Carbapenem and multidrug resistance in Gram-negative bacteria in a single centre in Italy: considerations on in vitro assay of active drugs

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ARTICLE INFO

Article history:

Received 15 April 2014

Accepted 16 April 2014

Keywords:

Acinetobacter baumannii

Klebsiella pneumoniae

Tigecycline

Colistin

Carbapenemase

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ABSTRACT

In intensive care units (ICUs), the most important causes of nosocomial bacterial infections are mainly multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains. Mortality related to these infection is very high due to lack of effective therapy and the severity of patient conditions. This study aimed to assess the prevalence of carbapenem resistance genes in 77 carbapenem-resistant Gram-negative bacteria isolated from severe infections (bloodstream, pulmonary and urinary tract) during the period 1 January to 31 July 2013 in a general ICU in Catania, Italy, and to examine their susceptibility to tigecycline and colistin using two different methods. In total, 52 *A. baumannii* belonging to the same sequence type (ST) 2 clone and carrying the *bla*$_{\text{OXA-23}}$ gene as well as 25 *K. pneumoniae* carrying *bla*$_{\text{KPC-3}}$ were isolated. Four distinct pulsotypes were identified in *K. pneumoniae*, which correlated with four distinct STs: ST258 and ST512, spread worldwide, and ST147 and ST395 detected for the first time in Italy. *Acinetobacter baumannii* isolates showed an XDR profile and were fully susceptible only to colistin; all KPC-producing *K. pneumoniae* isolated were MDR, whilst colistin was active against 19 of 25 strains. These results show that broth microdilution (BMD) is a reliable in vitro susceptibility test for colistin, above all *K. pneumoniae*, whilst both the gradient test and BMD are suitable for tigecycline susceptibility testing of *A. baumannii*.

1. Introduction

Gram-negative bacteria such as *Klebsiella pneumoniae* and *Acinetobacter baumannii* are among the most important causes of serious hospital-acquired bacterial infections for patients hospitalised in the intensive care unit (ICU). These Gram-negative bacilli are increasingly resistant to antibiotics and particularly to carbapenems, since these agents are often the last line of effective therapy available for the treatment of infections caused by multiresistant Gram-negative bacteria.

Acinetobacter baumannii is an important nosocomial pathogen associated with a wide range of infections, including respiratory tract, bloodstream, urinary tract and surgical site infections. Spread of *A. baumannii* between different patients in the hospital setting is difficult to control owing to the bacterium's ability to persist in the environment. Therefore, numerous nosocomial ICU outbreaks due to distinct clonal lineages of *A. baumannii* have been reported [1].

The carbapenemases described in *A. baumannii* include the metallo-β-lactamases (MBLs) (i.e. VIM, IMP, SIM and NDM types) and, more commonly, OXA type enzymes (i.e. OXA-23-like, OXA-24-like, OXA-51-like and OXA-58-like). Italian carbapenem-resistant *A. baumannii* (CRAB) diffusion is due to spread of the European clone II that has acquired OXA-23 alone or in association with OXA-58 [2,3].

Klebsiella pneumoniae carbapenemases (KPCs) are disseminated among nosocomial pathogens and have become the most frequent class A carbapenemases worldwide. KPC-producing *K. pneumoniae* have spread rapidly

and extensively in Italian hospitals, with a sharp increase reported by the Micronet surveillance network from 2% in 2009 to 19% in 2012 [4].

KPC-2 and -3 are the most common variants identified in Enterobacteriaceae: the KPC-3 variant, typically members of the sequence type 258 (ST258) lineage, is the predominant Italian clone and recently clinical isolates KPC-2 ST101 have appeared in a few areas [5].

Both CRAB and KPC-producing *K. pneumoniae*, causing high mortality among patients with bloodstream and pulmonary infections, have a multidrug-resistant (MDR) profile that evolves, increasingly, to an extensively drug-resistant (XDR) profile (susceptible only to colistin, tigecycline and one or more aminoglycosides) [6,7].

The objectives of this study were: (i) to analyse the spread and clonality of carbapenem-resistant *A. baumannii* and *K. pneumoniae* in a general ICU in Catania, Italy; (ii) to assess the actual prevalence of carbapenem resistance genes in the investigated isolates; and (iii) to examine susceptibility to colistin and tigecycline using two different methods, namely gradient test and microdilution (BMD), given the frequent difficulties of interpretation that are sometimes found for these molecules by the method of diffusion in agar.

