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Original Article

Physiological and molecular characteristics of carbapenem resistance in *Klebsiella pneumoniae* and *Enterobacter aerogenes*

Rito Santo Pereira[1], Vanessa Cordeiro Dias[1,2], Alessandra Barbosa Ferreira-Machado[1], Juliana Alves Resende[1], André Netto Bastos[2], Lucas Quinet de Andrade Bastos[2], Victor Quinet de Andrade Bastos[2], Ricardo Villela Bastos[2], Vânia Lúcia da Silva[1], Cláudio Galuppo Diniz[1]

[1] Department of Parasitology, Microbiology and Immunology, Institute of Biological Sciences, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil
[2] Cortes Villela Clinical Laboratory, Juiz de Fora, Minas Gerais, Brazil

Abstract

Introduction: Bacterial resistance is a growing concern in the nosocomial environment in which *Klebsiella pneumoniae* and *Enterobacter aerogenes* play an important role due to their opportunism and carbapenemase-production. This work aimed to evaluate physiological and molecular characteristics of carbapenem-resistant *K. pneumoniae* and *E. aerogenes* isolated in a Brazilian tertiary hospital.

Methodology: In total, 42 carbapenem-resistant bacteria isolated from clinical specimens were included (21 *K. pneumoniae* and 21 *E. aerogenes*). Drug-sensitive *K. pneumoniae* (n = 27) were also included. Antimicrobial susceptibility and biocide tolerance patterns, hemolytic activity, tolerance to oxidative stress, and aggregative ability were assessed. Genetic markers related to carbapenem resistance, or ESBL-production were screened by PCR.

Results: Compared to drug-sensitive strains, carbapenem-resistant *K. pneumoniae* were more tolerant to biocides and to oxidative stress, and they displayed an increase in biofilm formation. The genetic markers *bla*$_{KPC}$ (95.2%) and *bla*$_{TEM}$ (90.5%) were the most frequent. Among the carbapenem-resistant *E. aerogenes* strains, *bla*$_{KPC}$, and *bla*$_{TEM}$ were detected in all bacteria. Drug-sensitive *E. aerogenes* were not isolated in the same period. *bla*$_{SHV}$, *bla*$_{VIM}$, and *bla*$_{CTX}$ markers were also observed among carbapenem-resistant bacteria.

Conclusions: Results suggest that carbapenemase-producing enterobacteria might show peculiar characteristics regarding their physiology associated with their environmental persistency, virulence, and multidrug resistance. The observed phenomenon may have implications not only for antimicrobial chemotherapy, but also for the prognosis of infectious diseases and infection control.

Key words: Carbapenemases; enterobacteria; antimicrobial resistance; biocide tolerance.

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Introduction

It is accepted that *Enterobacteriaceae* pathogenicity is related to several virulence factors that allow them to overcome innate host immunity and to sustain tissue damage and invasion. Virulence factors include capsule and hypermucoviscosity, lipopolysaccharides, adhesins, iron acquisition systems, serum resistance, and biofilm formation [1- 3]. Differences that may be observed in a host's clinical features might be related with quality and quantity of expressed virulence factors [4].

β-lactam drugs have been the mainstay of treatment for serious infectious diseases, but are facing the antimicrobial resistance phenomena. Among them, carbapenems have the broadest activity spectra, and are still the most active drugs of this kind against multi-resistant Gram-negative bacteria, including extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* [5,6]. Unfortunately, mostly due to continuous exposure to antimicrobial drugs worldwide, carbapenem-resistant *Klebsiella pneumoniae* strains have been emerging and represent a major clinical problem, limiting chemotherapy in these situations [7,8]. Added to that, carbapenem-resistant enterobacteria are strongly related to hospital-acquired infections in which mortality rates seem to be higher than those of infections associated with other etiological agents [9].

Given the molecular epidemiology related to carbapenem resistance among enterobacteria, the extrachromosomal location of the genes encoding carbapenemases makes the issue even more difficult to deal with [10]. In addition, there are several gene variations leading to resistance phenotypes capable of

horizontal transference to other enterobacteria. The most relevant horizontal transference genetic markers to be investigated in our region are *bla*KPC, *bla*SIM, *bla*SPM-1, *bla*VIM, *bla*GIM and *bla*NDM-1 [9,10]. According to the literature, genes encoding for ESBL, such as *bla*TEM, blaSHV, and blaCTX-$_{M,}$ should also be considered for carbapenem-resistance. These genes may be overexpressed or even related to additional phenotypes along with efflux pumps or porin loss in carbapenem-resistant bacteria [6].

