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Carbapenem resistant *Klebsiella pneumoniae* isolated from bloodstream infection: Indian experience

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ABSTRACT

Increased incidence of multidrug resistant (MDR) Gram negative infection has resulted in high rates of morbidity and mortality. *Klebsiella pneumoniae* is one of the commonest MDR pathogens causing bacteraemia with limited therapeutic options such as colistin and tigecycline. Present study focused on molecular characterisation of MDR *K. pneumoniae* from bloodstream infection and their clinical outcome. A total of 115 *K. pneumoniae* from January 2015 to September 2016 were included in the study which comprised of phenotypically identified ESBL and carbapenem resistant (CR) isolates. Multiplex PCR was performed for detection of resistance genes encoding β-lactam resistance. This includes bla_{SHV}, bla_{TEM}, bla_{VEB}, bla_{PER}, bla_{CTX-M}, bla_{DHA}, bla_{CIT}, bla_{FOX}, bla_{ACC}, bla_{ACT}, bla_{NDM}, $bla_{OXA48-like}$, bla_{VIM} and bla_{KPC}. Co-expression of bla_{SHV}, bla_{TEM} and bla_{CTX-M} was predominant with 64% (74/115) prevalence. CTX-M-1 was the variant produced by all the isolates producing CTX-M. AmpC was uncommon, seen in 5% of the isolates (6/115). Among the carbapenemases co-expression of bla_{NDM} and $bla_{OXA48-like}$ was observed in 28% (32/115) and bla_{NDM} in 19% (22/115) and $bla_{OXA48-like}$ in 13% (15/115). bla_{KPC} was absent. Overall mortality was observed to be 57% (64/113) and mortality among CR *K. pneumoniae* (Kp) was 68% (50/73). The antibiotics that were administered for treatment of CRKp were colistin in 90% (66/73) and tigecycline in 7% (5/73) and in 99% combined with meropenem (72/73). Prevalence of community acquired and nosocomial infections were 5% (4/73) and 95% (69/73) respectively among CRKp. Minocycline and meropenem susceptibilities were comparable and hence minocycline can be a carbapenem sparing agent. The resistance to β-lactam antibiotics is steadily increasing and are plasmid mediated, their containment in healthcare setting is a challenge.

Introduction

The incidence of infections with extended spectrum β-lactamase (ESBL) producing Gram negative *Enterobacteriaceae* has steadily increased in nosocomial setting and is associated with significant morbidity and mortality. This situation has been worsened further by the emergence of carbapenemase producing strains that render carbapenem therapy suboptimal. *Klebsiella pneumoniae* and *Acinetobacter baumannii-calcoaceticus* complex (Abcc) are the most common multidrug resistant pathogens, and are frequently associated with a bacteremic illness. Other organisms are not as frequently reported to be resistant to commonly used antimicrobials as *K. pneumoniae* and *Acinetobacter* spp.

According to the Centre for Disease Dynamics, Economics and Policy (CDDEP), 80% of the Indian *K. pneumoniae* isolates are resistant to cephalosporins and up to 60% resistant to carbapenems. A review of data from our centre reveals a carbapenem resistance of 32 and 73% amongst *K. pneumoniae* and Abcc isolates respectively. India has witnessed a rise in carbapenem resistance rates from 9% in 2008 to 44% in 2010 [1]. In Italy, the prevalence of carbapenem resistant (CR) *K. pneumoniae* isolates, non-existent in 2008, has risen to 60% in 2013 (CDDEP). This rapid rise in carbapenem resistance reflects a worrisome trend.

Currently the *Klebsiella pneumoniae carbapenemase* (KPC), an Ambler class-A beta-lactamase is the most globally prevalent carbapenem resistance enzyme and is particularly widespread in Europe and North America. The New Delhi metallo-β-lactamase (NDM), an Ambler class-B enzyme, is the main cause of carbapenem resistance in India and has now spread across the globe [2]. This is slowly being accompanied by an increased incidence of the OXA48- like group, Ambler class D enzyme, in India [3–5].