2. Materials and methods

2.1. Study design

This study was performed from 1 January to 31 July 2013 in the ICU of Cannizzaro Hospital (Catania, Italy), a large emergency hospital of eastern Sicily.

In total, 77 carbapenem-resistant Gram-negative isolates responsible for severe infections were collected (52 *A. baumannii* and 25 *K. pneumoniae*) irrespective of patient source and clinical sample. Isolates were mainly obtained from lower respiratory tract infections as well as documented bloodstream infections and urinary tract infections. Isolates were collected by standard methods, isolated in pure culture on MacConkey agar plates and identified with API 20E for *K. pneumoniae* or API 20NE system for *A. baumannii* (bioMérieux, Marcy-l'Étoile, France).

2.2. Antimicrobial agents and minimum inhibitory concentration (MIC) determination

MIC determinations of the following antibiotics were performed by gradient test (Liofilchem, Roseto degli Abruzzi, Italy): meropenem; imipenem; ertapenem; piperacillin/tazobactam; ampicillin/sulbactam (SAM); amoxicillin/clavulanic acid; aztreonam; ceftazidime; cefotaxime; cefepime; amikacin; gentamicin; ciprofloxacin; trimethoprim/sulfamethoxazole (SXT); rifampicin; colistin; and tigecycline.

For tigecycline (Pfizer, Rome, Italy) and colistin sulfate (Sigma Chemical Co., St Louis, MO), MICs were also determined by the standard broth microdilution method.

Susceptibility and resistance categories were assigned according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [8]. Tigecycline breakpoints established by the US Food and Drug Administration (FDA) for Enterobacteriaceae (≤2/≥8 μg/mL for susceptible/resistant) were applied to *A. baumannii*. *Escherichia coli* ATCC 25922 was used as the quality control strain.

2.3. PCR of carbapenem resistance genes

Phenotypic screening for the presence of carbapenemases or overexpression of AmpC in combination with porin loss in *K. pneumoniae* strains was performed by a commercial synergy test (Rosco Diagnostica, Taastrup, Denmark).

To fully characterise the resistance profile of these strains, amplification and sequencing for detection of carbapenemases (KPC, IMP, VIM, OXA-48 and NDM) was performed using previously described primers [6,9].

PCR assays were carried out in *A. baumannii* using previously published primers for amplification of genes encoding carbapenemases (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA-23}$, *bla*$_{OXA-24}$ and *bla*$_{OXA-58}$). The *bla*$_{OXA-51}$ gene was also evaluated as an identification marker [2].

2.4. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

Klebsiella pneumoniae and *A. baumannii* isolates were examined for genetic relatedness by PFGE following extraction of genomic DNA and digestion with *Xba*I and *Apa*I, respectively, using conditions as previously described [2]. Restriction

fragments were separated on a CHEF DRII system (Bio-Rad Laboratories, Hercules, CA) and cluster designations were based on the criteria of Tenover et al. [10].

MLST of *K. pneumoniae* and *A. baumannii* isolates was performed as previously described [2,6], and STs were assigned using the MLST Pasteur website (http://www.pasteur.fr/mlst).

3. Results

3.1. Bacterial isolates

In total, 52 consecutive CRAB and 25 carbapenem-resistant *K. pneumoniae* were isolated from 57 patients admitted to the ICU between January and July 2013. All isolates were collected from various types of nosocomial infections, i.e. respiratory tract infections (70.1 %), bloodstream infections (16.9%) and, less frequently, urinary tract infections (13.0%).

3.2. Antimicrobial susceptibilities

Results of the in vitro susceptibility testing, expressed as MIC_{50} and MIC_{90} (MIC required to inhibit 50% and 90% of the isolates, respectively), are presented in Table 1.

The vast majority of *A. baumannii* strains showed an XDR phenotype, with resistance to all antibiotics: β-lactams (carbapenems, SAM); fluoroquinolones (ciprofloxacin); aminoglycosides (gentamicin, amikacin); rifampicin; and SXT. All isolates were susceptible to colistin, whilst tigecycline showing a 6% incidence of resistance with an MIC_{90} of 6 mg/L.