Facing the antimicrobial-resistance phenomenon and β-lactamase diversity, monitoring β-lactam resistance, and validating screening assays should be useful in supporting empirical therapy and clinical microbiology laboratories. In this regard, answering the calls for prospective studies on alterations in different bacteria populations driven by antimicrobial agents [11], our objectives in this study were as follows: to evaluate drug susceptibility and biocide tolerance patterns in carbapenem-resistant *K. pneumoniae* and *E. aerogenes*; to evaluate hemolytic activity, comparative biofilm formation, and tolerance to oxidative stress in carbapenem-resistant and drug-sensitive bacteria; and to screen carbapenemase- and ESBL-related genes in carbapenem-resistant bacteria.

Methodology

Study design, patients and bacteria collection

This is a cross-sectional study based on data routinely collected from patients admitted to a Brazilian tertiary hospital in Juiz de Fora – Minas Gerais, between January and December, 2012. This study was approved by the Ethics Committee of the Federal University of Juiz de Fora (certificate no. 346/2011). Bacterial samples (n = 3,437) were isolated from clinical specimens sent to the hospital-based clinical pathology laboratory (urine, blood, tracheal secretion, bronchoalveolar lavage and catheter tip), in accordance with the guidelines described by the Brazilian Health Surveillance Agency [12].

After initial bacteria isolation from monomicrobial cultures, all the presumptively identified enterobacteria were identified using the Vitek 2 System (BioMerieux, Marcy l'Etoile, France), according to the manufacturer's instructions. With regard to the further characterization tests, only non-replicate clinical isolates of carbapenem-resistant enterobacteria over the studied period were collected (*K. pneumoniae* and *E. aerogenes*). For quality control purposes, *Enterobacter cloacae* ATCC 700323 and *Stenotrophomonas maltophila* ATCC 17666 were included. Demographic and clinical characteristics of patients were recovered from the corresponding medical records.

Antimicrobial susceptibility patterns

Antimicrobial susceptibility assays were performed on Mueller–Hinton agar (BD-Difco, USA) using the disc-diffusion method and growth inhibition zones were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) [13] with the exception of tigecycline [14].

Disks of amikacin (30μg), gentamicin (10μg), ciprofloxacin (5μg), piperacillin-tazobactam (100/10μg), sulphazotrim (25μg), tetracycline (30μg), ampicillin-sulbactam (10/10μg), tigecycline (15μg), ertapenem (10μg), meropenem (10μg), and imipenem (10μg) were tested against all the identified *K. pneumoniae* and *E. aerogenes.* All the antimicrobial disks were of commercial grade (Laborclin, Sao Jose do Rio Preto, Brazil). *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, and *Staphylococcus aureus* ATCC 25923 were used for quality control, according to CLSI guidelines [13].

Additionally, as a complementary methodological approach, the modified Hodge test (MHT) was performed to confirm carbapenemase production, along with the resistance observed against ertapenem, meropenem, and imipenem. *Klebsiella pneumoniae* ATCC BAA1706 was taken as KPC-negative and *Klebsiella pneumoniae* ATCC BAA1705 was used as the positive control [13].

Biocides tolerance patterns for K. pneumoniae

Tolerance to disinfectants and antiseptics commonly used in hospitals: %, 1.5%, and 2% sodium hypochlorite; 5% benzalkonium chloride; 4.25% hydrogen peroxide; and 0.5% triclosan were determined for the isolated bacteria by adapting the disk diffusion technique [13]. Paper disks of 5mm diameter were soaked with 5μl of each biocide solution and placed on Mueller Hinton plates (Becton Dickinson-Difco, Franklin Lakes, USA) previously inoculated with 0.5 McFarland bacterial suspensions. After the incubation period (24 hours at 35ºC), growth inhibition halos were recorded.

The biocides used were of commercial grade, stored in regular conditions, and used within the validity periods. All tests were performed in duplicate and 27 *K. pneumoniae* strains sensitive to all antimicrobials were used as experimental controls.