Carbapenem resistant (CR) *K.pneumoniae* infections are associated with mortality rates as high as 30 to 44% [6–9]. Ben-David et al., report a 22 and 48% infection related mortality rate amongst patients infected with

ESBL and CR *K. pneumoniae* respectively [10]. In this context, a clear understanding of the prevalent mechanisms for carbapenem resistance is essential. Despite a high disease burden, reports from India, on the prevalence of resistance mechanisms in multi drug resistant *K. pneumoniae* isolates are limited. The objective of this study is molecular characterization of the enzymatic mechanisms of resistance to β-lactam antibiotics in *K. pneumoniae* blood stream isolates.

Materials and methods

Isolates and phenotypic characterisation

A total of 115 *K. pneumoniae* blood culture isolates from January 2015 to September 2016 at the department of Clinical Microbiology, Christian Medical College, Vellore, were included in the study. Semiautomated BacT/ALERT system (Biomerieux) was used for blood culture. The isolates were identified using standard culture and biochemical reactions [11]. Duplicate isolates from the same patient were excluded from the study.

Antimicrobial susceptibility testing for different classes of antimicrobials such as cephalosporins - cefotaxime (30 μg), ceftazidime(30 μg); β-lactam/β-lactamase inhibitors - piperacillin/tazobactam(100/10 μg), cefoperazone/sulbactam (75/30 μg); carbapenems- imipenem(10 μg), meropenem (10 μg); fluoroquinolones- ciprofloxacin (5 μg), levofloxacin (5 μg), aminoglycosides- amikacin (30 μg), gentamicin (10 μg), tetracycline- minocycline (30μg), glycylcycline- tigecycline (15μg), was performed for all the isolates by Kirby Bauer disk diffusion method as recommended by Clinical Laboratory Standards Institute (CLSI) and interpreted according to CLSI 2015 guidelines [12,13] except for cefoperazone/sulbactam interpreted according to manufacturer, Pfizer (http://www.thefilipinodoctor.com/brand_pdf/Sulperazone.pdf) and tigecycline interpreted as per FDA breakpoints (http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/021821s016lbl.pdf). ATCC *E. coli* 25922 was used as the control strain for susceptibility testing. The isolates were classified as ESBL producers if the zone diameter for ceftazidime was ≤22 mm and ≤27 mm for cefotaxime. They were screened for carbapenem resistance using meropenem and were included in the study if the zone diameter for meropenem was ≤19 mm.

In-house CarbaNP test as recommended by CLSI for the detection of carbapenemase activity was performed for a subset of randomly selected 73 isolates that were phenotypically resistant to imipenem and meropenem.

Genotypic characterisation

Bacterial DNA was extracted from 18 h cultures by Qiasymphony as per manufacturer's instructions. Multiplex PCR was performed for the detection of genes encoding beta-lactam resistance which includes EBSL, CTX-M, ampC and carbapenemases. Multiplex PCR for EBSL genes included bla_{SHV}, bla_{TEM}, bla_{PER}, bla_{VEB}, and bla_{GES} and was performed as previously described [14]. Multiplex PCR for CTX-M genes such as $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-8}$, $bla_{CTX-M-9}$ and $bla_{CTX-M-25}$ was performed according to Woodford et al. [15]. Multiplex PCR for AmpCβ-lactamases including bla_{ACT}, bla_{ACC}, bla_{CIT}, bla_{DHA} and bla_{FOX} was performed as previously described [16]. Carbapenemase genes detected by multiplex PCR were bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{SPM}, $bla_{OXA\ 48-like}$, and bla_{KPC} and was performed as previously described [14,17–19]. Known positive controls for each gene was used with every run (courtesy: IHMA, USA).

Whole genome sequencing for a subset of eleven isolates was performed using Ion Torrent PGM platform with 400 bp chemistry. The Raw reads were assembled *de novo* using Assembler SPAdes software v5.0 in Torrent suite server version 4.4.3. Annotation of the genomes was performed with RAST (Rapid Annotation using Subsystems Technology- http://rast.nmpdr.org/) and PATRIC (Pathosystems Resource Integration Centre - https://www.patricbrc.org/). The genomes were submitted to GenBank to obtain accession numbers. Using the annotated whole genome sequences (WGS), the presence of any antibiotic resistance genes was detected from the ResFinder version 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/).