All KPC-producing *K. pneumoniae* isolates were MDR: colistin was active against 19 of the 25 strains, and the results regarding the activity of tigecycline showed 32% resistance.

3.3. Agreement of in vitro method results

All strains were tested by gradient test and BMD using both colistin and tigecycline. In *A. baumannii*, the reference BMD method revealed a high agreement (MIC differences ± 1 dilution) of 81% for colistin and 86% for tigecycline. Most strains were intermediate (75–84%) with MIC values of 4 mg/L.

Of the 25 KPC-producing *K. pneumoniae*, there was only a 60% concordance between the two methods for colistin, whilst for tigecycline the agreement was 88%. The very major error rate was 4% for both antibiotics in *K. pneumoniae*, whilst very major error was not present for *A. baumannii*. The agreement data are presented in Tables 2 and 3.

3.4. Phenotypic analysis and resistance genes for carbapenemases

All *K. pneumoniae* resistant to meropenem and ertapenem were positive by carbapenemase phenotypic test, and PCR detection showed that all strains harboured the $bla_{\text{KPC-3}}$ gene and no MBL (bla_{IMP} and bla_{VIM}), $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes were detected.

All isolates of *A. baumannii* were positive for *bla*$_{OXA-51}$, the carbapenemase gene that is intrinsic to this species. The *bla*$_{OXA-23}$ gene was identified in all strains, whilst *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA-24}$ and *bla*$_{OXA-58}$ genes were absent.

3.5. Molecular typing by pulsed-field gel electrophoresis and multilocus sequence typing

All *A. baumannii* isolates, genotyped following *Apa*I digestion by PFGE, belonged to the same clone as they were indistinguishable from each other (100% identity) according to the criteria described previously by Tenover et al. [10]. By the MLST scheme, all *A. baumannii* isolates were attributed to ST2, an endemic Italian clone [2].

Epidemiological investigation by PFGE identified four pulsotypes among all of the KPC-producing *K. pneumoniae* (A, B, C and D). MLST of these isolates identified four distinct STs: pulsotype A strains belonged to ST258 and pulsotype B was categorised as ST512 detected in most isolates. Pulsotypes C and D were also identified, in a few strains, as ST147 and ST395, respectively.

4. Discussion

The global emergence of multidrug resistance among Gram-negative bacteria is alarming worldwide. During the last decade, *A. baumannii* has become a pathogen of increasing clinical importance due to its remarkable ability to cause outbreaks of infections and to acquire resistance to almost all currently used antibiotics, including carbapenems. The increasing prevalence of KPC-producing *K. pneumoniae* with

broad-spectrum β-lactam antibiotic resistance is a serious issue in Italy, which in 2012 reached 12%. Many KPC-producing isolates and *A. baumannii* producing carbapenemases are extensively drug resistant or pandrug-resistant, difficult-to-treat, dramatically limiting the therapeutic options leading to increasing reliance on last-resort antibiotics such as colistin and tigecycline [11,12].

These results showed the good in vitro activity of colistin against *A. baumannii* with a high rate of agreement between BMD and gradient test; 12% were non-susceptible to tigecycline if tested by BMD and 6% when the gradient test was used.

The resistance rates for colistin in KPC-producing *K. pneumoniae* isolates, tested by the gradient test and BMD methods, did not show significant differences; however, the agreement was only 60% with a 4% very major error rate because in some strains the MIC values were very different (i.e. strain no. 17 in Table 3: 8 mg/L by gradient test and >256 mg/L by BMD). This feature is probably due to the characteristics of the strain and the low diffusibility of colistin in agar owing to its high molecular weight, resulting in an underestimation of resistance [13,14]. This is an issue when the MIC determined by gradient test is intermediate because it hides the resistant phenotype.

These results show that the XDR phenotype is very diffused in *A. baumannii* isolated from severe infections in the ICU, prominently driven by the spread of CRAB isolates belonging to monoclonal ST2, the European clone II, producing the OXA-23 carbapenemase enzyme.

In a multicentre Italian study [2], OXA-58 was the most prevalent carbapenem resistance enzyme, which was gradually substituted by OXA-23, as recently described [1]. This trend is likely to be related to the higher carbapenemase activity of OXA-23 than OXA-58 with a consequent selective advantage [2].

