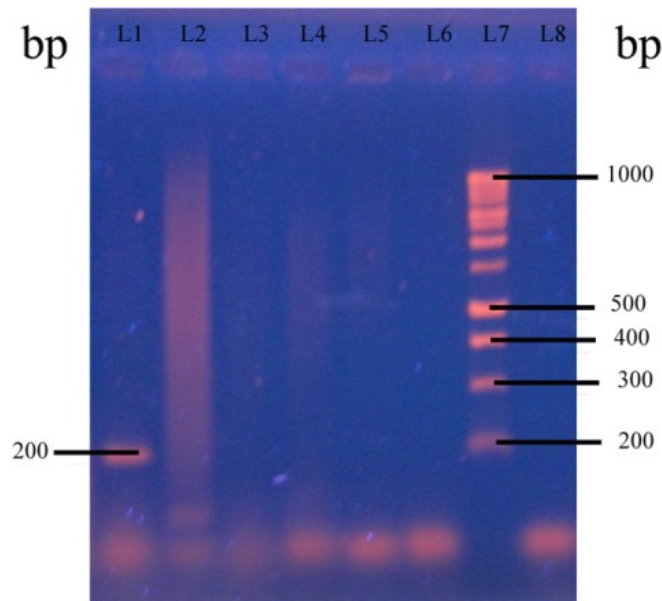
Results

A total of 35 imipenem-resistant strains by disk diffusion technique were included in this study. Fifteen *Acinetobacterbaumanni*, sixteen *Pseudomonas aeruginosa*, three *E. coli* and one *K. pneumonia* were isolated from the imipenem-resistant organisms. The MIC of imipenem of these isolates ranged from ≥ 256 µg/ml to ≤ 8 µg/ml (Figure).

Thirty-one (88.57%) MBL producers were detected by PCR, of which 8 (22.86%) were positive for *bla*NDM-1, 13 (37.15%) for *bla*VIM-1,23 (65.71%) for *bla*VIM-2, 18 (51.43%) for *bla*IMP-1 and 9 (25.71%) for *bla*IMP-2. All (100%) imipenem-resistant *Acinetobacter baumannii*, 14 (87.50%) of the 16 *Pseudomonas aeruginosa*, one (33.33%) of the 3 *E. coli* and the only *K. pneumoniae* were positive for *bla*MBL by PCR. Twenty-three (65.71%) isolates carried more than one MBL gene, 8 (22.86%) carried a single MBL gene, and 4 (11.43%) had no MBL gene.

The DDS tests and CD assays detected 24 (68.57%) and 30 (85.71%) MBL producers, respectively, among the 35 imipenem-resistant isolates. Out of the four negative amplified PCR products, three were positive by DDS test and all were

Figure. PCR detection of *bla*NDM-1 gene among 7 imipenem resistant strains



Hundred bp DNA ladder (lane 7), amplified DNA of 200 bp for *bla*NDM-1 (lane 1) and negative control (lane 5)

positive by CD assay. Sensitivity of the phenotypic methods considering PCR as gold standard was 67.74% for DDS test and 83.87% for CD assay.

Twenty-three (74%) of the MBL-producing isolates were positive for class 1 integron and 3 (75%) of the 4 non-MBL producers had class 1 integron. All the NDM-1 producing isolates had the class 1 integron (Table 1).

The combinations of different genes in single strains were observed. Table 1 shows that, among them, *blaVIM-2 + blaIMP-1 + class 1 integron* was the predominant variety, which was present in four (11.43%) of the isolates. *blaNDM-1* was present with other *blaMBLs* and class 1 integron in different combinations.

Table 2 illustrates MIC of imipenem among different species of MBL producers. Isolates that were negative for MBLs production by PCR had ≥ 256 $\mu\text{g/ml}$ MIC of imipenem for three (75%) isolates and 128 $\mu\text{g/ml}$ for one (25%) isolate.

All the MBL producers were 100% resistant to amoxicillin, cephradine, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, gentamicin and piperacillin, 96.77% to amikacin, 93.55% to ciprofloxacin, 87.09% to co-trimoxazole, 80.64% to the combination of tazobactam and piperacillin, and 67.74% to aztreonam. All the MBL-producing isolates were sensitive to colistin.