Clinical data collection

Data from eligible subjects were retrospectively collected using a systematic data abstraction form. Patient data included age, gender, admission date, discharge date or date of death and admission diagnosis. Data was entered in software EpiData Entry v 3.0 to rule out errors such as repeat entry. *K. pneumoniae* isolates were classified into community acquired vs. nosocomial based on Centers for Disease Control and Prevention (CDC) guidelines for the same. Antibiotic susceptibility profiles and dates for all positive blood cultures were recorded. For each antibiotic, the dose, frequency and duration were documented. In bacteremic patients repeat blood cultures following the index blood cultures were recorded to document microbiological clearance. Mortality data at hospitalization and 90 days post-discharge were also recorded. The data was analysed using EpiData Analysis software v 3.0.

Results

Susceptibility profile of *K. pneumoniae* from blood culture isolated during 2015 and 2016 is shown in Figure 1. During 2016, the susceptibility of most antimicrobials are comparable except for imipenem, meropenem, minocycline and tigecycline. Carbapenem susceptibility has reduced from 80 to 68% and minocycline to
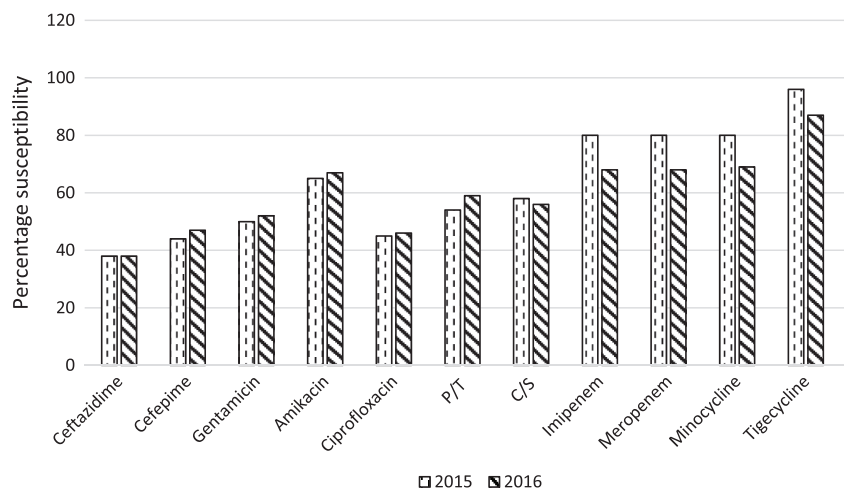
Figure 1. Antimicrobial susceptibility of *K. pneumoniae* from bloodstream infections at CMC.
P/T: Piperacillin/tazobactam; C/S: Cefoperazone/sulbactam.

69%. Susceptibilities of carbapenems and minocycline seem to be comparable. Susceptibility to tigecycline has reduced from 96 to 87% from 2015 to 2016.

CarbaNP test was found to be positive in 86% (*n* = 63/73) of *K. pneumoniae* tested. The results of multiplex PCR for β-lactamases are mentioned in Table 1. Among the ESBLs, 64% of the isolates showed co-expression of bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes. All the isolates tested for bla_{CTX-M} showed the presence of $bla_{CTX-M-1}$. AmpC was uncommon and seen in 5% of the isolates (6/115) which expressed bla_{DHA} and bla_{CIT}. Co-expression of bla_{NDM} and $bla_{OXA-48 like}$ was seen in 28% of the isolates. The bla_{KPC} gene was absent in the study isolates. Forty isolates that were phenotypically resistant to meropenem lacked carbapenemases.