The present study also described 25 *K. pneumoniae* isolates harbouring the *bla*$_{\text{KPC-3}}$ gene in isolates of four different clones, of which two were more widespread (A and B), belonging to the same clonal complex (ST258 and ST512) already found in other Italian hospitals.

Analysis of antimicrobial susceptibility patterns showed that *K. pneumoniae* isolates with PFGE type A ST258 all had a multiresistant antibiotype characterised by higher MICs to most antimicrobial agents compared with B, C and D pulsotypes.

The other two clones (ST147 and ST395) belonged to a different clonal complex and were identified for the first time in Italy carrying KPC-3. OXA-48-producing ST395 was previously isolated in The Netherlands [15,16], whilst ST147 was spread in Canada and Hungary carrying the *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{OXA-48}}$ gene, respectively [17].

To date, this is the first study to report the spread of KPC-3-producing *K. pneumoniae* ST147 and ST395 in Italy.

The capacity for rapid evolution of resistance determinants in these nosocomial pathogens and the difficulty of interpretation of molecules that represent the last

therapeutic option against superbugs led to the conclusion that a rapid and reliable method for colistin susceptibility testing of *K. pneumoniae* is required.

This evaluation of in vitro susceptibility testing for the polymyxin class drugs confirmed a continued serious testing error with the agar diffusion method [18,19]; therefore, clinical laboratories should use MIC methods to assist the therapeutic application of this drug, whilst these findings indicate that the gradient test and BMD are suitable for tigecycline susceptibility testing of *A. baumannii*.

Acknowledgment: The authors express their gratitude to Antony Bridgewood for language revision.

Funding: None.

Competing interests: None declared.

Ethical approval: Not required.

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Table 1

Susceptibilities and molecular characteristics of carbapenemase-producing Gram-negative bacteria

Species (*n*)	Specimen (*n*)	Gene	PFGE profile	ST (*n*)	MIC (mg/L)	Antibiotic (mg/L)															
						MEM	ETP	IPM	TZP	SAM	AMC	ATM	CAZ	CTX	FEP	AMK	GEN	SXT	CIP	RIF	TGC	CST
Acinetobacter baumannii (52)	Bronchial (42)	*bla*$_{OXA-23}$	A	2	Range	16 to >32	NT	4 to >32	32 to >256	>256	NT	NT	NT	NT	NT	16 to >256	2 to >256	1 to >32	2 to >32	0.5 to >256	2–12	0.25–2
	Urine (3)				MIC$_{50}$	32	–	32	>256	>256	–	–	–	–	–	32	8	8	32	>256	4	0.75
	Blood (7)				MIC$_{90}$	>32	–	>32	>256	>256	–	–	–	–	–	>256	64	16	>32	>256	6	1
Klebsiella pneumoniae (25)	Bronchial (12)	*bla*$_{KPC-3}$	A	258 (15)	Range	16 to >32	2 to >32	NT	32–256	NT	32–64	32–8	32–8	64–4	16–64	32–64	64–1	8 to >32	4 to >32	NT	1–8	0.75–64
					MIC$_{50}$	16	4	–	64	–	64	16	16	32	16	32	8	16	>32	–	3	1

Urine (7)			MIC_{90}	16	2	–	32	–	64	8	8	4	16	32	1.5	8	4	–	1.5	1
	B	512 (6)	Range	4–16	2	NT	32	NT	16–32	8	8	4	8	16–32	1	1–8	2–4	NT	0.75–1	0.5–0.75
Blood (6)			MIC_{50}	8	2	–	32	–	16	8	8	4	8	32	1	1	4	–	1	0.75
			MIC_{90}	8	2	–	32	–	16	8	8	4	8	16	1	8	4	–	1	0.5
	C	147 (1)	Range	4	2	NT	32	NT	16	8	4	2	4	2	0.5	0.19	2	NT	0.5	0.19
			MIC_{50}			–		–		–	–	–	–	–	–	–	–	–	–	–
			MIC_{90}			–		–		–	–	–	–	–	–	–	–	–	–	–
	D	395 (3)	Range	4	2	NT	32	NT	16	8	8	2–4	8	2–16	1	0.38–1	2	NT	0.75	0.25–0.5
			MIC_{50}	4	2	–	32	–	16	8	8	2	8	2	1	1	2	–	0.75	0.38
			MIC_{90}	4	2	–	32	–	16	8	8	2	8	2	1	0.38	2	–	0.75	0.25

PFGE, pulsed-field gel electrophoresis; ST, sequence type; MIC, minimum inhibitory concentration; MEM, meropenem; ETP, ertapenem; IPM, imipenem; TZP, piperacillin/tazobactam; SAM, ampicillin/sulbactam; AMC, amoxicillin/clavulanic acid; ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CEF,

cefepime; AMK, amikacin; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin; RIF, rifampicin; TGC, tigecycline, CST, colistin; NT, not tested; $MIC_{50/90}$, MIC required to inhibit 50% and 90% of the isolates, respectively.

