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Sequential Emergence of Resistance to Multiple Antimicrobial Agents in KPC-Producing-*Klebsiella pneumoniae*

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Introduction: *Klebsiella pneumoniae* is one of the most important gram-negative bacteria causing hospital-acquired infections.
Case Presentation: In this report is presented a patient with an abdominal infection caused by carbapenemase producing-*Klebsiella pneumonia (K. pneumonia)*. In spite of administrating the combination therapy, successive resistance to last-resort antimicrobial agents (colistin and tigecycline) was observed. The use of combination therapy, in four successive isolates recovered from this patient, was analyzed by killing curves. The genetic relatedness among the isolates was assessed.
Conclusions: All four isolates were *Klebsiella pneumoniae* carbapenemase (KPC) positive and showed resistance to all β-lactams including carbapenems and to other antimicrobial agents like aminoglycosides, fluoroquinolones, minocycline and trimetoprim-sulfametoxazol (TMS). Isolates 1 and 2 showed susceptibility to colistin, whereas isolate 3 was colistin-resistant and isolate 4 became tigecycline-resistant as well. Synergy was only observed with colistin plus rifampicin and with the triple combination of colistin, rifampicin and fosfomycin. The four isolates were indistinguishable genotypically. We described the sequential emergence of resistance to colistin and tigecycline in KPC-producing-*K. pneumonia* (KPC-Kp) isolates that occurred under treatment with these antimicrobial agents despite the use of combination therapy.

Keywords: Klebsiella Pneumonia; Carbapenemase; Rifampicin

1. Introduction

Klebsiella pneumoniae is one of the most prevalent nosocomial pathogens due to its ability to be recipient of resistance genes. Over the last years it has acquired resistance to multiple antimicrobial agents, mediated by the presence of extended spectrum β-lactamases and carbapenemases, porin loss and acquisition of efflux pumps. KPC-producing *K. pneumoniae* (KPC-Kp) isolates are widely disseminated in our country, especially in Buenos Aires city. In our hospital, KPC-Kp strains have a widespread presence since 2010 (1).

2. Case Presentation

We reported a patient with an abdominal infection caused by KPC-Kp that developed successive resistance to last-resort antimicrobial agents (colistin and tigecycline), during the treatment in spite of receiving a combination therapy. A 66-year-old-man with diabetes mellitus and gout disease was admitted. He underwent a urinary surgery in July 2012. Forty-eight hours later, he had a gastric ulcer perforation and was consequently operated. Vancomycin plus imipenem were administered as prophylaxis. A rectal swab was obtained and analyzed to confirm colonization by multidrug resistant gram-negative bacteria a week after his admission. KPC-Kp was recovered from this culture (isolate 1). His evolution was not favorable. Abdominal samples were taken to the laboratory, after each course of drainage. KPC-Kp was recovered (isolate 2) and the patient received the combination tigecycline (TGC) plus colistin (CT) for seven days, until isolate 3 (CT-resistant) was recovered, when rifampicin (RIF) was added. The triple combination therapy was maintained for 10 days. Isolate 4 (TGC-resistant) was recovered from the third abdominal sample and consequently TGC was replaced by fosfomycin (FOS). The patient died on September 27th, after nearly two months of hospitalization. The objective of this study was to analyze the use of combination therapy in the four successive isolates from this patient.

All isolates were identified using conventional methodology and minimal inhibitory concentrations (MICs) of imipenem (IMI), meropenem (MEM), rifampicin (RIF), CT, TGC and FOS were determined by agar dilution method and interpreted according to the Clinical and Laboratory Standards Institute (2), the European Committee

Table 1. In Vitro Activities of Different Antimicrobial Agents and Their Combinations Against the Four *Klebsiella. pneumoniae* Isolates and Therapy Installed[a]

Date	Isolate	MIC, mg/L						Combination Studies								Treatment
		IMI	MEM	CT	TGC	RIF	FOS	CT/ TGC	CT/ RIF	CT/ MEM	CT/ FOS	TGC/ RIF	FOS/ RIF	FOS/ TGC	CT/FOS/ RIF	
7/8	**1**	32	32	1	0.06	> 64	8	ND	ND	ND	ND	ND	ND	ND	ND	CT, TGC
29/8	**2**	32	32	1	0.06	> 64	8	I	I	I	ND	I	ND	ND	ND	CT, TGC,RIF
6/9	**3**	32	32	64	0.25	> 64	8	I	S	I	ND	ND	ND	ND	ND	CT, FOS, RIF
16/9	**4**	32	32	64	16	> 64	8	ND	ND	ND	I	I	I	I	S	