From the WGS analysis, apart from confirming the PCR results, the variants of β-lactamase genes such as bla_{SHV}, bla_{TEM}, bla_{CTX-M}, bla_{NDM} and $bla_{OXA-48-like}$ were identified. The variants of SHV obtained were SHV-1, SHV-2, SHV-11 and SHV-12. TEM1A and TEM-1B were the only two variants of TEM observed in this study. CTX-M-15 was the only member of CTX-M-1 group seen in this study. Among the carbapenemases, NDM-1 was common and in the group of OXA48-like enzymes, OXA181 and OXA232 were commonly seen. The whole genome sequences were deposited at GenBank with accession numbers as follows: *MOXM00000000, MIEJ00000000, MPCT00000000, MDZG00000000, MOXL00000000, MEBR00000000, MOXN00000000, LZYN00000000, MCFO00000000, MCFP00000000* and *MCFQ00000000*. Plasmid mediated colistin resistance due to *mcr-1* and *mcr-2* was absent.

Clinical data from 113 patients were collected and analysed. The overall mortality rate was noted to be 57% (64/113) while the mortality rate in patients with CR *K. pneumoniae* infections was 68% (50/73). Antimicrobials used in patients with CR *K.pneumoniae infection* included colistin 90% (66/73) and tigecycline 7% (5/73). Colistin or tigecycline were not initiated in 2 patients since they

succumbed to their illness within 3 h of presentation, before the availability of blood culture reports. Colistin or tigecycline were administered in combination with meropenem in 99% (72/73) patients. Amongst patients with CR *K. pneumoniae* infections, 5% (4/73) had a community acquired infection while 95% (69/73) had a nosocomial infection. Nosocomial CR *K. pneumoniae* infections presented either as infective complications following chemotherapy for hematologic or solid organ malignancies 20% (14/69), or occurred in ICU patients as ventilator or central venous catheter related complications 80% (55/69).

Discussion

Globally, the highest rate of carbapenem resistance has been reported in Greece with 68% resistance followed by India and eastern Mediterranean regions with 54% resistance [20] as mentioned in Table 2. USA, China and Africa have low resistance rates with 11, 11 and 4% respectively. The predominant enzymatic mechanism of resistance in Europe is KPC followed by OXA-48-like and NDM [21]. While in USA, it is KPC followed by NDM and minimal due to OXA-48-like [21].

While in India, amongst the ESBLs, SHV, TEM and CTX-M have been commonly reported (Table 1). Similar to this study observation, co-existence of SHV, TEM and CTX-M in *Enterobacteriaceae* have been reported by Manoharan et al. and Goyal et al. [22,23]. However, the number of isolates included in these studies are relatively less. In India, carbapenem resistance is predominantly due to NDM and OXA-48-like and KPC is hardly seen [4,24,25]. Though OXA-48 like group of carbapenemases are emerging in India, most Indian studies, have been unable to capture this trend adequately (Table 1). It is essential for all the hospitals in the county to monitor the carbapenemases prevalent in their regions so that the change in trend from NDM to OXA-48 like group of enzymes can be demonstrated. VIM metallo-β-lactamase

Table 1. β-lactamases seen in K. pneumoniae as reported by various studies from India including present study.