Table 2

Minimum inhibitory concentrations (in mg/L) of tigecycline and colistin for *Acinetobacter baumannii* sequence type 2 by gradient test and broth microdilution (BMD)

Isolates	Colistin		Tigecycline	
	Gradient test	BMD	Gradient test	BMD
1	0.75	1	4	16
2	1.5	1	4	4
3	1.5	2	3	2
4	1.5	1	4	16
5	1	1	3	4
6	1	0.5	3	4
7	1	1	4	4
8	1	1	4	4
9	0.75	1	4	4
10	1.5	1	2	2
11	1.5	0.5	3	4
12	1	1	4	4
13	1	1	3	16
14	1	1	2	2
15	1.5	1	4	4
16	0.5	0.75	4	4
17	1.5	1	4	4
18	1	1	6	4
19	1	1	3	4
20	1	0.5	6	4
21	1	0.5	8	4
22	0.25	1	8	4

23	0.75	1	4	2
24	0.5	1	4	4
25	0.75	2	4	4
26	0.5	1	4	16
27	0.75	1	6	4
28	2	1	12	4
29	0.5	1	4	4
30	1	1	4	4
31	0.25	1	4	4
32	0.5	2	4	16
33	0.38	2	4	4
34	1	1	2	2
35	0.38	1	4	4
36	0.25	1	6	4
37	0.75	0.5	6	4
38	0.75	1	3	4
39	0.5	1	4	16
40	1	1	3	4
41	0.75	1	2	4
42	0.75	0.5	2	4
43	0.5	1	6	4
44	1	1	3	4
45	0.75	1	4	4
46	0.5	1	4	4
47	1	1	3	4
48	0.38	1	3	2
49	0.25	1	3	4
50	0.5	1	3	2
51	0.75	1	3	4

52	0.75	1	3	4
Antimicrobial susceptibility testing and agreement with BMD				
%S	85	92	10	13
%I	15	8	84	75
%R	0	0	6	12
Agreement BMD (%)	81	NA	86	NA
Very major error	0	NA	0	NA

S, susceptible; I, intermediate; R, resistant; NA, not applicable.

Table 3

Minimum inhibitory concentrations (in mg/L) of tigecycline and colistin for *Klebsiella pneumoniae* by gradient test and broth microdilution (BMD)

ST	Isolate	Colistin		Tigecycline	
		Gradient test	BMD	Gradient test	BMD
258	1	1	0.25	0.5	0.5
	2	0.75	0.5	1 [a]	4
	3	64	256	2	2
	11	24	>32	1	2
	4	1	0.25	3	4
	5	0.38	0.5	4	4
	6	0.75	0.25	1	1
	7	0.75	1	0.75	2
	8	0.75	0.5	3	4
	9	1	2	1	2
	19	0.5	0.5	2	4
	21	2 [a]	64	6	8
	22	0.19	0.5	3	4
	23	0.5	1	0.75	1
	24	0.5	1	0.75	0.5
512	10	0.75	1	1.5	1
	12	1	0.5	3	4
	13	4	64	1	0.25
	14	1	1	2	4
	15	0.25	0.5	4	2
	16	1	0.5	8	4

395	17	8	>256	0.75	1
	18	16	>256	1.5	1
	20	16	>256	1	1
147	25	0.5	0.25	1.5	2
Antibiotic susceptibility testing and agreement with BMD					
%S		72	68	44	36
%I		4	4	24	24
%R		24	28	32	40
Agreement BMD (%)		60	NA	88	NA
Very major error (%)		4	NA	4	NA

ST, sequence type; S, susceptible; I, intermediate; R, resistant; NA, not applicable.

[a] Very major error.