[a] Abbreviations: S, synergy; I, indifference; ND, not determined; CT, colistin; MEM, meropenem; IMI, imipenem; RIF, rifampicin; FOS, fosfomycin; TGC, tigecycline; MIC, minimal inhibitory concentration.

on Antimicrobial Susceptibility Testing (3) for CT and FOS and Societe Française de Microbiologie (4) for RIF. Tigecycline susceptibility testing was based on the Food and Drug Administration breakpoints for *Enterobacteriaceae* (5). The double disc synergy test with phenylboronic acid (PBA) and carbapenems was performed for the phenotypic identification of KPC-Kp. The presence of blaKPC was confirmed by PCR amplification, performed using heat-extracted DNA as template and using specific primers: KPC-F: 5´ATGTCACTGTATCGCCGTCT 3' and KPC-R: 5' TTTTCAGAGCCTTACTGCCC 3' and conditions previously described (initial denaturation at 95°C for five minutes, annealing at 95° for one minute, at 55° for one minute, at 72° for one minute (30 cycles) and a final extension period at 72° for five minutes) (6). The amplified products were sequenced and nucleotide sequences were compared using BLAST (the National Center for Biotechnology Information, Bethesda, MD, USA, www.ncbi.nlm.nih.gov/Tools/). The genetic relatedness among the isolates was studied by DO-PCR, using primer AP/OD 19: GGTCGACYTTNGYNGGRTC and the following conditions: initial denaturation at 95° for five minutes, annealing at 93° for one minute, at 36° for 1.5 minutes, at 72° for two minutes (40 cycles) and an extension period at 72° for ten minutes (7). The activity of the different associations CT/RIF, CT/TIG, CT/MEM, CT/FOS, FOS/RIF, FOS/TGC and CT/FOS/RIF was determined by killing curves. The time-kill studies were performed twice and therefore, results were analyzed for each isolate, using the mean colony count values from the duplicate plates. The Mueller Hinton broth was inoculated with $5x10^5$ CFU/mL of each isolate in a log phase/mL and killing was assessed at 0, four and 24 hours following incubation at 37°C. The following concentrations were used for the time-kill experiments: 2 mg/L CT, 100 mg/L FOS, 4 mg/L TGC, 10 mg/L MEM and 4 mg/L RIF. Synergism was defined as $\geq$ 2-$\log_{10}$ CFU/ mL decrease in the viable count, with the combination compared to the most active single agent at different time points. Bactericidal activity was defined as $\geq$ 3-$\log_{10}$ CFU/mL decrease from the original inoculum at 24 hours and synergy was defined as $\geq$ 2-$\log_{10}$ CFU/mL decrease between the combination and the most active compound (8). The presence of resistant subpopulations (able to grow in the presence of > 2 mg/L of colistin and tigecycline) in the COL and TIG-susceptible isolates was evaluated by population analysis profile (PAP), using a previously reported method (9).

3. Discussion

The phenotypic double-disc test with PBA showed positive results in all the four *K. pneumoniae* isolates and the presence of the blaKPC-2 gene was lately confirmed by PCR, followed by sequencing. The isolates were resistant to all β-lactams, including carbapenems, and to other antimicrobial agents like aminoglycosides, fluoroquinolones, minocycline and TMS (data not showed in tables). The IMI, MEM, CT, TGC, RIF and FOS MIC values are demonstrated in Table 1. In time-kill studies colistin showed bactericidal activity at four hours, but regrowth was observed at 24 hours. Regarding the combination studies, the combinations of CT with RIF, MEM and TGC exhibited indifferent effects against isolate 2, whereas the combination of CT with RIF exhibited synergy against isolate 3 in spite of its CT MIC of 64 mg/L. Tigecycline did not have a synergistic effect with any of the antimicrobial agents tested. Although FOS alone and in combination with RIF and CT showed bactericidal activity at four hours, the bactericidal effect was only observed at 24 hours, when FOS was combined with CT and RIF simultaneously. Antagonism was not observed in any of the isolates studied (Table 1).