Reference	ESBL			CTX-M	ESBL and CTX-M			bla_{DHA}	AmpC		Class A carbapenemase	Class B carbapenemase (MBLs)			Class D carabpenemase	Class B and D carbapenemase		
	bla_{SHV}	bla_{TEM}	$bla_{SHV,TEM}$	bla_{CTX-M}	$bla_{TEM,CTX-M}$	$bla_{SHV,CTX-M}$	$bla_{SHV,TEM,CTX-M}$	bla_{DHA}	bla_{CIT}	bla_{MOX}	bla_{KPC}	bla_{NDM}	bla_{VIM}	$bla_{NDM,VIM}$	$bla_{OXA-48-LIKE}$	$bla_{NDM,OXA-48\ LIKE}$	$bla_{VIM,OXA-48\ LIKE}$	$bla_{NDM,OXA-48LIKE,OXA-VIM}$
Veeraraghavan et al. (n = 115) (this study)	16% (n = 18)	0 (0)	1.7% (n = 2)	0	2.6% (n = 3)	5.2% (n = 6)	64.3% (n = 74)	4.3% (n = 5)	0.8% (n = 1)	0	0	19% (n = 22)	1.7% (n = 2)	3.4% (n = 4)	13% (n = 15)	28% (n = 32)	0	0
Goyal et al., 2009 [23] (n = 21)	14% (n = 3)	19% (n = 4)	0	10% (n = 2)	29% (n = 6)	10% (n = 2)	19% (n = 4)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sharma et al., 2010 [38] (n = 25)	72% (n = 18)	60% (n = 15)	0	0	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND
Manoharan et al., 2011 [22] (n = 61)	7% (n = 4)	0	5% (n = 3)	8% (n = 5)	15% (n = 9)	8% (n = 5)	43% (n = 26)	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND
Nagaraj et al., 2012 [24] (n = 36)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	75% (n = 25)	13.8% (n = 5)	0	ND	ND	0	0
Mohamudha et al., 2012 [39] (n = 109)	ND	ND	ND	ND	ND	ND	ND	17.4% (n = 19)	10% (n = 11)	1.8% (n = 2)	ND	ND	ND	ND	ND	ND	ND	ND
Krishnamurthy et al., 2013 [40] (n = 28)	36% (n = 10)	7% (n = 2)	0	43% (n = 12)	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Chaudhary et al., 2013 [41] (n = 150)	45.33% (n = 68)	47.33% (n = 71)	0	37.33% (n = 56)	0	0	0	ND	ND	ND	7.9% (n = 19)	6.25% (n = 24)	17.3% (n = 26)	0	ND	ND	0	0
Roy et al., 2015 [42] (n = 6)	0	0	0	0	33% (n = 2)	0	67% (n = 4)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Anandan et al., 2015 [25] (n = 88)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	34% (n = 30)	0	0	44% (n = 39)	16% (n = 14)	0	0
Pragasam et al., 2016 [4] (n = 85)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	24% (n = 20)	0	2.3% (n = 2)	55% (n = 47)	16% (n = 14)	0	0
Sharma et al., 2016 [3] (n = 134)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	27% (n = 36)	1% (n = 2)	1% (n = 2)	36% (n = 48)	15% (n = 20)	10% (n = 14)	4% (n = 6)

Table 2. Epidemiology of carbapenem resistance in K. pneumoniae [20,21].

Region	Resistant rates (%)	Mechanism of resistance
Africa	4	OXA-48-like, NDM
America (USA)	11	KPC, NDM, OXA-48-like
Western pacific region (Mongolia, China)	11	KPC, NDM, OXA-48-like
Eastern Mediterranean	54	NDM, OXA-48-like
India	54	NDM, OXA-48-like, KPC
Europe (Greece, Georgia, Italy)	68	KPC, OXA-48-like, NDM

producing isolates are also being increasingly reported from India, overall this study detected only two VIM producing isolates. While KPC is the predominant carbapenemase produced in Europe it has been rarely reported from India.

A recent study by Falagas et al. showed that mortality in critically ill patients with CR *K. pneumoniae* infections continued to exceed 60% irrespective of whether they were on a combination regimen or monotherapy [26]. The mortality rate in the study was also noted to be high (68%) despite patients being on a combination of colistin and meropenem. Due to this compelling need for newer antimicrobial therapy in CR *K. pneumoniae* infections, older drugs such as minocycline are also being evaluated. On analysing isolates obtained between August 2015 and July 2016, we found that amongst ESBL *K. pneumoniae* isolates the susceptibility to minocycline and tigecycline was 85 and 91% respectively. Amongst CR *K. pneumoniae* isolates the susceptibility to minocycline and tigecycline was 55 and 73% respectively [27]. Minocycline will be of great use for ESBL positive isolates and can be a carbapenem sparing agent while among CR isolates its usefulness is low. Since the susceptibilities of meropenem and minocycline were same for the *K. pneumoniae* isolated in the present study centre for the past two years, minocycline can be used as an alternate for carbapenems. There is a need for studies from other parts of the country for meropenem and minocycline susceptibilities which can enable a changes in national antibiotic policies.

Eight colistin resistant isolates were identified among the study isolates [28]. Three of these had significant mutations in the *mgrB* gene while one isolate had a mutation in *pmrB* and *mgrB* genes as previously reported. These genes are responsible for lipid A modifications leading to colistin resistance. One of the colistin resistant isolates with a high MIC did not have an identifiable mutation but *arnT* gene had novel mutations when compared to mutations seen in other seven. Two isolates though had mutations in many genes, none were previously reported and their significance is yet to be established [28]. Among the ESKAPE pathogens, colistin resistance is rising in *Klebsiella* spp. than the other organisms. Highest resistance to colistin among *K. pneumoniae* has been reported from Netherlands with 55% followed by Italy (43%) and Greece (20%) [29–31].