Resistance to antimicrobials among the NDM-1 producing isolates was 100% for amoxicillin,

Table 1. Distribution of *blaMBLs* and class 1 integron among the imipenem resistant strains (n = 35)

Genotypes	Number amplified	Organisms
<i>blaVIM-2 + blaIMP-1</i>	2 (5.71%)	<i>A. baumannii</i> (1) <i>P. aeruginosa</i> (1)
<i>blaVIM-1 + blaVIM-2</i>	1 (2.86%)	<i>A. baumannii</i> (1)
<i>blaVIM-2 + blaIMP-2</i>	1 (2.86%)	<i>P. aeruginosa</i> (1)
<i>blaVIM-1 + class 1 integron</i>	1 (2.86%)	<i>P. aeruginosa</i> (1)
<i>blaVIM-2 + class 1 integron</i>	2 (5.71%)	<i>A. baumannii</i> (1) <i>P. aeruginosa</i> (1)
<i>blaNDM-1 + class 1 integron</i>	2 (5.71%)	<i>P. aeruginosa</i> (1) <i>A. baumannii</i> (1)
<i>blaVIM-2 + blaIMP-1 + blaIMP-2</i>	1 (2.86%)	<i>A. baumannii</i> (1)
<i>blaVIM-2 + blaIMP-1 + class 1 integron</i>	4 (11.43%)	<i>A. baumannii</i> (1) <i>P. aeruginosa</i> (3)
<i>blaVIM-1 + blaIMP-1 + class 1 integron</i>	2 (5.71%)	<i>A. baumannii</i> (2)
<i>blaVIM-2 + blaNDM-1 + class 1 integron</i>	1 (2.86%)	<i>P. aeruginosa</i> (1)
<i>blaIMP-1 + blaIMP-2 + class 1 integron</i>	1 (2.86%)	<i>P. aeruginosa</i> (1)
<i>blaVIM-1 + blaVIM-2 + blaIMP-1 + class 1 integron</i>	3 (8.57%)	<i>P. aeruginosa</i> (1) <i>A. baumannii</i> (2)
<i>blaVIM-2 + blaIMP-1 + blaIMP-2 + class 1 integron</i>	2 (5.71%)	<i>A. baumannii</i> (2)
<i>blaVIM-1 + blaVIM-2 + blaNDM-1 + class 1 integron</i>	1 (2.86%)	<i>K. pneumonia</i> (1)
<i>blaVIM-1 + blaVIM-2 + blaIMP-2 + blaNDM-1 + class 1 integron</i>	2 (5.71%)	<i>A. baumannii</i> (1) <i>P. aeruginosa</i> (1)
<i>blaVIM-1 + blaVIM-2 + blaIMP-1 + blaIMP-2 + blaNDM-1 + class 1 integron</i>	2 (5.71%)	<i>A. baumannii</i> (2)
<i>blaVIM-1</i>	1 (2.86%)	<i>P. aeruginosa</i> (1)
<i>blaVIM-2</i>	1 (2.86%)	<i>P. aeruginosa</i> (1)
<i>blaIMP-1</i>	1 (2.86%)	<i>E. coli</i> (1)
Class 1 integron	3 (8.57%)	<i>E. coli</i> (1) <i>P. aeruginosa</i> (2)
Absence of examined genes	1 (2.86%)	<i>E. coli</i> (1)

* Figures in parentheses in the last column indicate number of isolates.

Table 2. MIC of imipenem among MBL producers

MBL producers	MIC of imipenem ($\mu\text{g/ml}$)					
	≥ 256	128	64	32	16	≤ 8
<i>A. baumannii</i> (n = 15)	6 (40.00)	0 (0.00)	2 (13.33)	5 (33.33)	1 (6.67)	1 (6.67)
<i>P. aeruginosa</i> (n = 14)	4 (28.58)	3 (21.42)	4 (28.58)	1 (7.14)	1 (7.14)	1 (7.14)
<i>E. coli</i> (n = 1)	-	1(100.00)	-	-	-	-
<i>K. pneumoniae</i> (n = 1)	1 (100.00)	-	-	-	-	-
Total (n = 31)	11 (35.48)	4 (12.90)	6 (19.35)	6 (19.35)	2 (6.46)	2 (6.46)

* Figures in parentheses represent percentage.

cephradine, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, aztreonam, gentamicin and piperacillin, 87.5% for amikacin, 75% for ciprofloxacin, and 62.5% for co-trimoxazole and the combination of tazobactam and piperacillin.

Discussion

Acquired MBLs in Gram-negative bacteria are becoming an emerging resistant determinant worldwide [1]. The continuous monitoring and rapid detection of these virulent organisms may check their spread and play a vital role in infection control. To address this rising resistant determinant, we have observed the distribution of MBL encoding genes among the imipenem-resistant isolates in Bangladesh. The present study identified 31 (88.57%) MBL producers out of the 35 imipenem-resistant bacteria. All the imipenem resistant *Acinetobacter baumannii*, 87.50% of the *Pseudomonas aeruginosa*, 33.33% of the *E. coli* and the only *K. pneumoniae* had MBL genes. Though acquired MBL encoding genes are frequently found in *Pseudomonas* spp. and *Acinetobacter* spp., the existence of MBL encoding genes in the species of *E. coli* and *K. pneumoniae* in this study suggests that plasmid-mediated horizontal transfer of the MBL genes occurs continuously among Gram-negative bacilli, as reported previously [2].