Evaluation of K. pneumoniae physiological characteristics (oxidative stress tolerance, hemolytic activity, and biofilm formation)

The oxidative stress tolerance was evaluated by the disk-diffusion method, according to that previously described [15]. In brief, paper disks of 5 mm diameter were soaked with 5µl of 20% hydrogen peroxide and placed on Mueller Hinton plates (BD-Difco, USA) previously inoculated with 0.5 McFarland bacterial suspensions. After the incubation period (24 and 48 hours at 35ºC), growth inhibition zones were recorded.

Hemolytic activity was evaluated on sheep blood agar plates. Each bacterial sample was spot-inoculated and the plate was incubated at 35°C for 24 hours. The zone of clearance was recorded and hemolytic activity was evaluated [16]. The oxidative stress tolerance and hemolytic activity tests were performed in duplicate.

Bacterial adhesive properties were measured as the ability to form experimental biofilm aggregates. Briefly, bacteria were grown in polystyrene microtiter plates and the absorbance of incorporated dye (crystal violet 0.01% w/v, Newprov, Parana, Brazil) by bacterial aggregates, at the optical density 590 nm, was determined. Thus the absorbance was equivalent to the density of adherent bacteria and the results were reported as an average from three different experiments [17].

Regarding the physiological characteristics evaluation, drug-sensitive strains of *K. pneumoniae* (n = 27) were used as comparators.

DNA extraction and screening of carbapenem-resistance genetic markers

Bacterial genomic DNA were extracted from 1 ml of overnight cultures in Tryptic Soy Broth (BD-Difco) using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison,USA) following the manufacturer's instructions. The DNA extracts were quantified using NanoDrop (Thermo Fisher Scientific, Wilmington, USA) and stored in a freezer at -20°C, to be used as templates in polymerase chain reactions (PCR). The following carbapenemases and ESBL genes were screened by PCR according to previously established methods (Table 1): bla_{KPC}, bla_{SIM}, bla_{SPM-1}, bla_{VIM}, bla_{IMP}, bla_{GIM}, bla_{NDM-1}, bla_{TEM}, bla_{SHV}, and bla_{CTX-M}. All the PCR experiments were performed in duplicate.

The expected amplicons were visualized in 1.5% agarose gel stained with ethidium bromide. The 1kb DNA ladder was used as molecular weight standard (Life Technologies, Carlsbad, USA). Positive controls for PCR reactions were carried out by sequencing randomly selected amplicons comprising 10% of the total reactions. The PCR products were sequenced in an ABI Prism 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Student's t-test was used for comparison of biocides tolerance patterns and the experimental biofilm tests. The significance level was set as $p < 0.05$. The analysis of the association between carbapenem-resistance and biocide tolerance or carbapenem-resistance and

Table 1. Primers used, expected amplicons, and polymerase chain reaction (PCR) conditions.

Target	Primer sequence (5'-3')	Amplicon (bp)	PCR conditions	References
bla_{CTX-M}	F-5'-ATG TGC AGY ACC AGT AAA G-3' R-5'-GGT CAC CAG AAG GAG C-3'	562	94°C, 7 min; 35x (94°C, 1 min; 54°C, 45 seg; 72°C, 1 min); 72°C, 1 min	20
bla_{SHV}	F-5'-CTT TAC TCG CCT TTA TCG GC-3' R-5'-TTA CCG ACC GGC ATC TTT CC-3'	982		
bla_{TEM}	F-5'-GTG CGC GGA ACC CCT ATT-3' R-5'-TTA CCA ATG CTT AAT CAG TGA GGC-3'	968	94°C, 4 min; 30x (94°C, 1 min; 56°C, 1 min; 72°C, 1 min); 72°C, 5min	20
bla_{KPC}	F-5'-ATG TCA CTG TAT CGC CGT CT-3' R-5'-TTT TCA GAG CCT TAC TGC CC-3'	829		42
bla_{SPM-1}	F-5'-CCT ACA ATC TAA CGG CGA CC-3' R-5'-TCG CCG TGT CCA GGT ATA AC-3'	649	95°C, 5 min; 30x (95°C, 1min seg; 52°C, 1min; 72°C, 1min); 72°C, 5min	-45
bla_{NDM-1}	F-5'-GGT TTG GCG ATC TGG TTT TC-3' R-5'-CGG AAT GGC TCA TCA CGA TC-3'	621		27
bla_{IMP}	F-5'-GGA ATA GAG TGG CTT AAY TCT C-3' R-5'-CCA AAC YAC TAS GTT ATC T-3'	188	94°C, 5 min; 36x (94°C, 30 seg; 52°C, 40 seg; 72°C, 50 seg); 72°C, 5min	15
bla_{SIM}	F-5'-TAC AAG GGA TTC GGC ATC G-3' R-5'-TAA TGGC CTG TTC CCA TGT G-3'	570		
bla_{VIM}	F-5'-GAT GGT GTTT GGT CGC ATA-3' R-5'-CGA ATG CGC AGC ACC AG-3'	390		
bla_{GIM}	F-5'-TCG ACA CAC CTT GGT CTG AA-3' R-5'-AAC TTC CAA CTT TGC CAT GC-3'	477		