Earlier at the study centre, the novel β-lactam-β-lactam inhibitor combinations that are yet to be made available in the Indian market such as aztreonam-avibactam, ceftaroline-avibactam, ceftazidime-avibactam and ceftolozane-tazobactam were evaluated. Among these, susceptibility among the overall study isolates was found to be 84, 53, 58 and 53% for aztreonam-avibactam, ceftaroline-avibactam, ceftazidime-avibactam and ceftolozane-tazobactam respectively (unpublished data). Among the CR isolates, the susceptibility to aztreonam-avibactam, ceftaroline-avibactam, ceftazidime-avibactam and ceftolozane-tazobactam were 67, 17, 26 and 10% respectively (unpublished data). These combination drugs do not seem to be promising agents for the treatment of CRKp.

For CR isolates, tigecycline and colistin remain antimicrobials of choice. But with increased usage, resistance to tigecycline and colistin are rapidly emerging. From experience at the present study centre, the resistance to tigecycline has significantly increased from 2015 to 2016. However, in the present study, combination of meropenem and tigecycline was used for therapy only in 7% of the patients which is in contrast to meropenem-colistin combination being used in 90% of the patients. Though susceptibility to colistin remains about 98%, there has been emergence of plasmid mediated resistance [32,33] which has the potential to rapidly spread among many genera. With resistance to the last resort antibiotics, soon there will be a threat to management of infections especially in critically ill patients.

There is however, a paucity of data regarding the effectiveness of combination antimicrobial regimens when compared to monotherapy. This is primarily due to the fact that most of the existing evidence has been derived from retrospective data obtained from small patient numbers. In critically ill bacteremic patients with CR *K. pneumoniae*, evidence seems to suggest that a combination regimen may be associated with better survival than monotherapy alone. Three studies also show that meropenem addition to a colistin-tigecycline combination may result in a significant increase in survival, even in those patients who are infected with CR isolates, possibly due to potential colistin-carbapenem synergy [34–36]. The results of these studies however may be limited by small patient numbers.

Common combinations used in CR *K. pneumoniae* infections include tigecycline with colistin, tigecycline with gentamicin and carbapenem with colistin. Patients included in our study were predominantly treated with a combination of colistin with meropenem and with tigecycline-meropenem to a lesser extent. None were treated with monotherapy which limits the comparison of outcomes of monotherapy vs. combination therapy.

Among *K. pneumoniae*, there has been emergence of the hypervirulent strains which initially were prevalent among the isolates susceptible to antimicrobials.

Presently, they have been reported among MDR isolates [37], as also seen in the study centre, which further poses a challenge for treatment since virulence combined with antimicrobial resistance will be very worrisome leading to very high mortality rates.

Conclusion

Understanding the prevalent resistance mechanisms for β-lactam antibiotics is essential for individual treatment and hospital infection control. Steady increase in carbapenem and colistin resistance is seen in *K. pneumoniae* among the ESKAPE pathogens which is a worrisome condition. In this study, we predominantly see SHV, TEM and CTX-M-1 contributing to ESBLs while NDM and OXA48-like group are responsible for carbapenem resistance. Though previously uncommon, now OXA48-like group of carbapenemase is becoming widespread especially in the Indian setting. The mortality rate among the CRKp infections of 68% was similar to other studies reported worldwide. Colistin with meropenem was the most frequently used combination for treatment while tigecycline with meropenem was less frequent. But there is also simultaneous increase in resistance to colistin and tigecycline. Mostly infections in our setting were nosocomial in nature and management of MDR nosocomial infection is challenging. There is a need for better therapeutic options for CRKp infections. Among the β-lactam- β-lactam inhibitor combinations, aztreonam-avibactam is promising and minocycline can also be a potential carbapenem sparing agent.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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