Colistin-resistant subpopulations (MIC > 2 mg/L) were observed in isolates 1 and 2, whereas TGC-resistant subpopulations were not observed in isolates 1, 2 or 3. All four isolates showed similar band patterns in DO-PCR results, indicating clonal relatedness. Intra-abdominal infections often require both antibiotic therapy and surgical management, therefore, drainage of the source is considered critical in these infections. Combination therapy is strongly recommended to deal with severe infections, caused by multidrug resistant (MDR) strains. Several studies have supported the use of more than one antimicrobial agent and mortality rates were lower in most patients who received combination regimens (10). However, MDR strains are mostly resistant to at least one of the antimicrobial agents used in the clinical practice, the possibility of selection of resistance, influenced by infection and host factors, is undoubtedly high. Very few

in vivo studies have been developed to assess the efficacy of triple drug combinations to treat infections by MDR strains and most of these in vitro studies were conducted on *Acinetobacter* spp. or *Pseudomonas aeruginosa* strains (11). Urban et al. (12) assessed *K. pneumoniae* and *Escherichia coli* isolates and obtained bactericidal activity in 90% of the isolates studied, using the polymyxin, B-doripenem-RIF combination. In the present study the patient received double combination of TGC plus CT approximately for a week. Nevertheless, the selection of CT-resistance could not be prevented. Colistin heteroresistance is very frequent in KPC-Kp isolates (9, 13). We observed the presence of COL-resistant subpopulations in a previous study in almost 90% of the KPC-Kp isolates in our hospital. These resistant subpopulations may have a role in therapeutic failure, due to the absence of synergy between CT and TGC. In killing curves, CT plus TGC did not prevent the regrowth of these CT-resistant subpopulations at 24 hours of incubation. Isolate 4 became TGC-resistant, probably due to the selective pressure induced by the treatment with this drug (17 days) and by the poor microbiological clearance of the infection. Resistance to TGC has been described in *Enterobacteriaceae* (14, 15) and the mechanism proposed is an increased expression of some activators of the AcrAB efflux pump and porin OmpF (16).

In accordance with poor clinical results, we did not observe in vitro synergy with the TGC associations in any of the three isolates, contrary to what was shown by Pournaras et al. (17), who observed bactericidal activity with TGC plus CT combination, although a regrowth at 16-24 hours was detected in some of the studied strains. The CT and RIF combination had synergistic effects on CT-resistant isolates, as it was previously described (8). Nevertheless, this treatment scheme was not installed. The CT and MEM combination did not exhibit synergy, probably due to the high MEM MIC value (32 mg/L); previous studies stated that the combination of carbapenems with other antimicrobial agents would be effective in the treatment of carbapenemase-producing gram-negative bacteria, if the carbapenem MIC is $\leq$ 4 mg/L (18). FOS should always be used in association with other active agents due to its ability to select resistant mutants (19). Nevertheless, there are few reports of emergence of resistance to FOS, when used in double/ triple regimens against KPC-Kp (20), therefore additional in vitro studies should be performed to assess its role in the treatment of severe infections caused by these strains. In this case, we observed a rapid decrease in the CFU/mL at 4 hours, by time-killing studies, when tested in isolation, but at 24 hours an important regrowth was observed in all isolates. This regrowth was not prevented by adding other antimicrobial agents, like RIF, TGC or CT. Bactericidal activity was only obtained when a triple combination of agents was tested: FOS/CT/RIF, by means of the inhibition of these subpopulations. However, in vivo performance could not be evaluated. To the best of our knowledge, we described the first case of sequential emergence of resistance to colistin and tigecycline in KPC-Kp isolates, occurring under treatment with these antimicrobial agents. Undoubtedly, the impossibility to eradicate the source of infection by surgical measures promotes the selection of multiple mechanisms of antimicrobial resistance. The emergence of KPC-Kp isolates and the remarkable ability of these strains to become MDR, necessitate evaluating new combination studies and double therapy regimens should be progressively replaced by triple ones. We also reinforce the idea that strict control of infection policies should be implemented as a solution to these clinical situations.

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