physiological characteristics was evaluated by calculating the odds ratio (OR) [18]. The confidence interval used was 95%. An OR value of ≤ 1.0 indicated a negative correlation, *i.e.*, the probability is lower in the first group than in the second or the condition under study is equally likely in both groups. An OR value >1.0 indicated a positive correlation.

Results

In total, 3,437 samples were collected during the study period and 1,076 yielded monomicrobial positive cultures; considering only non-replicate clinical isolates of carbapenem-resistant enterobacteria, a total of 21 *K. pneumoniae* and 21 *E. aerogenes* strains were selected. In addition, 27 *K. pneumoniae* sensitive to all tested antimicrobials were also selected. *E. aerogenes* sensitive to these drugs were not observed.

Considering all carbapenem-resistant bacteria, 40.5% were isolated from urine samples, 26.2% from catheter tips, 16.7% from hemoculture, 14.3% from tracheal aspirate, and 2.4% from bronchoalveolar lavage. With regard to patients' demography and origin, 61.9% (n = 26) of the carbapenem-resistant bacteria were collected from patients hospitalized in the intensive care unit (ICU), 16.7% (n = 7) from the ward, 14.3% (n = 6) from the coronary care unit, and 7.1% (n = 3) from outpatients. The average patients' age was 72.6 years (ranging between 23 and 97 years). With regard to gender, 57.1% (n = 24) of the carbapenem-resistant bacteria were isolated from male patients,

Table 2. Antimicrobial resistance patterns of carbapenem-resistant *Klebsiella pneumoniae* and *Enterobacter aerogenes* isolated in a Brazilian tertiary hospital.