Interestingly, most of the cases from Europe, America, and Australia had a history of recent travel or hospital admission in the Indian subcontinent. NDM-1 producers are now alarmingly rising worldwide and pose potential therapeutic failures with current empirical treatments in place [9-11]. The results of the current study reflect the presence of NDM-1 producers in Bangladesh. Inappropriate and non-prescription antibiotic use might be the probable cause of development of high antimicrobial resistance in this subcontinent [22]. In addition, with NDM-1

producers, the current study found 13 (37.15%) VIM-1, 23 (65.71%) VIM-2, 18 (51.43%) IMP-1 and 9 (25.71%) IMP-2 producers among the imipenem-resistant bacteria. The presence of VIM-1, VIM-2, IMP-1 and IMP-2 in *Pseudomonas* spp., *Acinetobacter* spp., and the members of *Enterobacteriaceae* has been described previously [23-28]. The findings of the present study indicate the dissemination of *bla*MBL-carrying organisms in Bangladesh.

The present study observed 23 (74%) class 1 integron-carrying MBL producers. Most of the acquired *bla*MBLs were the part of integron and resided in large transmissible plasmids [29]. In agreement with the present findings, earlier studies observed the presence of the class 1 integron with *bla*MBLs [30-31]; the clonal relatedness of three VIM-1 producing *Pseudomonas* spp. isolated from three different hospitals, along with the observed presence of specific genotyped integrons within them suggest that they share a common ancestry [31]. Therefore, the presence of conserved integron associated with *bla*MBLs in epidemiologically unrelated strains indicates the possibility of worldwide dissemination. Though genome sequencing was not performed in this study, the existence of class 1 integron in MBL producers suggests the probable association among them. In this study, all the NDM-1 producers had class 1 integron. The results of a previous study that observed the presence of *bla*NDM-1 and *ISCR1* suggest that *bla*NDM-1 and *ISCR1* have the same genetic structure and that the strain also has class 1 integron [9]. However, the association between *bla*NDM-1 and the class 1 integron or *ISCR1* elements was not ascertained in this study.

A survey in rural Bangladesh demonstrated multiple transferable resistances in enteric flora [32], which may serve as reservoirs of antimicrobial resistance among the enteric pathogens. The present

study observed the presence of more than one MBL gene in 23 (65.71%) imipenem-resistant isolates. As acquired *bla*MBLs have the ability to transfer to other organisms via the integrons located in plasmids or transposons [29], reserved resistance determinants might relocate to other plasmids or other bacteria, explaining the existence of more than one antimicrobial resistance mechanism in a single strain.

The sensitivity of the DDS tests and CD assays for bacterial MBLs detection was 67.74% and 83.87%, respectively, considering PCR as the gold standard. As the zinc chelators also diminish the action of OXA carbapenemase [33], phenotypic methods might provide positive inference among the negative amplifiers. However, screening of OXA carbapenemase was not performed in this investigation.

In this study, among the MBL producers, the MIC of imipenem ranged from ≥ 256 $\mu\text{g/ml}$ to 8 $\mu\text{g/ml}$ (Table 2), which was similar to the findings of earlier studies [34-35]. The discrepancy in the level of imipenem resistance for MBL producers might be due to variations in the permeation capacity of imipenem through bacterial outer membranes [36]. In our study, four (11.53%) of the imipenem-resistant isolates showed negative amplification by PCR. The MIC of these strains was ≥ 256 $\mu\text{g/ml}$ for three (75%) isolates and 128 $\mu\text{g/ml}$ for one (25%) isolate. The high imipenem resistance among the non-MBL producers in this study might be due to resistance mechanisms other than MBL production [37]. However, the presence of *bla*MBLs other than the examined MBL genes among these strains also might be the reason behind it.

In this study, MBL producers were viewed as multidrug resistant. Selective pressure and/or the simultaneous presence of other drug resistance genes such as gene cassettes or other resistance mechanisms might be the reason for the co-resistance [2, 38-39]. High doses of aztreonam or tazobactam-piperacillin cure rats from experimental pneumonia by reducing MBL-producing strains [40]. But in this study, 67.74% of the MBL producers were resistant to aztreonam and 80.64% to the combination of tazobactam-piperacillin. A previous study demonstrated the overexpression of efflux genes and AmpC coupled with down-regulation of *oprD* in respect to antimicrobial resistance, and found the association of *bla*MBLs with efflux genes and/or AmpC β -lactamase genes, describing this resistance among the MBL producers [39]. The use of the most sensitive antibiotic, colistin, is limited due to its neurotoxicity and nephrotoxicity [41]. However,

very recently reversible colistin-induced nephrotoxicity was reported in carbapenem-resistant bacterial infection [42]. It is necessary to evaluate *in vitro* activities of two or more antimicrobial agents against MBL producers.

Conclusion

The high incidence of MBLs in our study among imipenem-resistant Gram-negative organisms highlights the emerging therapeutic challenge in Bangladesh. Early detection of this resistance mechanism, implementation of strict antimicrobial policies and infection control programs may avoid the rapid dissemination of these organisms.

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