Bacteria	Phenotype	Genotype	Samples	
			N	%
K. pneumoniae (n=21)	CP, PT, AB, TE, AK, TS	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*VIM	01	4.76
	CP, PT, AB, TE, TS, NI	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*VIM	01	4.76
	CP, PT, AB, TG, TS	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*VIM	01	4.76
	CP, PT, AB, TE, TS	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*VIM	01	4.76
	CP, AB, GM, NI	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*VIM	01	4.76
	CP, PT, AB, TE, GM, TS, NI	*bla*KPC, *bla*TEM, *bla*SHV, *bla*VIM	01	4.76
	CP, PT, AB, TE, TS, NI	*bla*KPC, *bla*TEM, *bla*SHV, *bla*VIM	01	4.76
	CP, PT, AB, TE, ST	*bla*KPC, *bla*TEM, *bla*SHV, *bla*VIM	01	4.76
	CP, AB, GM, NI	*bla*KPC, *bla*TEM, *bla*SHV, *bla*VIM	01	4.76
	CP, AB, TS, NI	*bla*KPC, *bla*TEM, *bla*SHV, *bla*VIM	01	4.76
	CP, PT, AB, TE, GM, TS, NI	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M	01	4.76
	CP, PT, AB, TE, TS, NI	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M	01	4.76
	CP, PT, AB, TE, TS	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M	01	4.76
	CP, PT, AB, TE, TS, NI	*bla*KPC, *bla*TEM, *bla*CTX-M, *bla*VIM	01	4.76
	CP, PT, AB, TS, TG, NI	*bla*KPC, *bla*TEM, *bla*SHV	01	4.76
	CP, PT, AB, TE, TS, NI	*bla*KPC, *bla*TEM, *bla*SHV	01	4.76
	CP, PT, AB, TS, NI	*bla*KPC, *bla*TEM, *bla*SHV	01	4.76
	CP, AB, TS, NI	*bla*KPC, *bla*CTX-M, *bla*VIM	01	4.76
	CP, PT, AB, TE, AK, TG, NI	*bla*KPC, *bla*TEM, *bla*SHV	01	4.76
	CP, PT, AB, TE, GM, TS, NI	*bla*KPC, *bla*VIM	01	4.76
	CP, AB, TS, NI	*bla*TEM, *bla*SHV	01	4.76
E. aerogenes (n=21)	CP, PT, AB, GM, TS	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M	01	4.76
	CP, PT, AB, TE, TS	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M	01	4.76
	CP, PT, AB, GM	*bla*KPC, *bla*TEM, *bla*SHV, *bla*CTX-M	02	9.53
	CP, PT, AB, TE, TS	*bla*KPC, *bla*TEM, *bla*SHV, *bla*VIM	01	4.76
	CP, PT, AB, GM, TS	*bla*KPC, *bla*TEM, *bla*SHV	02	9.53
	CP, PT, AB, GM	*bla*KPC, *bla*TEM, *bla*SHV	04	19.05
	CP, PT, AB, GM, TS	*bla*KPC, *bla*TEM, *bla*CTX-M	01	4.76
	CP, PT, AB, GM	*bla*KPC, *bla*TEM, *bla*CTX-M	01	4.76
	CP, PT, AB, TE, GM, TS	*bla*KPC, *bla*TEM	03	14.28
	CP, PT, AB, GM, TS, NI	*bla*KPC, *bla*TEM	01	4.76
	CP, PT, AB, GM, AK, TS	*bla*KPC, *bla*TEM	01	4.76
	CP, PT, AB, GM	*bla*KPC, *bla*TEM	02	9.53
	CP, PT, AB	*bla*KPC, *bla*TEM	01	4.76

CP (ciprofloxacin), PT (piperacillin-tazobactam), AB (ampicillin-sulbactam), TE (tetracycline), GM (gentamicin), AK (amikacin), TG (tigecycline), TS (trimethoprim-sulfamethoxazole), NI (nitrofurantoin).

while 42.9% (n = 18) were isolated from females. The rate of intra-hospital mortality associated with infection or colonization with these microorganisms was 81%.

The most effective antimicrobial drugs against the evaluated bacteria were amikacin and tigecycline, for which resistance of only 7% was observed. Resistance to at least three of the tested antimicrobials was observed for all carbapenem-resistant bacteria: 2.4% were resistant to three antimicrobials, 21.42% to four, 23.80% to five, and 23.80% and 9.52% resistant to six or seven antimicrobials, simultaneously (Table 2).

All carbapenem-resistant *K. pneumoniae* showed to be carbapenemase producers; bla_{KPC} was the most frequently detected genetic marker (95.2%). With regard to the ESBL markers, bla_{TEM} was detected in 90.5% of strains; additionally, bla_{SHV}, bla_{VIM}, and bla_{CTX-M} were also detected (85.7%, 61.9% and 47.6%, respectively). Within the carbapenem-resistant *E. aerogenes*, all strains were positive to bla_{KPC} and bla_{TEM}. Although bla_{SHV}, bla_{CTX}, and bla_{VIM} were observed in 52.4%, 28.6% and 4.8%, respectively (Table 2), all the strains were negative for bla_{NDM-1}, bla_{IMP}, bla_{GIM}, and bla_{SPM-1} screening. Overall, the number of genetic markers detected in each isolated bacteria ranged between one and four and the majority of the strains were positive to three or four of the tested genes.

The physiological characteristics were accessed only for *K. pneumoniae* once no drug-sensitive *E. aerogenes* were recovered. Biocide tolerance tests showed that carbapenem-resistant *K. pneumoniae* were less tolerant to 1%, 1.5%, and 2% sodium hypochlorite, and also less tolerant to 5% benzalkonium chloride ($p < 0.05$). With regard to 4.25% hydrogen peroxide and 0.5% triclosan, no significant difference in bacterial tolerance was observed, considering both carbapenem-resistant and drug-sensitive bacteria. The carbapenem-resistant strains showed a significant increase in oxidative stress tolerance when compared to the drug-sensitive strain ($p = 0.004$). No hemolytic activity was observed among any *K. pneumoniae.* Carbapenem-resistant bacteria showed higher aggregative properties if compared to drug-sensitive ones ($p = 4.67\times10^{-25}$) (Table 3).

The analysis of the association between carbapenem-resistance and biocide tolerance or carbapenem-resistance and physiological characteristics retrieved OR values >1.0, indicating the possibility of a positive correlation between carbapenem resistance and biocide tolerance or carbapenem-resistance and physiological characteristics (Table 4). Overall, carbapenem-resistant *K. pneumonia* showed to be more tolerant to oxidative stress. The mean value recorded for growth inhibition halos were 12.36 ± 0.67 mm for carbapenem-resistant and 13.41 ± 0.23 mm for carbapenem-sensitive bacteria ($p = 0.04$). Regarding the ability of biofilm formation, bacterial aggregative properties were highly altered in carbapenem-resistant bacteria. The mean value recorded for absorbance of incorporated dye by

Table 3. Biocide tolerance patterns of carbapenem-resistant and carbapenem-sensitive *K. pneumoniae* isolated in a Brazilian tertiary hospital.

Biocides solutions (% v/v)	Inhibition halo diameter (mm)		*p* value
	Carbapenem-sensitive (n=27)	Carbapenem-resistant (n=21)	
Sodium hypochlorite (1.0%)	8.79; ± 0.57	4.83; ± 4.72	5.94×10^{-07}
Sodium hypochlorite (1.5%)	10.7; ± 0.66	7.95; ± 3.74	5.65×10^{-06}
Sodium hypochlorite (2.0%)	12.23; ± 1.03	11.12; ± 1.88	0.04
Benzalconium chloride (5.0%)	28.0; ± 2.14	24.39; ± 2.42	5.29×10^{-06}
Hydrogen peroxide (4.25%)	26.93; ± 3.54	24.93; ± 2.46	0.12
Triclosan (0.5%)	18.29; ± 9.11	14.31; ± 14.20	0.10

Table 4. Correlation between physiological characteristics or biocide tolerance, and carbapenem-resistance in *Klebsiella pnemoniae* isolated in a Brazilian tertiary hospital.

Evaluated parameters		*Odds ratio*[a] (range)
Physiological characteristics	Oxidative stress[b]	1.25 (1.07-1.83)
	Biofilm formation	18.50 (2.10-158.7)
Biocide tolerance	NaOCl (1%)	1.85 (0.57-6.04)
	NaOCl (1.5%)	3.52 (0.82-15.05)
	NaOCl (2%)	2.50 (0.65-9.55)
	BZK (5%)	1.42 (0.40-4.98)
	H_2O_2 (4.25%)	0.74 (0.20-2.72)
	TCS (0.5%)	1.6 (0.50-5.05)

[a] *Odds ratio* with 95% confidence interval: OR = 1, absence of correlation; OR > 1, positive correlation; OR < 1, negative correlation. [b] Oxidative stress by 20% hydrogen peroxide exposure. NaOCl (sodiumhypochlorite), BZK (*b*enzalkonium chloride), H_2O_2 (h*y*drogen peroxide), TCS (tricosan).

bacterial aggregates, at the optical density 590 nm, were 0.469 ± 0.042 for carbapenem-resistant and 0.310 ± 0.016 mm for carbapenem-sensitive bacteria ($p = 4.67\times10^{-25}$).

Discussion

KPC-producing bacteria are predominantly involved in nosocomial and systemic infections, although they are mostly enterobacteria [19]. Their emergence and dissemination into the community as opportunistic pathogens have also been observed. Our results may corroborate the observations regarding their emergence as community-related bacteria, based on the fact that 7.1% of the total carbapenem-resistant *K. pneumonia* and *E. aerogenes* were isolated from outpatients. In this study, 61.9% of patients were admitted to ICU and the average age was 72.6 years old, representing a group at increased risk for carbapenem-resistant bacteria colonization. Advanced age, prior ICU admission, and prior surgical procedures are considered among the risk factors for colonization by carbapenem-resistant *K. pneumoniae* [20-23]. The ICU has been described as a major reservoir for selecting antimicrobial-resistant bacteria due to its extremely vulnerable population of critically ill patients and the high use of invasive procedures [22].

Although mortality rates observed in this study were 81%, several investigators have reported rates ranging from 24% to 65% in patients infected with carbapenem-resistant strains. Resistance to carbapenems may be considered an independent predictor of death [9, 23, 24].

Considering hemolytic activity, biofilm formation ability, and tolerance to biocides as components of bacterial virulence and / or persistency in different environments, such as health care units, a group of drug-susceptible bacteria isolated in the same hospital was used as a comparator. None of the carbapenem-resistant bacteria showed hemolytic activity on sheep blood agar. This is consistent with other studies, which highlight that *Klebsiella* spp. possess selective hemolytic activity on rabbit erythrocytes [25]. On the other hand, antimicrobial resistance was associated with biocide increased tolerance and biofilm formation.

Biofilm formation is an important step in the development of a bacterial infection [26, 27]. Bacteria in biofilms are less susceptible to antimicrobials and disinfectant and shielded from opsonization and phagocytosis, and may even develop a communication between them leading to the expression of virulence properties [28].

In this study, such increased biofilm formation ability and reduced biocide tolerance applies to carbapenem-resistant bacteria that are also resistant to other antimicrobial drugs. The most efficient antimicrobials observed were amikacin and tigecycline. Different authors have reported that tigecycline and colistin became the last-resort treatments for infections by multidrug-resistant Gram negative bacteria [20, 29-33].

Regarding the genetic markers screening, the presence of bla_{KPC} among the carbapenem-resistant enterobacteria has already been described worldwide, including reports from Brazil [34-37]. Of the studied bacteria samples, the majority of carbapenemase-producing strains also carried ESBL-related genes. It is suggested that the frequent association of TEM, SHV, and CTX-M with KPC in *K. pneumoniae* and *E. aerogenes* is associated with the acquisition of transmissible plasmids carrying the bla_{KPC} gene by local endemic strains harboring the other genes [38]. The bla_{KPC} was detected in only one ertapenem-resistant strain of *K. pneumoniae*, perhaps due to other possible resistance mechanisms, such as an association between TEM, SHV, or CTX-M production and porin loss, which is also responsible for decreased susceptibility to carbapenem [6].

Limited information is available about the molecular epidemiology of VIM-producing *Enterobacteriaceae* in Brazil. The production of VIMs has mostly been described in *Pseudomonas aeruginosa* infection and remains relatively rare among members of the *Enterobacteriaceae* [39]. The exceptions are *K. pneumoniae*, *E. aerogenes*, and *E.coli*, which were detected in Mediterranean Europe (*i.e.*, VIM-producing *K. pneumoniae* were detected in Greece, Italy, and Spain and VIM-producing *E. aerogenes* in Spain), Taiwan, and Japan. Metallo-beta-lactamases (MBL) have the ability to hydrolyze a wide variety of β-lactams, such as penicillins, cephalosporins, and carbapenems, but not the monobactams (*i.e.,* aztreonam). VIMs are often associated with class one integrons that may harbor several gene cassettes that render bacteria resistant to different antimicrobial agents [39,40]. This circumstance is most probably due to the horizontal transfer of bla_{VIM} between these bacteria [41]. Medical records showed that the KPC + VIM producers were isolated throughout the study period from various ward unities similarly to the rest of the isolates. Thus any epidemiological link could not be supported. Data indicate that *K. pneumoniae*-producing KPC + VIM has been established in this setting.

Taken together, our results may suggest that carbapenemase-producing enterobacteria might show particular characteristics regarding their physiology that may be linked to their environmental persistency, virulence and multidrug resistance. The observed phenomenon has implications not only for antibiotic therapy, but also for the prognosis of infectious diseases and infection control. This study provides additional information on the biology of carbapenem-resistant *K. pneumoniae* and *E. aerogenes* circulating in southern Brazilian hospitals, and reinforces the need for continuous surveillance over time, as antimicrobials are still widely used.

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Corresponding author

Cláudio Galuppo Diniz,
Laboratory of Bacterial Physiology and Molecular Genetics,
Department of Parasitology, Microbiology and Immunology,
Institute of Biological Sciences, Federal University of Juiz de Fora,
36.036-900, Juiz de Fora, MG, Brazil.
Phone: + 55 32 2102-3213
Email: claudio.diniz@ufjf.edu.br

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